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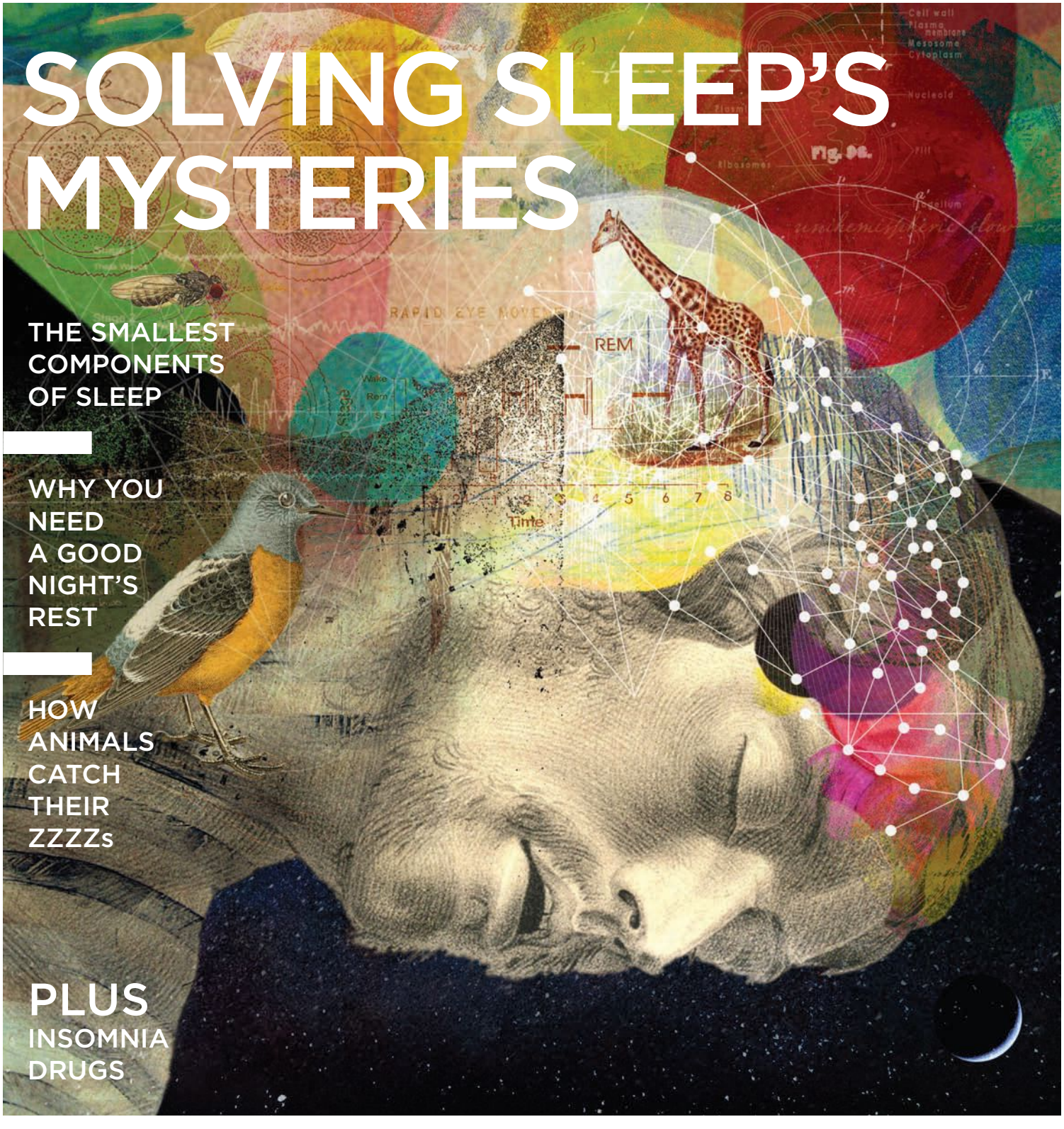

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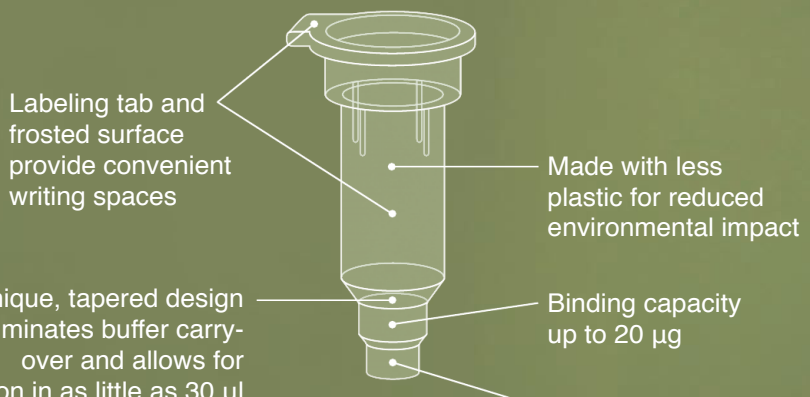

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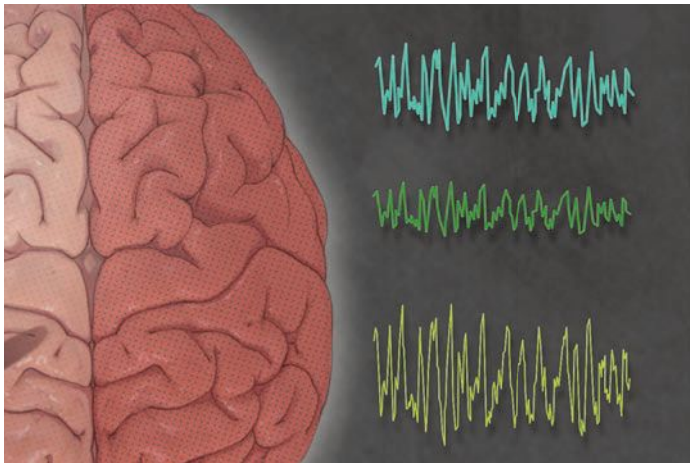

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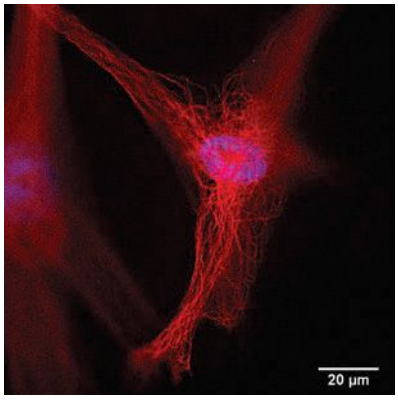

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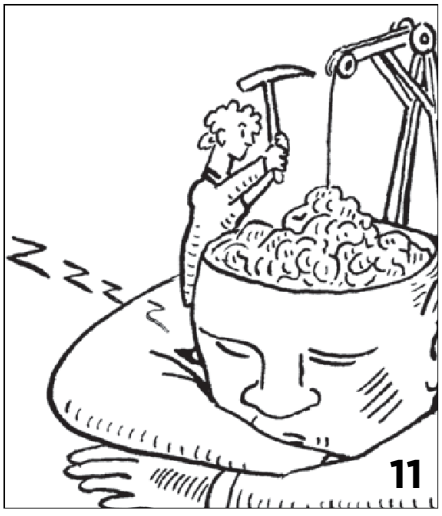

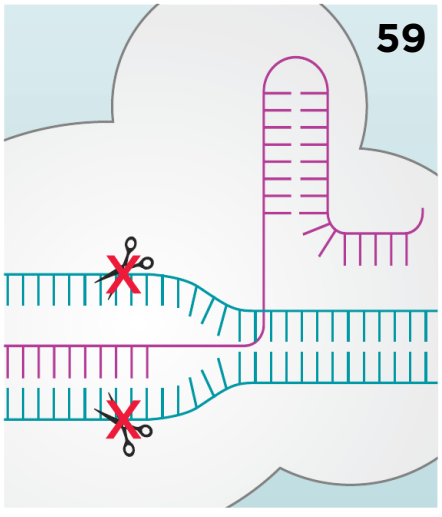

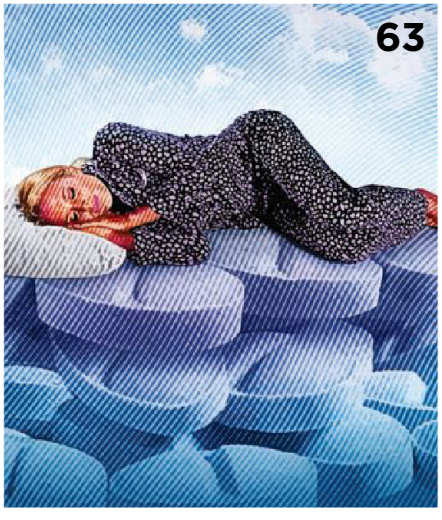

CORRECTIONS:

In "Giraffe Diplomacy" (*The Scientist*, February 2016), the giraffe Marius was 24 months old when euthanized, not 18 months old, and the lion cub in the photo is not eating the body of the giraffe. Because the photograph in "Holding Their Ground" (p 27) was flipped, the handedness of the DNA is incorrect.

The Scientist regrets the errors.

Online Contents

THIS MONTH AT THE-SCIENTIST.COM:

VIDEO
Water Bed
Watch zebrafish as they engage in sleeplike behavior.

VIDEO
A Month in Mammoth
In 1938, two sleep researchers braved the subterranean environment of a Kentucky cave to see if they could train their bodies to abandon the cyclical rhythms of the 24-hour day.

VIDEO
Night Night, Flipper
See underwater footage of a dolphin sleeping in the wild.

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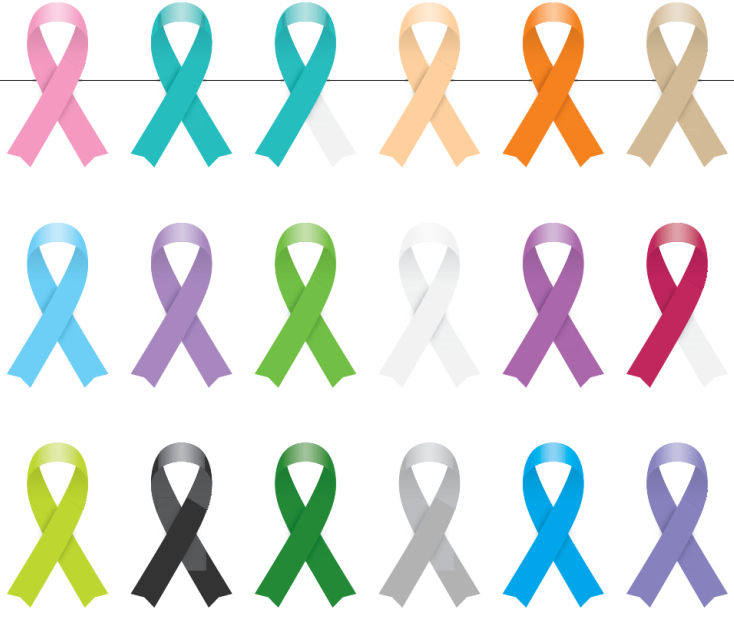

HERE'S WHAT YOU'LL FIND IN NEXT MONTH'S ISSUE:

- Altered metabolism in cancer cells
- Cancer and the microbiome
- Physical forces in cancer
- Exosomes and metastasis
- Liquid biopsies

AND MUCH MORE

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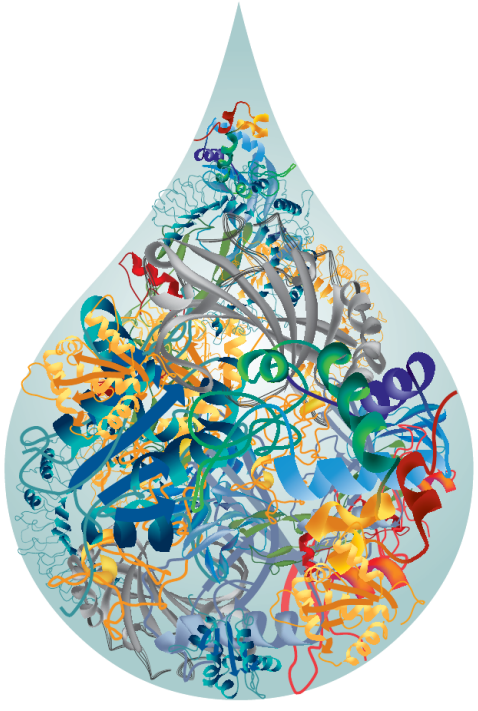

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Contributors

As an undergraduate at the City University of New York in the 1960s, **Jerome Siegel** thought he wanted to be an electrical engineer. But during his studies he became interested in human consciousness, and realized that electrical engineering approaches could be used to study the human brain. "It seemed to be the perfect combination of my inclinations and my more general interest in science," Siegel says of sleep research. Graduating with a PhD in psychology from the University of Rochester in 1973, he moved to the University of California, Los Angeles, where he made the first recordings of single brain cells in relation to REM sleep and narcolepsy in animals. Now a professor of psychiatry at UCLA's Semel Institute of Neuroscience and Human Behavior and director of its Center for Sleep Research, he has recently expanded his research to study sleep in pre-industrial human societies. Siegel discusses sleep across the animal kingdom in "Who Sleeps?" (page 28).

As a PhD student studying catfish physiology at the University of Pennsylvania in 1974, **James Krueger** first encountered sleep research during an interview for a postdoc in the lab of John Pappenheimer at Harvard University. "He was well-known in the field of respiration, and I thought that's what he was interviewing me for," says Krueger. "I'd never even thought about sleep." Yet when he joined the lab, Krueger began work on a project to isolate a "sleep factor" in cerebrospinal fluid. The project took six years, and led to a series of fruitful research pathways, including the first study to demonstrate the sleep-promoting properties of cytokines. In 1997, Krueger joined Washington State University as chair of the Department of Veterinary and Comparative Anatomy, Physiology, and Pharmacology, where he is now a Regents Professor working on sleep regulation and the links between sleep and infectious disease. His current research focuses on the control of sleep in cells grown in culture.

Sandip Roy graduated in 1998 from the University of Illinois, Urbana-Champaign, with a degree in electrical engineering. After receiving his doctorate from MIT in 2003, he joined the faculty of Washington State University to work on the management of large infrastructures, from air traffic networks to electric power grids.

Several years later, he was approached by Krueger to apply similar network approaches to the biology of sleep. An associate professor at Washington since 2009, Roy has collaborated with Krueger's group on both theoretical and experimental approaches to the study of sleep—projects that he says provide interesting contrasts but also parallels to his research on man-made infrastructures. "It's a much more complicated system in many ways than the others I look at," he says of the sleeping brain. Krueger and Roy explore the emergence of sleep as a network property in "Sleep's Kernel" (page 35).

David Gelernter had an early introduction to the world of computers: his father, Herbert, was a pioneer of computer science research in the 1950s. "I grew up in an atmosphere in the '60s and '70s surrounded by computers—which was unusual at the time," says Gelernter. After obtaining a master's degree in classical Hebrew literature from Yale University, Gelernter earned a PhD in computer science from Stony Brook University in 1982. Joining Yale's faculty, Gelernter researched parallel computing throughout the 1980s, developing the Linda programming system and ideas that would later be incorporated into cloud computing. He has been a member of the National Council on the Arts and has written several books, including *Mirror Worlds*, which predicted the rise of public data sharing, and *The Muse in the Machine: Computerizing the Poetry of Human Thought*. In recent years, Gelernter has combined his research on artificial intelligence with work on consciousness, human thought and religion. "Both science and scholarship have always appealed to me, and still do," he says.

In an essay ("In Your Dreams," page 66) based on his newest book, *The Tides of Mind: Uncovering the Spectrum of Consciousness*, Gelernter makes the case for sleep as a window into the human mind.

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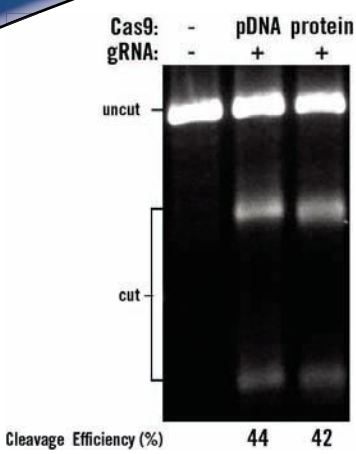

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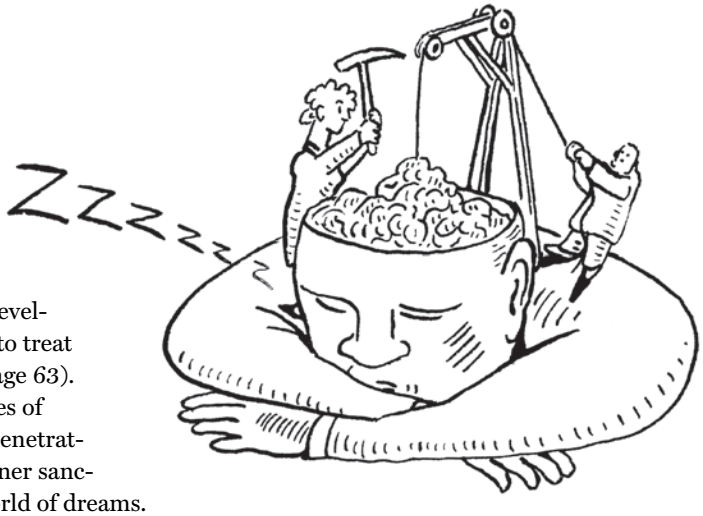
Things That Go Bump

Scientists still don't know why animals sleep or how to define the ubiquitous behavior.

BY BOB GRANT



ANDRZEJ KRAUZE

Strange things happen in the night. Distant stars flicker above darkened towns. Nocturnal creatures prowl. Things go bump. And every night (if we're lucky), we sleep through it all. But for all its ubiquity, for all its regularity, and for all the intimacy we feel toward a phenomenon that literally lays us flat every day of our lives, sleep is mysterious. It may, in fact, be one of the broadest biological enigmas left. We're not entirely sure how other animals sleep, or even if they all do. We don't know *why* any of us sleep. We know neither exactly how sleep benefits us nor how skipping it harms us. We don't even have a clear definition of the process.

But as with many enduring scientific mysteries, there are intrepid researchers who seek to crack sleep open and understand its intricacies. This issue celebrates them.

Like early geologists scraping through the uppermost layers of the Earth's crust, researchers in the relatively small field of sleep science are devising methods, technologies, and experimental designs that aim to lay bare what is undoubtedly a massive trove of game-changing discoveries lurking beneath the surface. From attempts to document the circadian rhythms of humans cut off from sunlight and temperature cycles in the depths of a Kentucky cave (page 72) to modern optogenetic (page 50) and molecular (page 51) manipulations of the brain's sleep centers, researchers have been probing into the brains and cells of snoozing humans (page 22) and nonhuman animals (page 28), hoping to gain basic insights into how and why we sleep. Other scientists have sought to pin down more-fundamental, emergent properties of sleep, inciting sleep-like behavior in isolated bits of cortical tissue and even in networks of neurons and glial cells in vitro (page 35).

Even without a solid handle on the biological functions of sleep, researchers and clinicians have been addressing sleep pathologies in patients for decades. On page 42, Senior Editor Kerry Grens takes a peek inside clinical sleep labs to explore the latest research on the neural, metabolic, and long-term effects of sleep disruption. And a Bio Business article by Anna Azvolinsky surveys the landscape of companies developing drugs to treat insomnia (page 63).

Some lines of inquiry are penetrating sleep's inner sanctum—the world of dreams. If we know little about how sleep jibes with biology, we know even less about how and why the sleeping brain engages in time travel, shape shifting, and emotional animation on a nightly basis. But again, researchers are mapping the human brain's dream centers (page 16), and at least one scientist calls for sleep and dreaming to be incorporated into models of the human mind in order to form a more complete concept of that still-amorphous entity (page 66).

As with any emerging field, sleep science raises its share of controversial questions: Is depriving an organism of sleep the best way to ferret out sleep's function? Is it even ethical to deprive people of sleep in clinical studies, given the myriad problems that such disruption seems to evoke? How strong is the evidence that sleep plays a role in memory consolidation or learning?

There is broad concurrence on this last point, though some sleep scientists challenge the notion that sleep evolved as a means of cementing long-term memories (page 28). The epic poet Homer declared in the *Odyssey*, "There is a time for many words, and there is also a time for sleep." In these pages are a very many insightful words from the front lines of science's voyage to the heart of sleep's terra incognita. Enjoy them all. Then, given the evidence (page 15) that sleep *can* aid your memory and hone your learning, maybe take a nap when you're done. ■

Bob Grant
Senior Editor
Special Issue Coordinator

Speaking of Science

Look, sleep is the most demanding master there is; no one can stop it when it has chosen to arrive. We were the first to recognize the sheer economic might of sleep.

—**Jamaal Bhai**, a Delhi entrepreneur who rents quilts to homeless laborers in the sprawling metropolis, quoted in a new documentary about the practice called *Cities of Sleep* (*The New York Times*, January 4)

If sleeping and dreaming do not perform vital biological functions, then they must represent nature's most stupid blunder and most colossal waste of time.

—Jungian analyst and psychiatrist **Anthony Stevens**, in his 1995 book *Private Myths: Dreams and Dreaming*

DOWN TIME: Human beings spend a third their lives sleeping, on average. That means for a person who lives 71 years, more than 23 of those are spent abed. For all the time we spend sleeping, the function of the phenomenon remains largely unknown to science.

If the association between microcephaly and Zika virus is confirmed, there will be an ethical imperative to protect women of childbearing age from contracting the infection. The public will demand well-funded, proactive leadership from the World Health Organization.

—**Lawrence Gostin**, public health and law expert at the O'Neill Institute for National and Global Health Law at Georgetown University, speaking about the decision by World Health Organization Director-General Margaret Chan to convene an emergency meeting on the emerging epidemic of the Zika virus in the Americas (January 28)

Dengue viruses, which are also transmitted by these two mosquito species, caused 2.3 million cases of dengue fever and far more serious dengue hemorrhagic fever in 2013 in the same countries in the Americas that have been, or will be, affected by Zika. These included more than 37,000 severe illnesses and 1,300 deaths. And yet these numbers hardly raised an eyebrow in the United States.

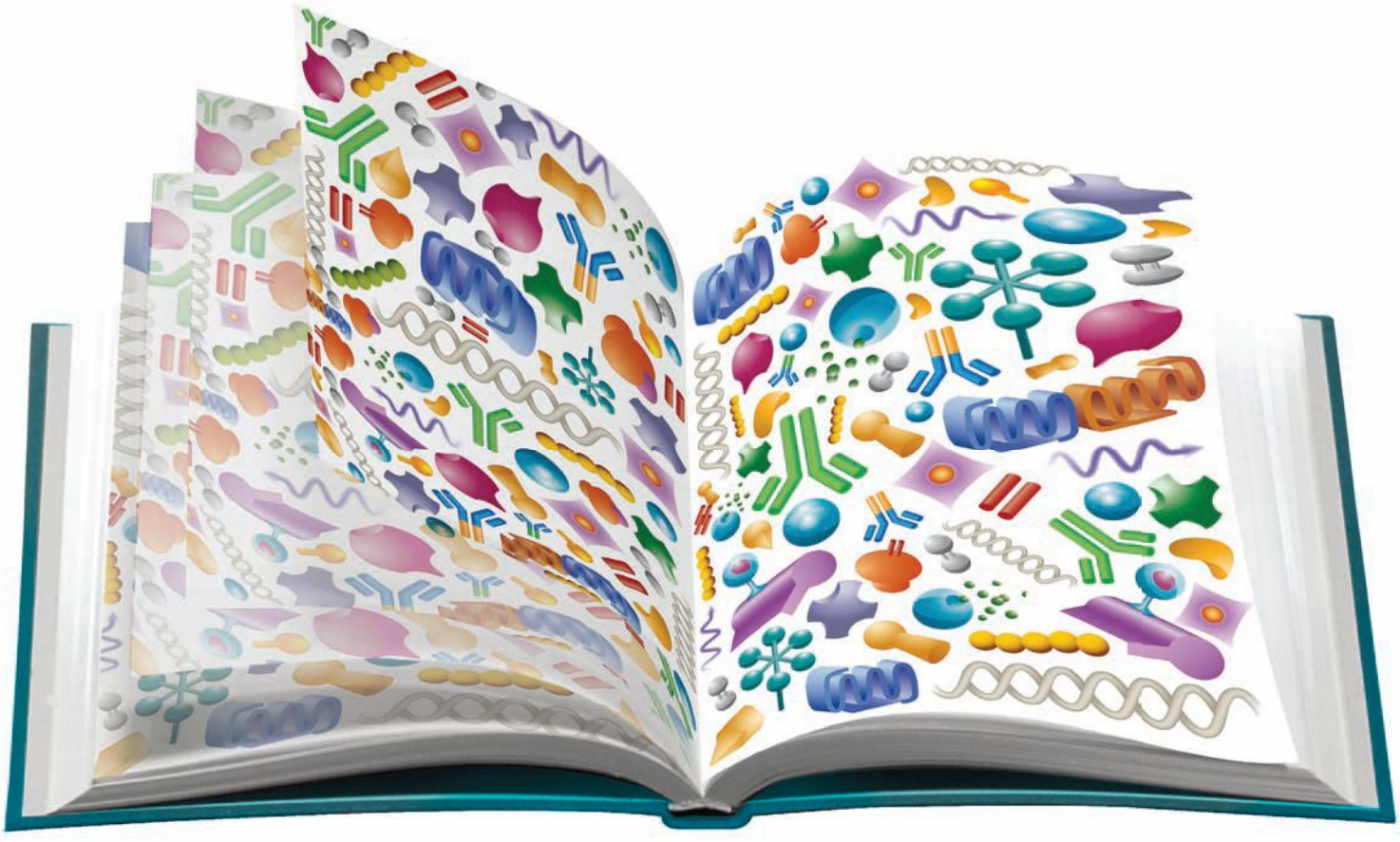
—**Michael Osterholm**, director of the Center for Infectious Disease Research and Policy at the University of Minnesota (*The New York Times Sunday Review*, January 28)

Not only is a discrimination-free environment the right setting for all people, it also fosters important learning, mentoring and research that are imperative to the advancement of science.

—**The US National Science Foundation**, in a recent announcement affirming its commitment to "preventing harassment and to eradicate gender-based discrimination in science." (January 25)

There is concern among some front-line researchers that the system will be taken over by what some researchers have characterized as "research parasites."

—**Dan Longo** and **Jeffrey Drazen**, editors at the *New England Journal of Medicine*, from an editorial on the dangers of data sharing (January 21)



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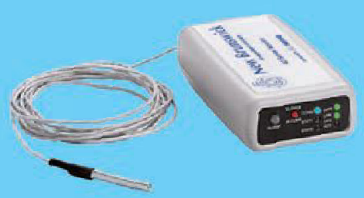

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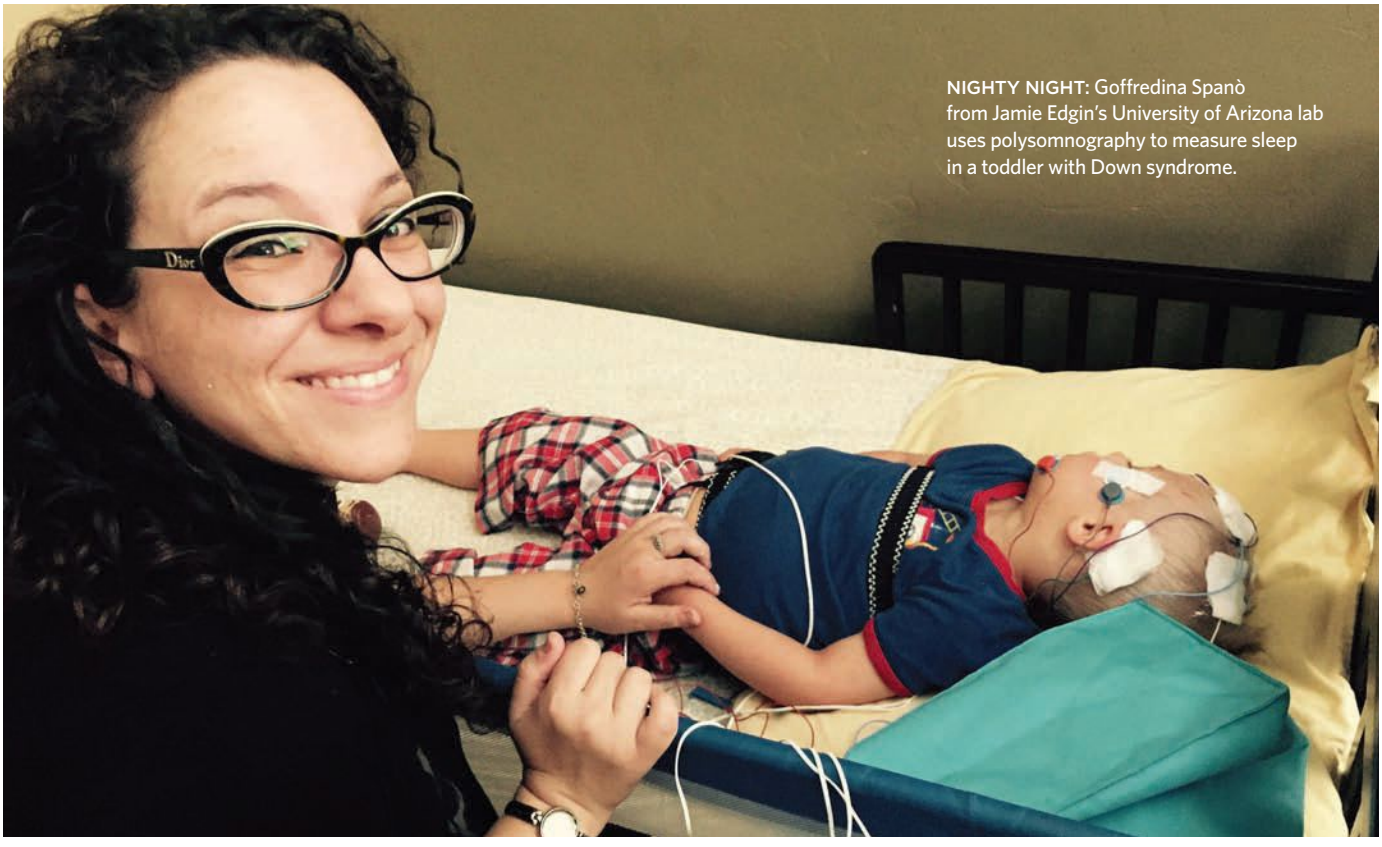


NIGHTY NIGHT: Goffredina Spanò from Jamie Edgin's University of Arizona lab uses polysomnography to measure sleep in a toddler with Down syndrome.

PAMELA SPEDER

Learning with the Lights Out

By the early 2000s, scientists had found that sleep helps young adults consolidate memory by reinforcing and filing away daytime experiences. But the older adults that Rebecca Spencer was studying at the University of Massachusetts Amherst didn't seem to experience the same benefit. Spencer wondered if developmental stage altered the relationship between sleep and memory, and chose nearby preschool children as subjects. She found that habitual nappers benefitted the most from daytime rest, largely because their memories decayed the most without a nap. "By staying awake, they have more interference from daytime experiences," Spencer explains.

Until recently, most of the research into the relationship between memory and sleep has been conducted using young adults or animal models. These studies have suggested that dampened sensory inputs during sleep allow the brain to replay the day's events during a period relatively free of distracting information, helping to solidify connections and transfer daytime hippocampal memories into long-term storage in the cortex. But how sleep and memory interact at different ages has been an open question.

In children younger than 18 months, learning is thought to occur in the cortex because the hippocampus isn't yet fully developed. As a result, researchers hypothesize that infants don't replay memories during sleep, the way adults do. Instead, sleep merely seems to prevent infants from forgetting as much as they would if they were awake. "The net effect is that sleep permits infants to retain more of the redundant details of a learning experience," says experimental psychologist Rebecca Gómez of the University of Arizona. By the time they are two years old, "we think that children have the brain development that supports an active process of consolidation," she adds.

At that age, adequate nighttime sleep becomes critical for learning. Toddlers who sleep less than 10 hours display lasting cognitive deficits, even if they catch up on sleep later in their development (*Sleep*, 30:1213-19, 2007). The effects are particularly strong in children with developmental disorders, who often suffer from sleep dis-

ruptions. "Kids with Down syndrome that are sleep-impaired look like they have very large differences in language," says Jamie Edgin of the University of Arizona who studies sleep and cognition in such children. When comparing Down syndrome children who are sleep deprived with those who sleep normally, she has observed a vocabulary difference of more than 190 words on language tests, even after controlling for behavioral differences.

Understanding the impact of sleep on memory could also help another at-risk group of learners at the other end of the age spectrum. Previous research has suggested that older adults don't remember newly acquired motor skills as well as young adults do, perhaps because the posttraining stages of the learning process appear diminished. But neuroscientist Maria Korman and her colleagues at the University of Haifa in Israel recently demonstrated that a nap soon after learning can allow the elderly to retain procedural memories just as well as younger people (*Neurosci Lett*, 606:173-76, 2015). Korman hypothesizes that by shortening the interval between learning and consolidation, the nap prevents intervening experiences from weakening the memory before it solidifies. Overnight sleep might be even better, if the motor skills—in this case a complex sequence of finger and thumb movements on the nondominant hand—are taught late enough in the day, something she is testing now.

Optimizing the timing of sleep and training in the elderly takes advantage of something Korman sees as a positive side of growing old. "As we age, our neural system becomes more aware of the relevance of the task," Korman says. Unlike young adults who solidify all the information they acquire throughout the day, older people consolidate "those experiences that were tagged by the brain as very important."

Tests for older adults' memory acuity are generating new findings about the relationship between sleep and memory at other ages as well. After learning at a conference about a memory test for cognitive impairment and dementia in older adults, neuroscientist Jeanne Duffy of Brigham and Women's Hospital in Boston wondered if sleep could help strengthen the connection between names and faces. She and her colleagues found that young adults who slept overnight after learning a list of 20 names and faces showed a 12 percent increase in retention when tested 12 hours later compared with subjects who didn't sleep between training and testing (*Neurobiol Learn Mem*, 126:31-38, 2015). The findings have "an immediate real-world application," Duffy says, as they address a common memory concern among people of all ages.

How sleep and memory interact at different ages has been an open question.

A poll by the National Sleep Foundation found that adolescents have a deficit of nearly two hours of sleep per night during the school week compared with the weekend, suggesting the potential for serious learning impairments, according to Jared Saletin, a postdoctoral sleep researcher at Brown University. In fact, one study found that restricting 13- to 17-year-olds to six and a half hours of sleep a night for five nights reduced the information they absorbed in a school-like setting (*J Adolesc Health*, 47:523-25, 2010). However, other studies have suggested that four nights of just five hours of sleep didn't impair 14- to 16-year-olds' performance on tests of skills and vocabulary (*Sleep Med*, 12:170-78, 2011). A lack of consistency in study design and the ages of the subjects makes these conflicting results difficult to interpret, Gómez writes in a review, and much remains to be discovered about the true impact of sleep deficits on teenagers' learning (*Trans Issues in Psych Sci*, 1:116-25, 2015).

Developing a fuller picture of what happens to memories during sleep—and how best to tweak sleep habits to aid the recall process—could benefit some of society's most sleep-deprived members of every age. "We need to understand this role of sleep in memory because there is such potential for intervention," Spencer says. "Now that we have a well-founded concept of what sleep can do for memory, it's time to put it to the test."

—Jenny Rood

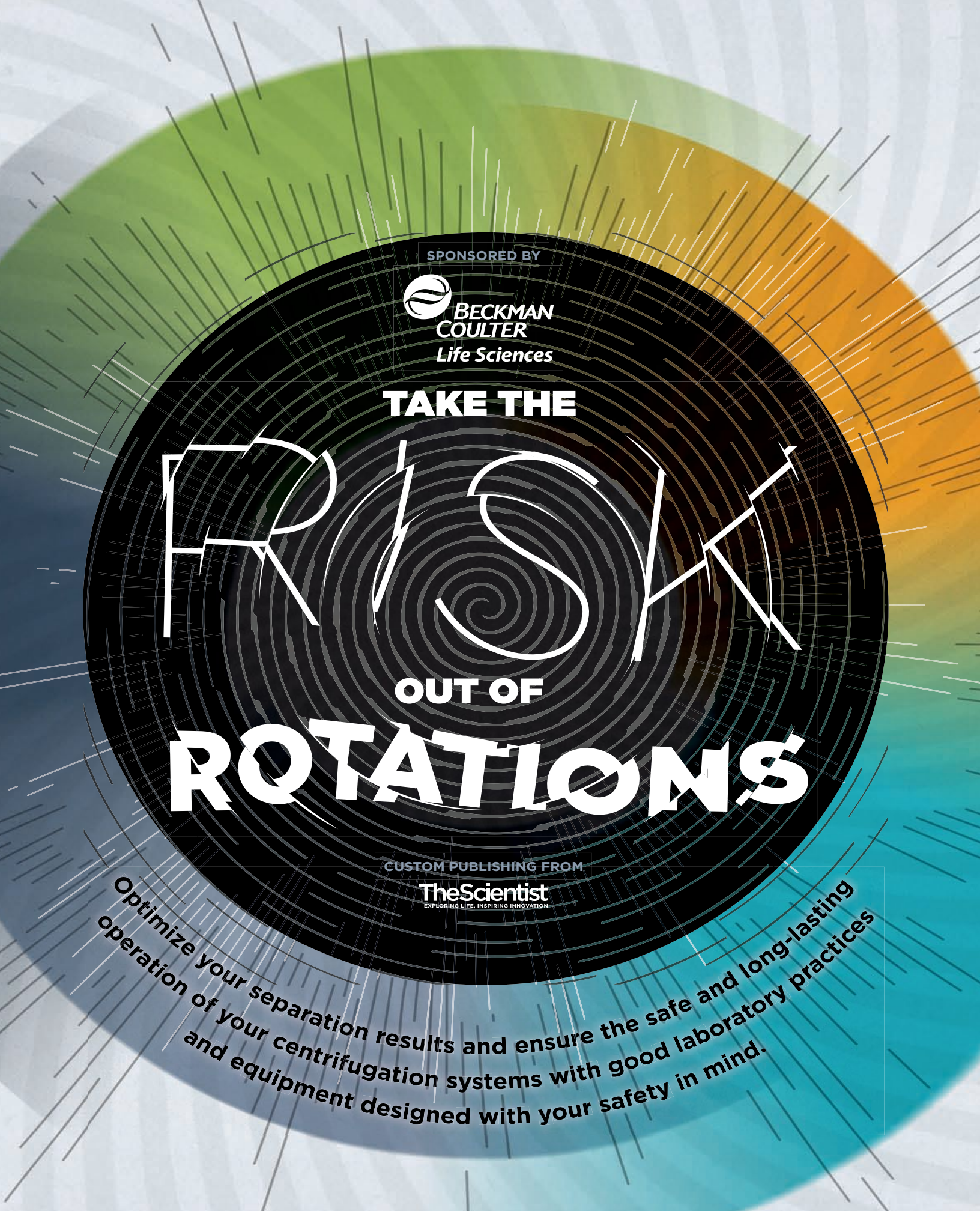
Perchance to Dream

Prefrontal leucotomies—surgeries to cut a section of white matter in the front of the brain, thus severing the frontal lobe's connections to other brain regions—were all the rage through the 1950s as treatments for psychoses. The operations drastically altered the mental state of most patients. But along with personality changes, dulled initiative, and reduced imagination came a seemingly innocuous effect of many of these procedures: the patients stopped dreaming.

Mark Solms, a neuropsychologist at the University of Cape Town in South Africa, uncovered the correlation in historical data from around the globe as part of a long-term study to assess the impact, on dreams and dreaming, of damage to different parts of the brain. Between 1985 and 1995, Solms interviewed 332 of his own patients at hospitals in Johannesburg and London who had various types of brain trauma, asking them about their nightly experiences.

Solms identified two brain regions that appeared critical for the experience of dreaming. The first was at the junction of the parietal, temporal, and occipital lobes—a cortical area that supports spatial cognition and mental imagery. The second was the ventromesial quadrant of the frontal lobes, a lump of white matter commonly associated with goal-seeking behavior that links the limbic structures to the frontal cortex. "This lesion site rang a historical bell in my mind—that's where the prefrontal leucotomy used to be done," says Solms, adding that the operation controlled the hallucinations and delusions that came with psychosis. "That sort of struck me as, 'Gosh, that's what dreaming is.'" Lesions in other areas could intensify or reduce certain aspects of dreams, but damage to either of the regions Solms pinpointed reportedly caused dreaming to cease completely (*Psychoanal Q*, 64:43-67, 1995).

Advances in neuroimaging have lent more support to Solms's brain map, and pinned down other areas that researchers now understand play a part in dream devel-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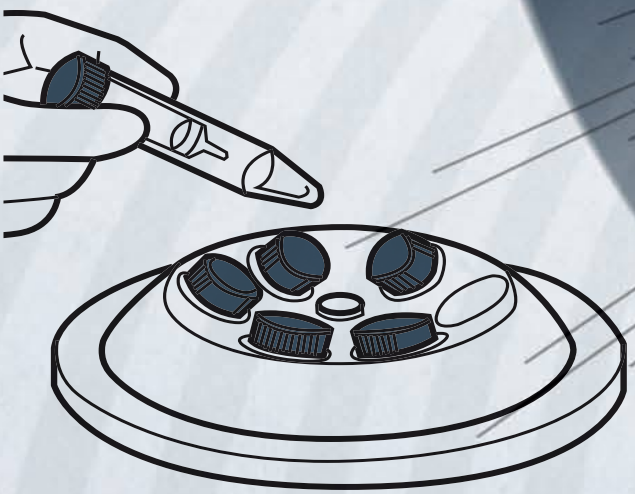

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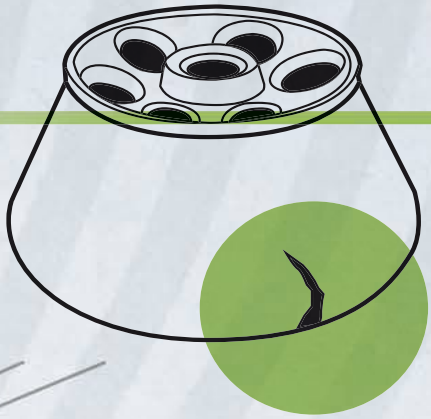

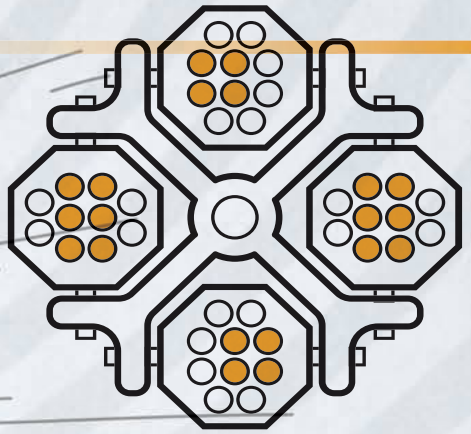

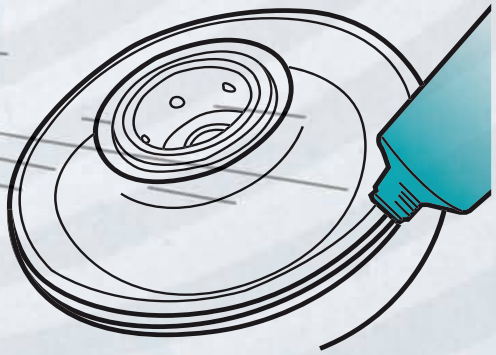

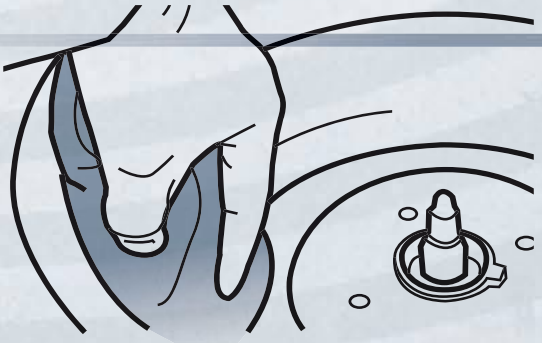

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Inspect the rotor at least monthly for rough spots, cracks, pitting, white powder deposits or heavy discoloration. Regularly check the condition of O-rings and replace any that are worn or damaged. For heavy usage replace O-rings twice a year, otherwise replace as required.

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Wash rotors and rotor components, including O-rings, with a mild detergent immediately if they come into contact with salts or other harmful materials; do not allow these materials to dry on rotor surfaces. Lubricating rotors, rotor components and accessories thoroughly on a regular basis will help extend their useful life. Use silicone vacuum grease for O-rings and Spinkote lubricant (Beckman Coulter) for rotor metal threads and rotor drive pins.

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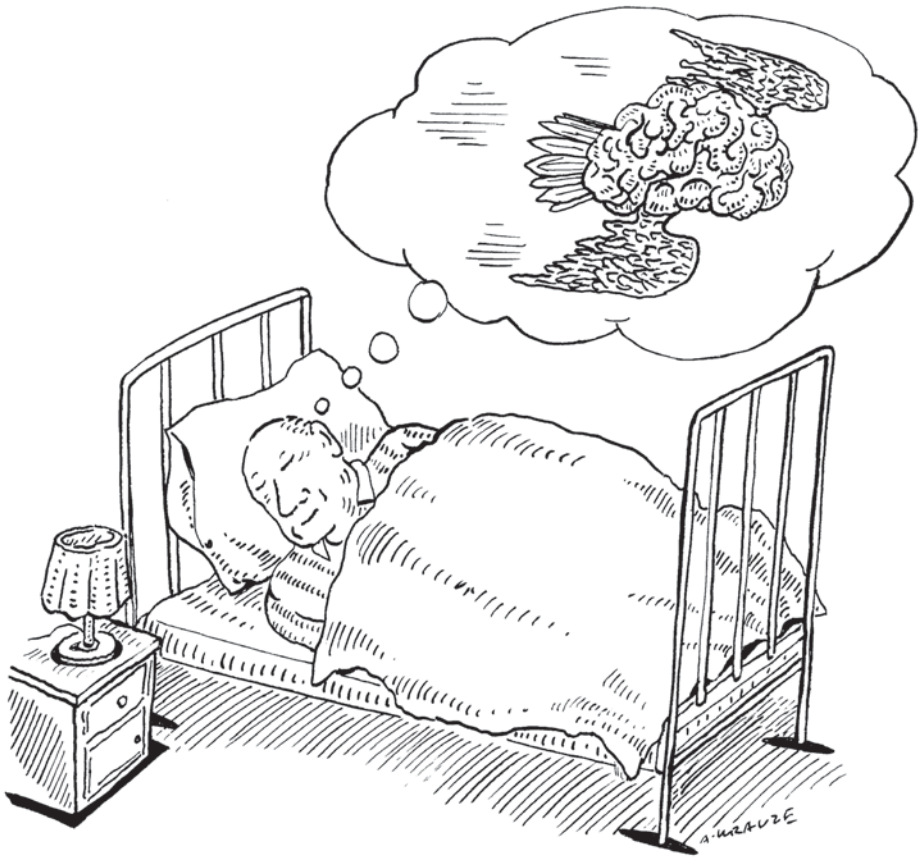
opment. In 2013, Bill Domhoff, a psychologist from the University of California, Santa Cruz, and colleagues from the University of British Columbia published results that combined neuroimaging scans from separate studies of REM sleep and daydreaming. They discovered that brain regions that light up when there's a high chance that one is dreaming overlapped with parts of the brain's default mode network—regions active when the brain is awake but not focused on a specific external task (*Front Hum Neurosci*, 7:412, 2013). "It very much lines up," says Domhoff. "It's just stunning."

The default mode network allows us to turn our attention inward, and dreaming is the extreme example, explains Jessica Andrews-Hanna, a cognitive scientist at the University of Colorado Boulder. The network takes up a large amount of cortical real estate. Key players are regions on the midline of the brain that support memories and future planning; these brain sections connect to other areas affecting how we process social encounters and imagine other individuals' thoughts. "When people are sleeping—in particular, when they're dreaming—the default mode network actually stays very active," says Andrews-Hanna. With external stimuli largely cut off, the brain operates in a closed loop, and flights of fancy often ensue.

We usually take the bizarre nature of these experiences at face value. "Even in a completely crazy dream, we all think that it's normal," says Martin Dresler, a cognitive neuroscientist at Radboud University in the Netherlands. Dresler and many other researchers attribute this blasé acceptance to the deactivation of a brain region called the dorsolateral prefrontal cortex. When we sleep, the dorsolateral prefrontal cortex powers down, and higher executive control—which would normally flag a nonsensical concern, such as running late for a class when you haven't been in school for a decade, as unimportant —evaporates. "You have this overactive default mode network with no connectivity, with no communication with regions that are important for making sense of the thoughts," says Andrews-Hanna.

In healthy sleeping subjects, these executive functions can be unlocked in what's known as lucid dreaming, when the prefrontal cortex reactivates and sleepers gain awareness of and control over their imagined actions. A lucid dreamer can actually "direct" a dream as it unfolds, deciding to fly, for example, or turning a nightmarish monster into a docile pet.



ANDRZEJ KRAUZE

Records of lucid dreaming are limited to REM sleep, the sleep stage where the brain is most active. REM sleep normally induces paralysis to prevent people from acting out their dreams, but the eye muscles are exempt, and this gives skilled lucid dreamers a way to signal their lucidity to researchers.

Dresler's team is using this phenomenon as a tool to ask specific questions about dreams. Before trained lucid dreamers fall asleep in Dresler's lab, they agree to flick their eyes from left to right as soon as they realize within a dream that they're asleep. The dreamed movement causes their actual eyes to move in a similar way under their closed eyelids. Researchers mark this signal as the beginning of a lucid dream, and then track brain patterns associated with specific dreamed actions. Dreaming also occurs in non-REM sleep, but with the brain less active, the eye muscles won't respond to dream input—so there's no robust way to tell if lucid dreaming takes place.

When subjects achieved lucidity and consciously dreamed that they performed a predetermined hand movement, Dresler's research team observed activity in the sensorimotor cortex matching what would occur if the subjects actually moved their hands while awake (*Curr Biol*, 21:1833-37, 2011). "It's probably the case that, for most of what we are dreaming about, the very same machinery and the very same brain regions are active compared to wakefulness," says Dresler. "It's just that the motor execution is stopped at the spinal level."

Beyond sleep research, tracking lucid and normal dreaming offers an investigative model to study aspects of psychosis, according to some researchers. "These regions that are activated during lucid dreaming are typically impaired in patients with psychosis," explains Dresler. "Having insight into your non-normal mental state in dreaming shares neural correlates with having insights into your non-normal state of consciousness in psychosis." Dresler proposes training patients in early stages of psychosis to dream lucidly, in the hope that it might grant them some therapeutically relevant understanding of their illness.

While executive functions are impaired in many patients suffering from psychosis, their

default networks seem to be overactive, says Andrews-Hanna. But how much similarity exists between the brain states of dreaming and psychosis remains controversial. Domhoff emphasizes the unique nature of dreams. "They're not like schizophrenia, they're not like meditation, they're not like any kind of drug trip," he says. "They're an enactment of a scenario that is based upon various wishes and concerns."

Ultimately, says Solms, deciphering dreaming furthers the field's knowledge of what the brain does, as much as studies conducted during waking hours. "If you're a clinician, and you understand what the different parts of the brain do in relation to dreaming, then it's one of the things you can use as a road map for evaluating your patients."

—Karen Zusi

Slumber Numbers

Humans typically sleep between six and eight hours per night. Some bats doze for a whopping 15 or 20 hours. Large animals, such as giraffes and elephants, snooze less than four hours a day. What explains such diversity in sleep times across mammals? Although a concrete answer is still lacking, ideas abound about how and why sleep patterns evolved.

One popular hypothesis is that, because larger animals have to eat so much to maintain their big bodies, they don't have time to sleep a lot. "An elephant has to spend between 17 and 19 hours per day eating," says Suzana Herculano-Houzel of Brazil's Institute of Biomedical Sciences at the Federal University of Rio de Janeiro. "Because there's only 24 hours in a day, elephants and [other] very large animals sleep very little."

Another hypothesis links sleep duration with metabolic rate. In October 2013, Maiken Nedergaard of the University of Rochester Medical Center and her colleagues published evidence that sleep helps clear away metabolites generated in the brain during waking (*Science*, 342:373-77). After reading the study, Herculano-Houzel suspected that the greater density of neurons in the brains of small animals leads to a faster buildup of metabolites, causing the animals to fall asleep. "There are these hilarious stories [of] pygmy shrews: they start eating a cricket, [and] they fall asleep mid-lunch," she says. "I can't help but think that's really the price you pay for having such a dense concentration of neurons."

As brains evolved to be bigger, they became less dense, so "those sleep-inducing metabolites are going to take a little bit longer to accumulate," Herculano-Houzel reasons. Sure enough, she found a nice correlation between daily sleep duration and the ratio of cortical neuronal density to surface area across mammals (*Proc Biol Sci*, 282:20151853, 2015). "That actually tells a pretty compelling story," she says.

But when discussing variation in sleep habits across the animal kingdom, total time snoozing isn't the only important variable, notes Isabella Capellini of the University of Hull in the U.K. Another key difference in how animals sleep is whether they do it all at once, as humans usually do, or whether they nap on and off throughout the day. In 2008, Capellini and her colleagues showed that small mammals tended to sleep for less time in a given bout, but for more time overall, than large mammals (*Functional Ecology*, 22:847-53). This variation may also stem from differences in the animals' energy needs, the team suggested: because small animals have high metabolic rates, they may need to eat more frequently (even if the total amount of food a shrew consumes is far less than that ingested by an elephant), while large animals such as elephants, with slower metabolisms, can enjoy longer sleep bouts.

"We proposed that one advantage of becoming bigger over evolutionary time might be that you can also consolidate your sleep," says Capellini. "And the moment you consolidate your sleep, you can start getting rid of those transitional stages that bring your brain from active to sleep," which take time that is dedicated neither to the restorative stages of sleep nor to the productive stages of waking.

Ecological variables also play a role, Capellini notes. Predation risk, for example, may put pressure on prey animals to be awake and vigilant more often than beasts at the top of the food chain. Another possibility is that the presence of parasites may influence mammalian sleep patterns. Capellini and her colleagues found no link between predation risk and either total sleep time or daily sleep patterns in mammals, but they did find that higher risk of infection is associated with longer bouts of sleep, which boost immune function (*BMC Evol Biol*, 9:7, 2009). Animals in environments where parasites are common may have evolved longer sleep times to protect themselves, Capellini says. "We proposed the idea that sleep essentially allows the body to divert energy to expensive physiological processes like maintaining the immune system."

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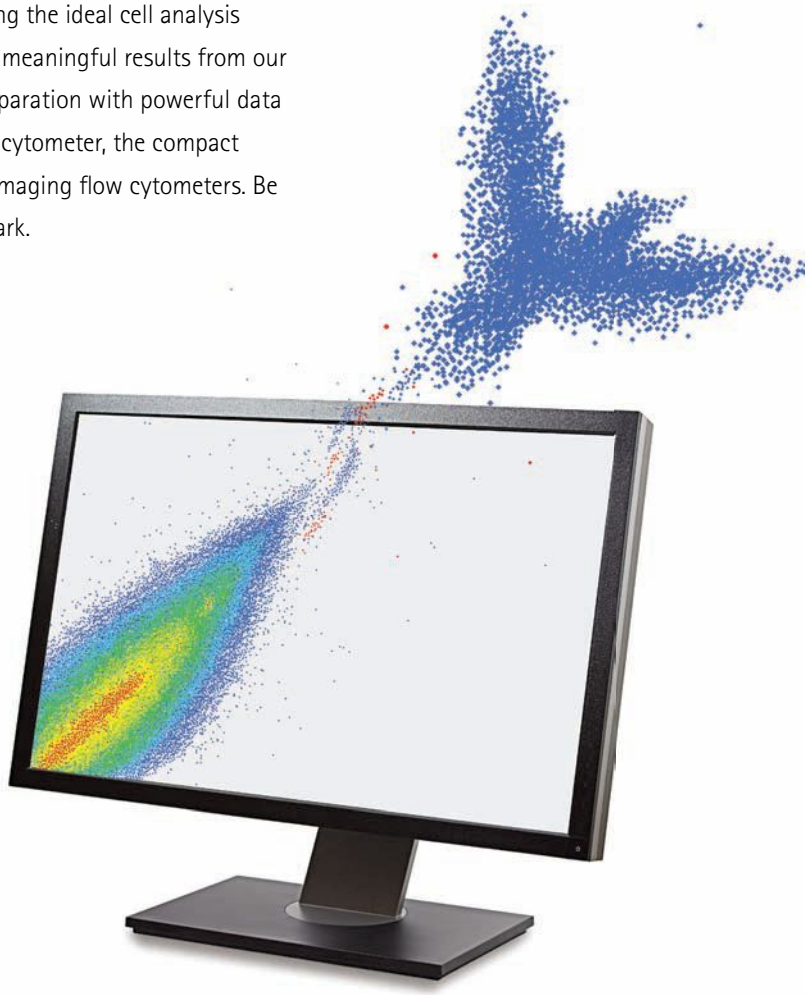

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Another open question about the evolution of sleep patterns is how humans, with our unusually large brains, differ from other primates, and why. Duke University's

What explains the diversity in sleep times across mammals?

Charles Nunn, who collaborated on Capellini's studies, and his postdoc David Samson recently culled data on sleep times among primates from an ecological informatics database and saw a surprising trend. "We looked at the data and realized, wow, humans are the shortest-sleeping primate," Nunn says. Accounting for variables such as body mass, metabolic rate, and brain size, the researchers built a model to predict what human sleep duration would be if the species were a typical primate—and got an answer of more than 10 hours per day, confirming the initial impression that humans are exceptionally short sleepers (*Evol Anthropol*, 24:225-37, 2015).

Nunn and Samson hypothesize that humans evolved shorter sleep times in conjunction with the transition from an arboreal lifestyle to life on the ground—where our ancestors needed to be more vigilant for predators and hostile conspecifics, and when "fire and other things enabled us to stay up late, past dark, and be effective," Nunn says. Less sleep would also have given early hominins more time to establish beneficial social relationships and to learn new skills and knowledge, though they would still have needed sleep to help them maintain that information. Supporting this hypothesis, Nunn and Samson also found that humans spend an unusually long time in rapid eye movement (REM) sleep, which is believed to reinforce learning and memories. "If our sleep is really shortened partially because of the benefits of social learning, you would expect humans to have a higher proportion of REM sleep, which is what we find," Nunn says. Essentially, humans sleep more "efficiently" than other primates, he and Samson suggest.

Herculano-Houzel thinks that mastering fire may have also helped human ancestors meet the increased energy demands of their bigger brains. "Cooking is actually a way of predigesting your food before you put it in your mouth," she says. "The fire breaks it apart, breaks molecules down, makes meat tender." Herculano-Houzel has calculated that, to accommodate a body of 70 kg and a brain of 86 billion neurons, a typical primate would have to spend 9.5 hours eating raw foods, and one study estimated that cooking reduced total time spent chewing from 4–7 hours a day to only an hour (*Evol Anthropol*, 19:187-99, 2010).

With less time spent eating, and less time spent sleeping, says Herculano-Houzel, we now have "more time available to do something more interesting with [our] day."

—Jef Akst

Sleeping for Two

There's an old wives' tale that the sleep problems that often beset pregnant women naturally prepare the mother for the extreme sleep disruption that arrives with the baby. Part of this appears to be true: sleep issues—whether it's waking up in the night, being unable to fall asleep, or more serious conditions such as sleep apnea—are indeed common complaints of expectant moms, especially as heartburn, back pain, and frequent trips to the toilet set in. But recent research is challenging the assumption that sleep woes represent an advantageous mechanism to gear up for subsequent sleepless nights.

David Gozal, who studies pediatric sleep disorders at the University of Chicago, says he was inspired to look into the effects of sleep disturbances in expectant women about a decade ago, when reports began to appear that pregnancy is accompanied by an increased risk for developing sleep perturbations. "People started talking about whether this could have effects on blood pressure and preeclampsia, things related to the pregnancy itself, but not to the fetus," says Gozal. "I'm a pediatrician, so I'm interested in what comes out of the pregnancy."

Yet no one had studied the consequences of a mom's sleep apnea—which involves microarousals that occur many times each hour because of snoring or other interruptions in normal breathing—for the baby, beyond immediate birth outcomes such as weight and mode of delivery. And for good reason: to do rigorous studies on children's long-term health and development is incredibly time-intensive, expensive, and logistically difficult. So Gozal turned to mice.

In one experiment, his team subjected rodents during the last third of pregnancy to something akin to sleep apnea—they were in a cage that shook every two minutes during the period when mice sleep. The mice slept just as much as control mice, but their sleep was fragmented, similar to that experienced by women with apnea. To isolate the effects of disturbed sleep per se, the researchers did not induce hypoxia, the low blood oxygen that is another important component of sleep apnea.

At first, Gozal and his team didn't notice anything different between pups born to mothers with fragmented sleep and those whose mothers slept normally. But after a couple of months, the offspring of the sleep-disrupted mice started eating more and gaining weight (*Diabetes*, 63:3230-41, 2014). "At 24 weeks. . . these mice were insulin resistant and had features of metabolic syndrome," says Gozal.

The mechanistic connection between sleep apnea in moms and metabolic changes in pups is still speculative. Gozal suggests a number of possibilities, including changes in the body's sensitivity to the satiety-related hormone leptin, which is altered during sleep loss. Or there may be changes in other maternal hormones, in the mother's microbiome, or in inflammation. In a subsequent study, the team compared methylation profiles in the DNA of visceral fat cells between control animals and mice born to sleep-fragmented mothers. The researchers found more than 2,000 differentially methylated regions, some of which are implicated in obesity and metabolic syndrome (*International Journal of Obesity*, 39:1135-42, 2015). The significance of these epigenetic

DON'T WAKE MOMMY: Do unborn babies suffer the lasting effects of their mothers' sleep problems?

changes is not yet clear, but they raise the possibility of long-term repercussions of sleep disorders during pregnancy.

"Certainly, it's very plausible," says Louise O'Brien, who studies sleep during pregnancy at the University of Michigan (she trained with Gozal as a postdoc, but was not involved in his work on the mice). "It's been known for decades that what happens in utero can affect the long-term health of the baby." Observations of Dutch women who were pregnant during the food rationing of World War II and their children demonstrated that the mother's well-being, in this case her nutrition, could affect offspring over a lifetime. Sons born to women who experienced famine during the first half of pregnancy were more likely to become obese later in life, for instance (*NEJM*, 295:349-353, 1976). "Do we know that in terms of sleep apnea? We don't, but it's a very plausible mechanism," says O'Brien.

Other problems with sleep are linked with untoward pregnancy outcomes in women. O'Brien and her colleagues have shown that snoring brought on by pregnancy is tied to a greater likelihood of emergency cesarean sections, and babies whose moms snored before and during pregnancy were smaller than babies of nonsnoring moms (*Sleep*, 36:1625-32, 2013). O'Brien is now looking through those babies' growth charts to see whether maternal snoring is linked with long-lasting effects on the children, and whether their genomes also display different methylation patterns.

"Snoring is not just an annoying problem," says Bilgay Izci-Balsarak, who studies pregnancy and sleep at the University of Illinois Chicago College of Nursing. "The consequences of sleep disturbance during pregnancy can be very serious." She cites a study reporting that women who slept just six hours a night during late pregnancy underwent four times as many C-sections as women who slept longer. Sleep disturbances also correlate with gestational diabetes, and even sleep position may make a difference. O'Brien and colleagues, in addition to two other independent teams working different parts of the world, have found that women who reported sleeping on their backs during pregnancy were much more likely to have a small or stillborn baby (*International Journal of Gynecology & Obstetrics*, 121:261-65, 2013).

Izci-Balsarak says a number of factors predispose pregnant women to sleep disturbances. Body temperature is increased, "which isn't good for sleep quality," she says, and estrogen levels rise, which can cause nasal congestion and snoring. Iron deficiency—common as women's bodies ramp up their blood volume to supply the developing fetus with oxygen—may lead to restless legs syndrome.

Despite these warning signs, few clinicians screen pregnant women for sleep troubles, says O'Brien. "I think historically health-care providers said, 'Oh, it's preparing you for the baby.' Is this really what [sleep problems are] doing? I think we have to be very careful about pooh-poohing it and saying, 'Oh, it's normal.'" —Kerry Grens

What Lies Sleeping

Why hasn't science defined this most basic biological process?

BY PHILIPPE MOURRAIN

Sleep researchers must grapple with a major conundrum: we don't really know what sleep is. This may come as a shock to the uninitiated, but a conclusive definition of sleep still eludes scientists and probably will continue to do so until the function of sleep is fully established. That's not to say science doesn't have a working definition of sleep.

A cortical definition of sleep

Researchers have used electrophysiology to characterize sleep since the middle of the 20th century. In animals with a developed neocortex, including mammals and birds, sleep states manifest as telltale patterns of brain activity, which can be detected by electroencephalography (EEG), along with distinct eye movements and changes in muscle tone. Rapid eye movement (REM) sleep and non-REM sleep are differentiated by behavioral attributes and characteristic patterns of electrical activity in the cortex. But it is impossible to distinguish REM sleep from the waking state based on EEG alone, so REM sleep is also known as paradoxical sleep (REM/PS).

Using this electrophysiological working definition of sleep, researchers have made major progress in deciphering the mechanisms regulating sleep and wake states, and it is still the most precise method to quantify sleep in animals that possess a neocortex. This definition is so well-entrenched in the sleep research community that many of us have forgotten how deceptive and restrictive it can be. First, EEG only records signals from the neocortex, which covers and hides the rest of the brain, where the actual neuronal loci of sleep generators and regulators reside. Second, because EEG alone cannot distinguish REM/PS sleep from the awake state, it's clear that recording the surface of the brain is not enough to define sleep.

A behavioral definition of sleep

In the early 1980s, Irene Tobler of the University of Zurich established a broader definition of sleep applicable to all animals, from cockroaches to elephants, using six behavioral criteria: reduced mobility, site preference (e.g., bed), specific posture (e.g., lying), rapid reversibility (unlike coma), and, most importantly, increased arousal threshold (no perception of the environment) and homeostatic control (sleep rebound after sleep deprivation). As of today, using those six criteria, researchers have identified sleep in hundreds of vertebrates and invertebrates, and there is currently no clear evidence of any animal species that does not sleep.

Furthermore, Tobler's behavioral criteria have allowed the recent introduction of well-established (and neocortex-lacking) genetic models such as *Drosophila*, *C. elegans,* and zebrafish. Still, behavioral criteria are limited because they do not allow the quantification of sleep as do EEG and muscle measurements. And so these sleep studies mostly rely on assessments of movement. Determining whether a fruit fly is asleep, for example, requires physically disturbing it and likely waking it up in the process. Only a brain recording–based definition of sleep can allow proper quantification without disrupting an animal's natural behavior.

Toward a subcortical definition of sleep

Here is the paradox: although it seems sleep is conserved across the animal kingdom, our most precise definition of the phenomenon relies on recordings of the neocortex, the least-conserved part of the vertebrate nervous system (and altogether absent in invertebrates). Using electrophysiological and behavioral criteria, major progress has been made in deciphering the mechanisms regulating sleep and awake states in vertebrates. Researchers have pinpointed brain nuclei, circuits, and neurotransmitters involved in sleep-wake regulation, and they've identified centers of state-switching in the brain

stem and hypothalamus, structures conserved across all vertebrates.

Instead of relying on the neocortical output of sleep, a definition of sleep should emerge across vertebrates based on the activity of the subcortical structures generating and regulating sleep. A region of primary interest is the brainstem (a.k.a. hindbrain), as it contains regions critical for sleep induction (pons), arousal (locus coeruleus, parabranchial nucleus, and others), and muscle-tone control. Decades of developmental biology studies using many vertebrate models, including mouse, chicken, quail, frog, and zebrafish, have demonstrated the strong conservation of the neural segments (rhombomeres) and circuits composing the vertebrate hindbrain/brainstem. With the development of whole-brain imaging methods allowing the comparison of two distant vertebrate sleep models, such as mouse and zebrafish, we should soon be able to define sleep for all vertebrates based on the dynamics of conserved networks in deeper brain regions.

Toward a synaptic and functional definition of sleep

While a subcortical definition of sleep across vertebrates would already be a leap forward, it would still not easily apply to the invertebrate nervous system, and hence would not provide a truly universal definition of sleep for all animals. Evolutionary conservation lends support to the hypothesis that sleep serves a fundamental physiological need, and the best way to define sleep would be to identify this need. So instead of relying on an output (EEG, behavior) or a regulatory (neuronal circuits) definition of sleep, the ultimate definition will directly answer the simple question: why do we sleep?

Sleep likely has multiple functions, but a large and growing body of evidence supports a primary role for sleep in the regulation of nervous system plasticity. Sleep deprivation impairs performance on motor and cognitive tasks, and sleep strengthens cognitive functions, memory consolidation, and learning.

Importantly, work in mammals, nonmammalian vertebrates (zebrafish), and invertebrate models has highlighted the importance of sleep for synaptic remodeling. In view of these commonalities, sleep might be compared to a neurodevelopmental state: a functional state that has been preserved from simple circuits to neocortical complex networks. In this hypothesis, the sleep state(s) allows critical plasticity mechanisms to be brought online to facilitate the making and breaking of connections within neural circuits that, during the unpredictable synaptic environment of the wake state, could disrupt behavior or learning. With the experimental knowledge gathered to date, it is safe to acknowledge that sleep, on a synaptic level, is a specific type of plastic state likely conserved across circuits, developmental stages, and evolution. This critical state is not only important for the proper function of the nervous system, but is itself dependent on the prior activity and connectivity of the nervous system.

In the years to come, experiments focusing on the synaptic changes specific to sleep across the entire brain should uncover the core ancestral function of sleep and establish a definition that not only could apply to any species but also to most types of neuronal networks requiring enhanced plasticity. (See "Sleep's Kernel," page 35.)

It will also be interesting to see whether we can identify neocortical REM/PS and non-REM synaptic sleep signatures in nonneocortical plastic networks, allowing a molecular definition of non-REM sleep and REM/PS. It may sound provocative today because REM/PS is often associated with dreaming and is at the root of mammalian-centric conceptions of sleep, but REM/PS could have an ancestral form in nonmammalian and invertebrate species in its most reduced synaptic form. ■

Philippe Mourrain studies neurobiology and gene regulation at the Stanford Center for Sleep Sciences and Medicine.

Getting Animal Research Right

Regulatory and compliance expectations for animal-based research are demanding, while public and political scrutiny of animal research is rising.

BY ANDREW JEFCOAT

A new reality circumscribes animal-based research. Regulatory agencies and mandates pervade the scientific community, and animal-rights activist groups are becoming increasingly vocal about their objections to animal studies. In addition, the Internet and social media continue to influence public and political perception of such research, spreading unfiltered and/or selectively abridged information around the world in seconds. Life scientists who use animal models must recognize this new political and social climate, and continue to reduce and refine animal usage, while fostering positive public relations about their work.

The number-one priority for animal researchers must be maintaining regulatory compliance. A single instance of noncompliance can lead to fines, funding disruptions, and substantial negative publicity. The best way to ensure compliance is to take advantage of research animal veterinarians and Institutional Animal Care and Use Committees (IACUCs), which are well versed in the requirements. Veterinarians and IACUCs are charged with protecting animal well-being, but also function to support scientific advancement. They should be viewed as resources and not merely as regulators.

One thing veterinarians or IACUC members can help with is drafting the animal-use protocol, a contract describing every important aspect of an animal-based research project. The protocol is the protective document for a principal investigator and the institution should any questions about the work arise from a regulatory agency or outside group. All areas of an animal-use protocol are important, but key aspects that require special attention are humane endpoints, anesthesia and analgesia appropriate for the species and situation, justification of species used and number of animals required, and the balance of societal benefit versus potential harm to animals.

Once the project is underway, all activities performed using research animals must be documented. It is not enough to state in a protocol that all animals will receive analgesic medication after surgery; investigators must maintain a log of every time the analgesic is given. A statement of intent in a protocol is a promise; proof of keeping promises must exist in laboratory records.

And it's important to keep those promises. If an animal-use protocol describes administration of analgesic twice per day for five days, the analgesic must be given in that exact regimen. Methods stated in a protocol are not negotiable by laboratory members on a case-by-case basis. If there is legitimate reason to modify the experiment, a protocol amendment or IACUC-approved veterinary exemption to alter the regimen is required.

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Finally, beyond all the logistics of adhering to research regulations, investigators must make an effort to ensure that the importance of their research is made clear to the public. A future may one day exist when it will not be necessary to use animals in biomedical research, but that day is not here yet, regardless of opposing declarations. Animal models are still critical to understanding and treating human disease. All members of the lab must be able to quickly, accurately, and persuasively explain their work to a lay audience, and if the work is basic science, they must be sure to emphasize the translational benefit.

It is difficult enough for scientists to secure funding and produce biologically relevant and reproducible results. Complicating matters by courting trouble with regulatory agencies or public opinion is unwanted and unnecessary. Adhering to regulatory protocols and effectively communicating with the public about one's research can help an investigator stay focused on achieving scientific, professional, and personal goals while using animal models in the most appropriate and humane way. ■

Andrew Jefcoat is Senior Program Veterinarian at the School of Medicine and Public Health, University of Wisconsin–Madison.

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While You Were Sleeping

Assessing body position in addition to activity may improve monitoring of sleep-wake periods.

BY RUTH WILLIAMS

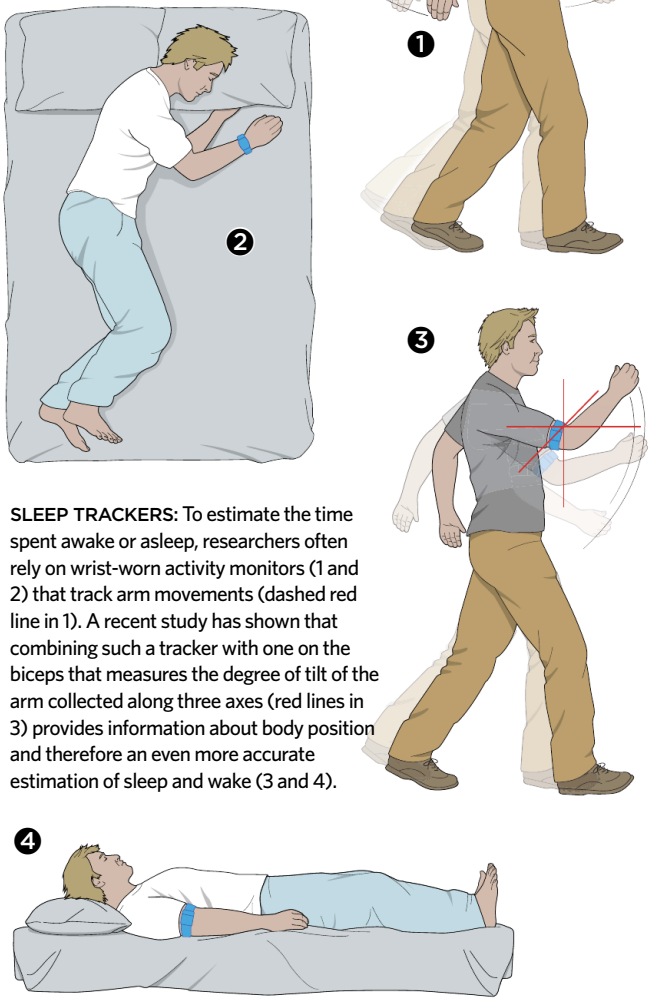
Polysomnography—the combined assessment of brain waves, heart rate, oxygen saturation, muscle activity, and other parameters—is the most precise way to track a person's sleeping patterns. However, the equipment required for such analyses is expensive, bulky, and disruptive to natural behavior.

By contrast, a wrist-worn activity monitor allows sleep-wake data to be gathered as a person goes about normal life—but, unsurprisingly, the information is far less accurate.

Researchers are thus searching for ways to improve the accuracy of wearable devices while maintaining user-friendliness. Maria Angeles Rol of the University of Murcia in Spain and her colleagues have now discovered that by using a device strapped to the patient's upper arm that measures both arm activity and position (the degree of tilt), they can more precisely detect periods of sleep. That is, when individuals wore both a wrist activity monitor and the upper-arm monitor for a period of seven days, data from the latter correlated better with the subjects' own sleep diaries.

In the study, the team also assessed the subjects' wrist temperature, which rises during sleep. These temperature measurements were also in good agreement with the subjects' own records of sleeping. Rather than replace one technique with another, however, Rol says, "we recommend combining them," because "the more information you get, the more precise the sleep detection."

The researchers studied just 13 people in this pilot study, says Barbara Galland of the University of Otago in New Zealand, but adds that nonetheless it "provide[s] an opening for further investigations to demonstrate the value of this novel technique." (*Chronobiol Int*, 32:701-10, 2015)



SLEEP TRACKERS: To estimate the time spent awake or asleep, researchers often rely on wrist-worn activity monitors (1 and 2) that track arm movements (dashed red line in 1). A recent study has shown that combining such a tracker with one on the biceps that measures the degree of tilt of the arm collected along three axes (red lines in 3) provides information about body position and therefore an even more accurate estimation of sleep and wake (3 and 4).

AT A GLANCE

SLEEP/WAKE MONITORING DEVICE	HOW IS DEVICE WORN?	BODY POSITION ASSESSMENT?	ACTIVITY MEASUREMENT?	SLEEP APPROXIMATION
Actiwatch, Cambridge Neurotechnology Ltd	On nondominant wrist, like a watch	No	Yes. Detects acceleration	In good agreement with subjects' diaries
HOBO Pendant G Data Logger, Onset Computer Corporation	On nondominant upper arm, held in place with a sports band	Yes. The device measures tilt on three axes.	Yes. The device takes a reading of tilt every 30 seconds, then calculates change in tilt to determine activity.	Device-detected sleep and subjects' diaries match more precisely.

The A B Zzzzs

The first stage of human sleep shows an electroencephalogram (EEG) pattern of high-amplitude waves in the cortex. This stage represents the deepest level of sleep and is characterized by slow and regular heart and respiration rates. Throughout slumber, we also experience periods of rapid-eye movement (REM) sleep, during which low-voltage waves of brain activity resembling those of the waking brain can be seen on an EEG. During REM sleep, which is when humans are known to dream most vividly, the eyes dart around rapidly and the body experiences numerous physiological changes, such as increased heart rate and irregular breathing. People will switch between REM and slow-wave, or non-REM, sleep throughout a normal night's sleep.

WAKING

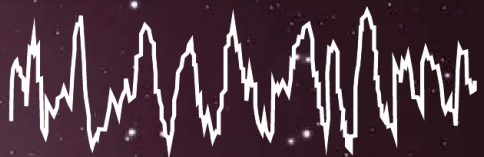
- Low-voltage, fast cortical EEG waves
- Controlled eye tracking
- Slow, regular respiration and heart rates
- Normal muscle tone

RAPID-EYE MOVEMENT (REM) SLEEP

- Low-voltage, fast cortical EEG waves
- Eye twitches
- Irregular respiration and heart rate and other physiological changes
- Very low or absent muscle tone

NON-REM SLEEP

- High-voltage, slow cortical EEG waves
- No eye movement
- Slow, regular respiration and heart rates
- Low muscle tone



SLEEP REGULATION

Sleep and waking are under both circadian and homeostatic regulation. Individuals of a species tend to sleep at about the same time each day, as dictated by their circadian clocks, but sleep deprivation can cause animals to sleep even during normal waking hours. Animals are harder to wake when they are recovering from sleep loss, and exhibit slow waves with higher-than-normal voltage during non-REM sleep and more activity of the eyes and body during REM sleep. These effects are known as sleep rebound.

CREDIT LINE

WHO SLEEPS?

Once believed to be unique to birds and mammals, sleep is found across the metazoan kingdom. Some animals, it seems, can't live without it, though no one knows exactly why.

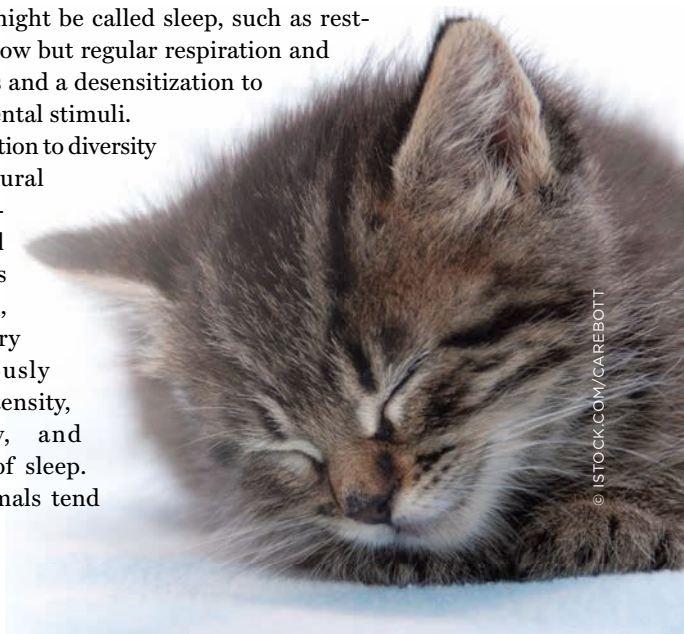
BY JEROME SIEGEL AND *THE SCIENTIST* STAFF

Some sleep researchers are fond of saying that all animals sleep; that sleep is maladaptive because it takes time away from activities that appear more adaptive, such as mating, seeking food, and looking out for predators; and that no one knows the function of sleep. A good case can be made that each of these statements is false.

To say whether an animal sleeps requires that we define sleep. A generally accepted definition is that sleep is a state of greatly reduced responsiveness and movement that is homeostatically regulated, meaning that when it is prevented for a period of time, the lost time is made up—an effect known as sleep rebound. Unfortunately, the application of this definition is sometimes difficult. Can an animal sleep while it is moving and responsive? How unresponsive does an animal have to be? How much of the lost sleep has to be made up for it to be considered homeostatically regulated? Is the brain activity that characterizes sleep in humans necessary and sufficient to define sleep in other animals?

Apart from mammals, birds are the only other animals known to engage in both slow-wave and rapid eye movement (REM) sleep. Slow-wave sleep, also called non-REM sleep, is characterized by slow, high-amplitude waves of electrical activity in the cortex and by slow, regular respiration and heart rate. During REM sleep, animals exhibit a waking-like pattern of cortical activity, as well as physiological changes including jerky eye twitches and increased variability of heart rate and respiration. (See "The A, B, Zzzzs" on page 27.) But many more animals, including some insects and fish, engage in behaviors that might be called sleep, such as resting with slow but regular respiration and heart rates and a desensitization to environmental stimuli.

In addition to diversity in the neural and physiological correlates of sleep, species vary tremendously in the intensity, frequency, and duration of sleep. Some animals tend



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to nap intermittently throughout the day, while others, including humans, tend to consolidate their sleep into a single, long slumber. The big brown bat is the current sleep champion, registering 20 hours per day; giraffes and elephants doze less than four hours daily. What can account for such differences in sleep times? While brain size and brain-body weight ratio do not strongly correlate with the total amount of sleep or with the amount of REM sleep (*Nature*, 437:1264-71, 2005), persuasive evidence now links food intake to sleep duration. Herbivores such as the elephant and the giraffe must eat and chew constantly because of the low caloric density of their food. Not surprisingly, they have evolved to sleep relatively little. Lions, on the other hand, sleep long—for 14 hours or more—and deeply, especially after they have consumed prey. (See "Slumber Numbers" on page 18.)

Breathing also greatly influences sleep time. Because dolphins, whales, and certain other marine mammals need to surface every few minutes to breathe, they cannot have slow waves in both hemispheres of their brain at once. Some marine mammals and birds are able to remain continuously active for weeks with no apparent sleep rebound that would suggest the animals even get sleepy. Although it's unclear if these animals experience unihemispheric slow waves during these periods of activity, I would argue that, if an animal is swimming, turning to avoid obstacles, and vocalizing, it can't be asleep.

For animals that do sleep for significant periods of time, one approach to determining the function of sleep is to keep them awake. But because extended sleep deprivation requires repeated awakening, which in humans triggers a surge of cortisol throughout the body, it becomes very difficult to separate the effects due to the loss of sleep from those due to the stress associated with repeated arousal. An additional issue is the effect of interference with task recall resulting from the additional period of learning during the deprivation procedure. A recent, extensive study showed that human sleep parameters were unrelated to memory consolidation on all tasks examined, undermining a vast literature on memory consolidation as a major function of sleep (*Sleep*, 38:951-59, 2015).

So why do animals sleep? It may be in part simply to conserve energy. At rest, an awake human brain uses 20 percent of the energy consumed by the body, even though it is only about 2 percent of body weight. If a safe sleeping site is found, or if you are a top predator that has no fear of other predators, there is a huge benefit to reducing that 20 percent cost, a reduction that occurs during sleep (*Nat Rev Neurosci*, 10:747-53, 2009). Recent work by my team with "preindustrial" humans, living under the conditions in which our species evolved, supports this idea that sleep helps organisms save energy, finding that these hunter-gatherers consistently sleep during the coldest portion of the night, rather than their sleep being tightly linked to the light level (*Curr Biol*, 25:2862-68, 2015). In this way, sleep is on a continuum with hibernation or torpor. By reducing activity and body temperature when the daily temperatures are lowest, substantial energy savings may be achieved.

So it's fair to say that not all animals sleep in the traditional sense, if they sleep at all (*Trends Neurosci*, 31:208-13, 2008). And while it is true that we don't know all the functions of sleep, recent research provides strong support for the hypothesis that energy conservation—a clearly adaptive function—is an important factor. As we continue to study sleep and sleep-like states across the animal kingdom, from insects to mammals, let us keep an open mind about this still-enigmatic phenomenon.

Jerome Siegel is a professor of psychiatry and biobehavioral sciences at the University of California, Los Angeles.

BIRDS

For the last 15 or so years, scientists around the world have discovered that birds, which are more closely related to reptiles than mammals, exhibit sleeping brain activity similar to that of people (and mice and manatees), engaging in both REM and non-REM sleep. "There must be something about being a bird and being a mammal that causes their brains to need the same kinds of sleep," says John Lesku, a lecturer at La Trobe University in Melbourne, Australia.

But there are also differences in the brain activity of sleeping mammals and birds, says Niels Rattenborg, who leads the avian sleep group at the Max Planck Institute for Ornithology in Seewiesen, Germany. Unlike mammalian REM sleep, which usually occurs in relatively few episodes that can span several minutes to an hour or more, avian REM sleep typically occurs in hundreds of brief spurts, each lasting just seconds (*Curr Biol*, 24:R12-R14, 2014).

While the functions of sleep in birds, as in mammals, have been hotly debated, Lesku and Rattenborg say the evidence implicating memory formation and storage is strong. "Several studies suggest that the brain rhythms we see during sleep are playing a role in processing information acquired during the previous day," says Rattenborg. For example, neurons involved in song production are active in the brains of sleeping juvenile songbirds that learn tunes from adult tutors while awake during the day (*J Neurophysiol*, 96:794-812, 2006). And researchers have speculated that slow-wave sleep promotes a reduction in synapse strength, weakening less-important

memories from the recent past, "so that, at the end of sleep, you're ready for a new day of learning," says Lesku.

Like many marine mammals, some birds are capable of unihemispheric sleep, in which one half of the brain remains alert while the other half exhibits electrical signals of slow-wave sleep. The eye connected to the alert half of the brain remains open, while the other typically closes. (REM sleep only occurs in both hemispheres at the same time.) Some bird species must get by on very little sleep altogether. Polygynous male pectoral sandpipers attempt to breed with as many females as possible during a 19-day breeding period, so they stay awake for about 95 percent of this time, Lesku says. "The sleep that they do have is packaged into hundreds of episodes just a few seconds long."

Other species, such as bar-tailed godwits, will fly nonstop for days or weeks on end to migrate thousands of miles as seasons change. It's not yet known whether migratory species sleep during flight, however. While no EEG recordings have been performed during flight to date, preliminary work has suggested that slow-wave sleep—even in both hemispheres at the same time—may be possible while on the wing. —Tracy Vence

A lot of things are conserved throughout evolution, so we can use the fly to really understand the genes and molecules that regulate sleep. —Mark Wu, Johns Hopkins University

INSECTS

In the late 1990s, Joan Hendricks of the University of Pennsylvania School of Veterinary Medicine spent hours sitting in a dark, hot room tapping petri dishes that housed *Drosophila*. Whenever the flies settled down, Hendricks would disturb their rest, which typically lasted for seven to eight hours each night if left undisturbed. But eventually the flies stopped responding to her taps, to the point that Hendricks was sure they must be dead. But they weren't dead; they were sleeping (*Neuron*, 25:129-38, 2000). "It was unbelievably compelling," she recalls. "I'm bothering these poor flies, they're clearly at risk by this giant perturbing their universe, and they fell asleep."

On the other side of the country, Paul Shaw, then a postdoc in Giulio Tononi's lab at the Neurosciences Institute in San Diego, was undertaking a very similar experiment. And just as Hendricks had observed, his flies exhibited a distinct sleep rebound—following deprivation, they fell asleep more easily and were harder to wake up (*Science*, 287:1834-37, 2000). "The behavior is just so obvious and so clear," says Shaw, now an associate professor at Washington University in St. Louis. He, too, concluded that the flies were sleeping.

The *Drosophila* experiments sent shock waves through the sleep research community, which at that time held that only birds and mammals slept. But Hendricks's and Shaw's results were not unprecedented. As early as the 1980s, researchers such as Walter Kaiser and Jana Steiner-Kaiser of the Technische Universität (then Hochschule) Darmstadt in Germany and Irene Tobler of the University of Zurich had documented sleep-like behavior in honeybees, cockroaches, and scorpions. While the *Drosophila* studies did meet with some resistance, it wasn't long before the field began to assume that most, if not all, animals sleep, and to recognize that many of the mechanisms regulating sleep-wake cycles are conserved from insects to humans. Even in those first studies, Hendricks's group found that drugs known to affect sleep in mammals had similar effects in *Drosophila,* and Shaw and his colleagues observed that several molecular markers known to fluctuate with mammalian sleep-wake states also cycle in flies.

In the last 15 years, research on invertebrate sleep has exploded, with *Drosophila* now serving as a powerful model system to probe the function of sleep. In insects, as in mammals and birds, most researchers believe sleep serves an important role in memory formation and retention. In 2011, Shaw's group found that

stimulating a group of neurons in the fly brain called the dorsal fan-shaped body could put flies to sleep, and in doing so induce the formation of a long-term memory (*Science*, 332:1571-76, 2011). And last year Shaw and colleagues were able to reverse memory deficits in a *Drosophila* strain that was learning-disabled, as well as in a fly model of Alzheimer's disease, simply by inducing sleep (*Curr Biol*, 25:1270-81, 2015). "Somehow sleep is able to fix the brain," Shaw says.

Research has also revealed the importance of sleep for learning and memory in honeybees, which must remember the locations of multiple food sources and communicate that information to their hive mates. Last year, Randolf Menzel of the Free University of Berlin and his colleagues presented sleeping honeybees with an odor cue they had experienced during awake learning, and this improved their memory performance after they woke up (*Curr Biol*, 25:2869-74, 2015). "It's a brilliant study," says Shaw. "Just like humans and rats—it's incredible."

Invertebrate sleep researchers are now probing the brain circuitry that regulates sleep-wake cycles: How do wake- and sleep-promoting neurons interact, and what causes an animal to fall asleep or awaken? Other avenues of research focus on how sleep affects brain plasticity and functions beyond memory, and on the molecular mechanisms at play in sleep regulation.

"We're screening thousands of [*Drosophila*] lines," says Amita Sehgal of the University of Pennsylvania Perelman School of Medicine. "[It's] a totally unbiased approach toward [identifying] what genes or proteins are required to maintain daily levels of sleep." And so far, the results have pointed to the same genes in flies and in people. "In the limited amount of human work that's been done, the same molecules are coming up," says Sehgal.

"A lot of things are conserved throughout evolution, so we can use the fly to really understand the genes and molecules that regulate sleep," agrees Johns Hopkins University's Mark Wu, who in 2014 characterized the function of *WIDE AWAKE* in flies and found that the gene has a mouse homolog whose expression is enriched in the suprachiasmatic nucleus, the master circadian pacemaker in the mammalian brain (*Neuron*, 82:151-66, 2014). "Flies do lots of things that are very similar to humans," Wu says. "Neurotransmitters are similar [and] do similar things. The neural circuits, although they look different at first glance, often do similar things."

And Shaw points out at least one way that insects may even be a better system for studying human sleep than rodents. "The mouse is like a narcoleptic human; they can't stay asleep," he says. "In some ways the fly looks more like us than the mouse does."

—Jef Akst

FISH

Despite anecdotes from scuba divers, fishermen, and aquarium owners, science knows very little about fish sleep. What is known, from less than a decade of work on zebrafish (*Danio rerio*), is that the fish sleep—from a behavioral perspective and, it's increasingly understood, on genetic and neural levels—in ways that are remarkably similar to mammals, birds, and other animals.

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In 2001, for example, Boston University researcher Irina Zhdanova (then at MIT) and her colleagues found that zebra-fish exhibit characteristic behaviors such as motionlessness, decreased sensitivity to sensory stimulation, and a sleep rebound response following rest deprivation (*Brain Research*, 903:263-68). Then, in 2010, Caltech neuroscientist David Prober (then at Harvard) and colleagues screened about 6,000 small molecules and found that the vast majority of those that were known to affect mammalian sleep had similar impacts on sleep in larval zebrafish (*Science*, 327:348-51). More recently, Prober and his labmates also found that melatonin is required for the circadian control of sleep in zebrafish, just as it is in humans and other mammals (*Neuron*, 85:1193-99, 2015).

"There are huge similarities in terms of gene networks, neural transmitter systems, ontogeny, and development of sleep," says Karl Karlsson, a Reykjavik University neuroscientist who studies sleep in zebrafish.

Scientists are now working to nail down more-definitive markers of sleep in the species, says Prober, but zebrafish are already proving to be excellent models of sleep in humans. "It doesn't even matter if it's 'sleep,'" he says. "The question is: 'Can we discover anything using the fish that's useful for humans?' We think the answer to that is yes."

One advantage of studying zebrafish is that, as with *Drosophila*, mice, and rats, their genome has been completely characterized. Zebrafish are also vertebrates (unlike fruit flies), diurnal (unlike mice and rats), and, as larvae, transparent. "It's a luxury to be able to look into a whole vertebrate," says Philippe Mourrain of Stanford University. "You can look at the vasculature. You can look at the cell migration. You can look at the creation and disappearance of synapses. You can look at the entire brain. It's a very powerful system for neuroscience."

Taking advantage of this transparency, Mourrain and his colleagues pioneered the documentation of sleep-dependent impacts on the accumulation and pruning of synapses in the zebrafish's brain. "We could look through different sleep/wake cycles directly at synapses and see if they emerge or disappear during those wake and sleep phases." Based on this work, Mourrain's group provided evidence that zebrafish sleep serves to prune away less-important synaptic connections forged during waking hours, a process that likely strengthens overall cognition (*Neuron*, 68:87-98, 2010). "We were the first ones to demonstrate that in zebrafish you have this kind of decrease during sleep of the synapses," he says.

And zebrafish are likely to continue providing clues about the largely mysterious phenomenon of sleep, Prober says. "It's still a relatively small field," he says. "There are many more people who work on rodents and flies than on fish, but the fish community is growing very rapidly as people are appreciating some of the advantages that it brings."

—Bob Grant

MARINE MAMMALS

Most marine mammals, unlike their terrestrial cousins, can enter a state of being literally half awake: while one hemisphere of the brain remains alert, the other produces the slow brain waves akin to those seen in terrestrial mammals during non-REM sleep.

John Lilly, director of the Communication Research Institute in the Virgin Islands in the 1960s, first suggested that dolphins sleep unihemispherically when he observed them closing one eye at a time while at rest either at the surface or on the bottom of their pools. In 1972, EEG recordings from a female pilot whale revealed that neural activity characteristic of slow-wave sleep alternated between the whale's brain hemispheres.

A few years later, researchers from the USSR observed that dolphins experienced wakefulness and intermediate states, hovering between waking and sleeping, in one or both hemispheres, but slow-wave sleep in only one hemisphere at a time. The animals could also swim slowly and surface to breathe without waking the sleeping hemisphere. When the researchers recorded dolphins' EEG patterns over 24-hour periods, they discovered that the average total sleep time for each hemisphere was approximately 4.5 hours per day, though individual sleep sessions lasted only about 40 minutes (*Neurophysiology*, 20:398-403, 1988).

Unihemispheric sleep may allow these marine mammals to stay alert for long periods of time without detrimental effects. In 2012, a team led by researchers from the National Marine Mammal Foundation in San Diego found that bottlenose dolphins can remain responsive continuously for periods of at least 15 days, swimming, echolocating, and reacting to stimuli at random intervals without any noticeable cognitive deterioration (*PLOS ONE*, 7:e47478, 2012). "It makes you wonder whether the unihemispheric slow wave that you see in dolphins really should be called 'sleep' at all," says Jerome Siegel of the University of California, Los Angeles, and the author of the introduction to this feature. But dolphins do show evidence of sleep rebound within each hemisphere when tracked with implanted electrodes: if the dolphin is periodically disturbed so as to consistently wake up one hemisphere, the deprived half of the brain will attempt to fall asleep more often and stay asleep longer (*J Sleep Res*, 1:40-44, 1992).

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For other marine mammals, researchers have turned up behavioral evidence suggesting they, too, sleep half a brain at a time. Siegel's group has observed captive killer whales and a captive gray whale calf at Sea World, both of which exhibited resting behaviors that resembled those of dolphins. "[The gray whale] seemed to open one eye at a time, so that is consistent with what we've seen in the killer whale and the dolphin," says Siegel. "In all likelihood, they have unihemispheric sleep, but nobody's actually measured."

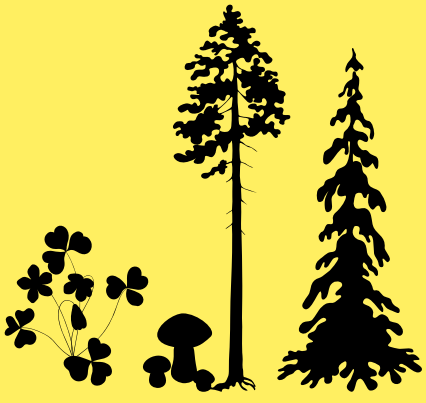
In the wild, tagging studies of sperm whales offer further clues. These animals take resting dives in which they remain still and silent under the water for up to 30 minutes, says Saana Isojunno, a fellow in the sea mammal research unit at Scotland's University of St Andrews. Sperm whales in a resting cycle surface to breathe for several minutes and then dive again, repeating the process as many as 10 times in a row unless they are disturbed by sounds such as killer whale vocalizations. But the whales take these dives only about 5 percent of the time during tagging studies, says Isojunno. "Resting behavior is rare."

Some semiaquatic marine mammals such as fur seals also display unihemispheric slow-wave sleep, but with a twist. While on land, fur seals experience REM sleep like other terrestrial mammals, but in the water, they transition to a unihemispheric sleep pattern in which they swim on their sides with the "awake" flipper paddling in the water to keep them afloat. And, like dolphins, fur seals don't seem to experience any negative effects from missing out on REM sleep.

"You bring them back up on land, and they just go back to the normal mode of land sleep," says Siegel. "They kind of break all the rules as to what we think of as normal in terms of sleep regulation."

—Karen Zusi

OUTSIDE THE ANIMAL KINGDOM



Nonanimal organisms such as plants, fungi, and bacteria do not have central nervous systems, which is how sleep manifests in animals. And many animals will continue to sleep, albeit irregularly, even if their circadian clocks are disrupted. For these reasons, most researchers would agree that plants and other nonanimals do not sleep in the traditional sense, but they do stick to a tight daily schedule, dictated by molecular clocks akin to those in animals.

As early as the 18th century, French astronomer Jean-Jacques d'Ortous de Mairan noted that mimosa plants continued to open their leaves each day and close them each night even when kept in total darkness. That was the first inkling that plants (and other organisms) keep time internally to anticipate environmental changes, rather than simply reacting to light in the environment.

Many activities fluctuate with a plant's circadian rhythms. Most species time their photosynthesis to peak at noon with the brightest sunlight, and undergo growth spurts at night, elongating their stems using the stores of starch accumulated during the day. Just before dawn, *Arabidopsis thaliana* mounts immune defenses, anticipating the arrival of downy mildew spores with the sunrise. Later in the day, the plant's immunity is dialed down, so it doesn't stunt overnight growth. (See "Holding Their Ground," *The Scientist,* February 2016.)

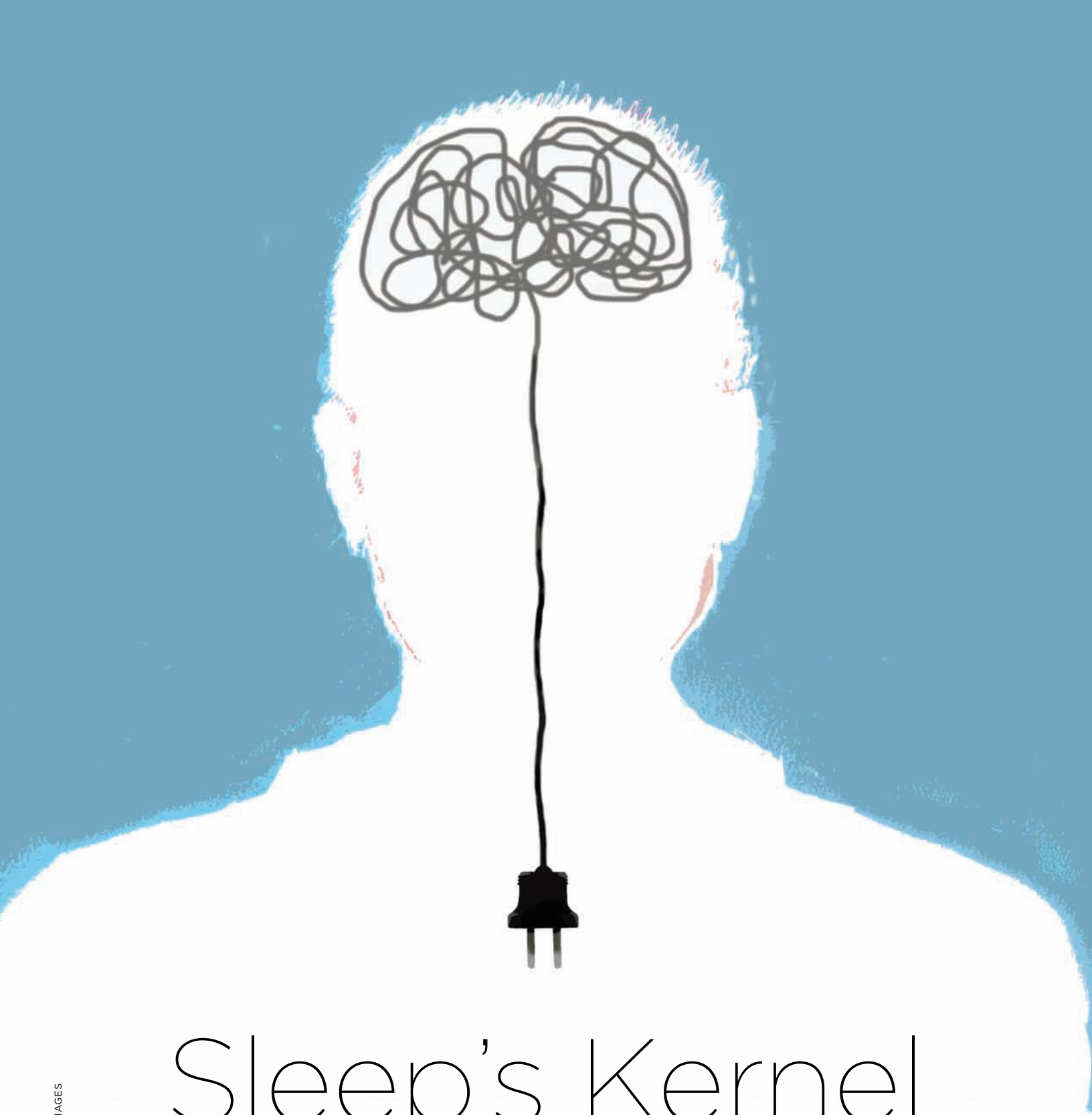
"We've got this beautiful data now in plants to show how clock proteins are involved in every major decision a plant has to make," says Steve Kay, a molecular geneticist at the Scripps Research Institute in La Jolla, California.

Examples of circadian rhythms exist in every kingdom of life. The green alga *Chlamydomonas reinhardtii* swims up toward the light during the day and toward nitrogen sources at night. Some fungi release spores in a daily cycle as temperature and humidity fluctuate. The salt-loving archaeon *Halobacterium salinarum* shows daily rhythms in gene expression, most likely to adjust its metabolism to the levels of oxygen dissolved in its environment. Some bacteria even maintain daily routines in metabolism (*PLOS ONE*, 4:e5485, 2009). Circadian biologists once thought that bacteria would never keep a 24-hour clock, as a bacterium may live less than a day. This assumption was incorrect, says Susan Golden, who studies circadian rhythms in cyanobacteria at the University of California, San Diego. "The great-grandchildren know what time great-grandma thought it was," and daily cycles emerge when observing populations of bacteria.

Importantly, all of these species maintain their circadian rhythms when subjected to constant darkness or light, suggesting that the clock is internally driven, just like sleep in animals. Indeed, sleep is simply an output of circadian timekeeping that animals evolved alongside other clock-driven states, such as body temperature and expression of metabolic genes.

"The circadian clock in general has evolved in biological organisms to allow organisms to deal with the perpetual changes in our environment," says Janet Braam, a plant biologist at Rice University. "Sleep is an output [of the clock] more specific to certain organisms, like certain animals."

—Kate Yandell



Sleep's Kernel

Surprisingly small sections of brain, and even neuronal and glial networks in a dish, display many electrical indicators of sleep.

BY JAMES M. KRUEGER AND SANDIP ROY

Sleep is usually considered a whole-brain phenomenon in which neuronal regulatory circuits impose sleep on the brain. This paradigm has its origins in the historically important work of Viennese neurologist Constantin von Economo, who found that people who suffered from brain infections that damaged the anterior hypothalamus slept less. The finding was a turning point in sleep research, as it suggested that sleep was a consequence of active processes within the brain. This stood in stark contrast to the ideas of renowned St. Petersburg physiologist Ivan Pavlov, who believed that sleep resulted from the passive withdrawal of sensory input. Although the withdrawal of sensory input remains recognized as playing a role in sleep initiation, there is now much evidence supporting the idea that neuronal and glial activity in the anterior hypothalamus leads to the inhibition of multiple excitatory neuronal networks that project widely throughout the brain.

But we also know from millions of stroke cases that cause brain damage and from experimentally induced brain damage in animal models that, regardless of where a lesion occurs in the brain, including the anterior hypothalamus, all humans or animals that survive the brain damage will continue to sleep. Further, a key question remains inadequately answered: How does the hypothalamus know to initiate sleep? Unless one believes in the separation of mind and brain, then, one must ask: What is telling the hypothalamus to initiate sleep? If an answer is found, it leads to: What is telling the structure that told the hypothalamus? This is what philosophers call an infinite regress, an unacceptable spiral of logic.

For these reasons, 25 years ago the late Ferenc Obál Jr. of A. Szent-Györgyi Medical University in Szeged, Hungary, and I (J.K.) began questioning the prevailing ideas of how sleep is regulated. The field needed answers to fundamental questions. What is the minimum amount of brain tissue required for sleep to manifest? Where is sleep located? What actually sleeps? Without knowing what sleeps or where sleep is, how can one talk with any degree of precision about sleep regulation or sleep function? A new paradigm was needed.

What is sleep?

There is no direct measure of sleep, and no single measure is always indicative of sleep. Quiescent behavior and muscle relaxation usually occur simultaneously with sleep but are also found in other circumstances, such as during meditation or watching a boring TV show. Sleep is thus defined in the clinic and in experimental animals using a combination of multiple parameters that typically correlate with sleep.

The primary tool for assessing sleep state in mammals and birds is the electroencephalogram (EEG). High-amplitude delta waves (0.5–4 Hz) are a defining characteristic of the deepest stage of non–rapid eye movement (non-REM) sleep. However, similar waves are evident in adolescents who hyperventilate for a few seconds while wide awake. Other measures used to characterize sleep include synchronization of electrical activity between EEG electrodes and the quantification of EEG delta wave amplitudes. Within specific sensory circuits, the cortical electrical responses induced by sensory stimulation (called evoked response potentials, or ERPs) are higher during sleep than during waking. And individual neurons in the cerebral cortex and thalamus display action potential burst-pause patterns of firing during sleep.

Regardless of where a lesion occurs in the brain, all humans or animals that survive the brain damage will continue to sleep.

Using such measures, researchers have shown that different parts of the mam-

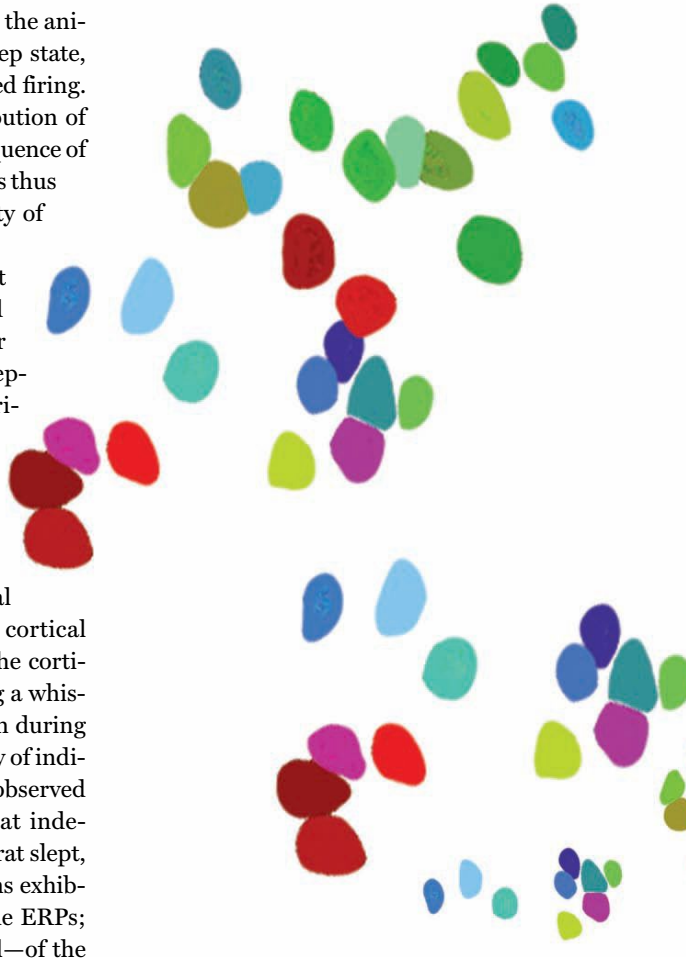
malian brain can sleep independently of one another. Well-characterized sleep regulatory substances, or somnogens, such as growth hormone releasing hormone (GHRH) and tumor necrosis factor α (TNF-α), can induce supranormal EEG delta waves during non-REM sleep in the specific half of the rat brain where the molecules were injected. Conversely, if endogenous TNF-α or GHRH production is inhibited, spontaneous EEG delta waves during non-REM sleep are lower on the side receiving the inhibitor. A more natural example of sleep lateralization is found in the normal unihemispheric sleep of some marine mammals. (See "Who Sleeps?" on page 28.)

Much smaller parts of the brain also exhibit sleep-like cycles. As early as 1949, Kristian Kristiansen and Guy Courtois at McGill University and the Montreal Neurological Institute showed that, when neurons carrying input from the thalamus and surrounding cortical tissue are surgically severed, clusters of neurons called cerebral cortical islands will alternate between periods of high-amplitude slow waves that characterize sleep and low-amplitude fast waves typical of waking, independently of surrounding tissue.[1] This suggests that sleep is self-organizing within small brain units.

In 1997, Ivan Pigarev of the Russian Academy of Sciences in Moscow and colleagues provided more-concrete evidence that sleep is a property of local networks. Measuring the firing patterns of neurons in monkeys' visual cortices as the animals fell asleep while performing a visual task, they found that some of the neurons began to stop firing even while performance persisted. Specifically, the researchers found that, within the visual receptive field being engaged, cells on the outer edges of the field stopped firing first. Then, as the animal progressed deeper into a sleep state, cells in more-central areas stopped firing. This characteristic spatial distribution of the firing failures is likely a consequence of network behavior. The researchers thus concluded that sleep is a property of small networks.[2]

More recently, David Rector at Washington State University and colleagues provided support for the idea of locally occurring sleep-like states. In a series of experiments, they recorded electrical activity from single cortical columns using a small array of 60 electrodes placed over the rat somatosensory cortex. The sensory input from individual facial whiskers maps onto individual cortical columns. As expected, ERPs in the cortical columns induced by twitching a whisker were higher during sleep than during waking. But looking at the activity of individual columns, the researchers observed that they could behave somewhat independently of each other. When a rat slept, most—but not all—of the columns exhibited the sleep-like high-amplitude ERPs; during waking, most—but not all—of the columns were in a wake-like state. Interestingly, the individual cortical columns also exhibited patterns that resembled a sleep rebound response: the longer a column was in the wake-like state, the higher the probability that it would soon transition into a sleep-like state.[3]

To test how cortical-column state can affect whole-animal behavior, Rector and his team trained rats to lick a sucrose solution upon the stimulation of a single whisker, then characterized the whisker's cortical-column state. If the column receiving input from the stimulated whisker was in a wake-like state (low-magnitude ERP), the rats did not make mistakes. But if the column was in the sleep-like state (high-magnitude ERP), the animals would fail to lick the sucrose when stimulated and would sometimes lick it even when their whisker was not flicked.[4] Even though the animal was awake, if a cortical column receiving stimulation was asleep, it compromised the animal's performance. These experiments indicate that even very small neuronal networks sleep and that the performance of learned behavior can depend on the state of such networks.



CHARACTERIZING SLEEP

Sleep-like patterns of neural activity are apparent not just at the level of the whole brain, but also in isolated neural circuits. Researchers have even documented sleep-like behavior in cultures of glial and neural cells. By increasing the number of electrophysiological measurements we use to characterize sleep states, the homology between sleep-like states in culture and sleep in intact animals becomes stronger.

WHOLE BRAIN

Mammalian sleep is characterized by several stages, typically measured using an electroencephalogram (EEG), which involves the recording of brain activity from an array of electrodes on the scalp. Rapid-eye movement (REM) sleep, the stage during which vivid dreams occur, is characterized by EEG waves similar to those observed during waking. High-amplitude delta waves (0.5–4 Hz) occur at the deepest stage of non-REM, or slow-wave, sleep. Both the presence and amplitude of these delta waves are used to characterize sleep in whole animals. When treated with the somnogen tumor necrosis factor α (TNF-α), the brain produces higher-amplitude delta waves, indicating a deeper stage of sleep.

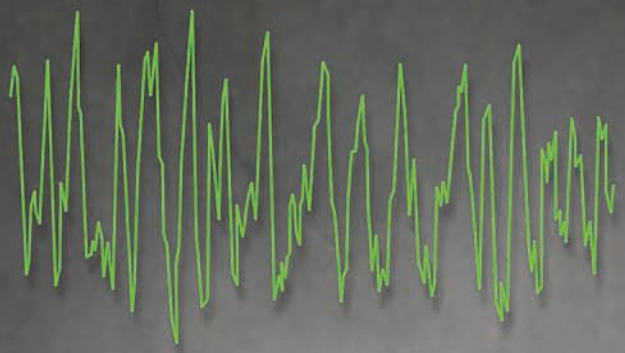
Awake

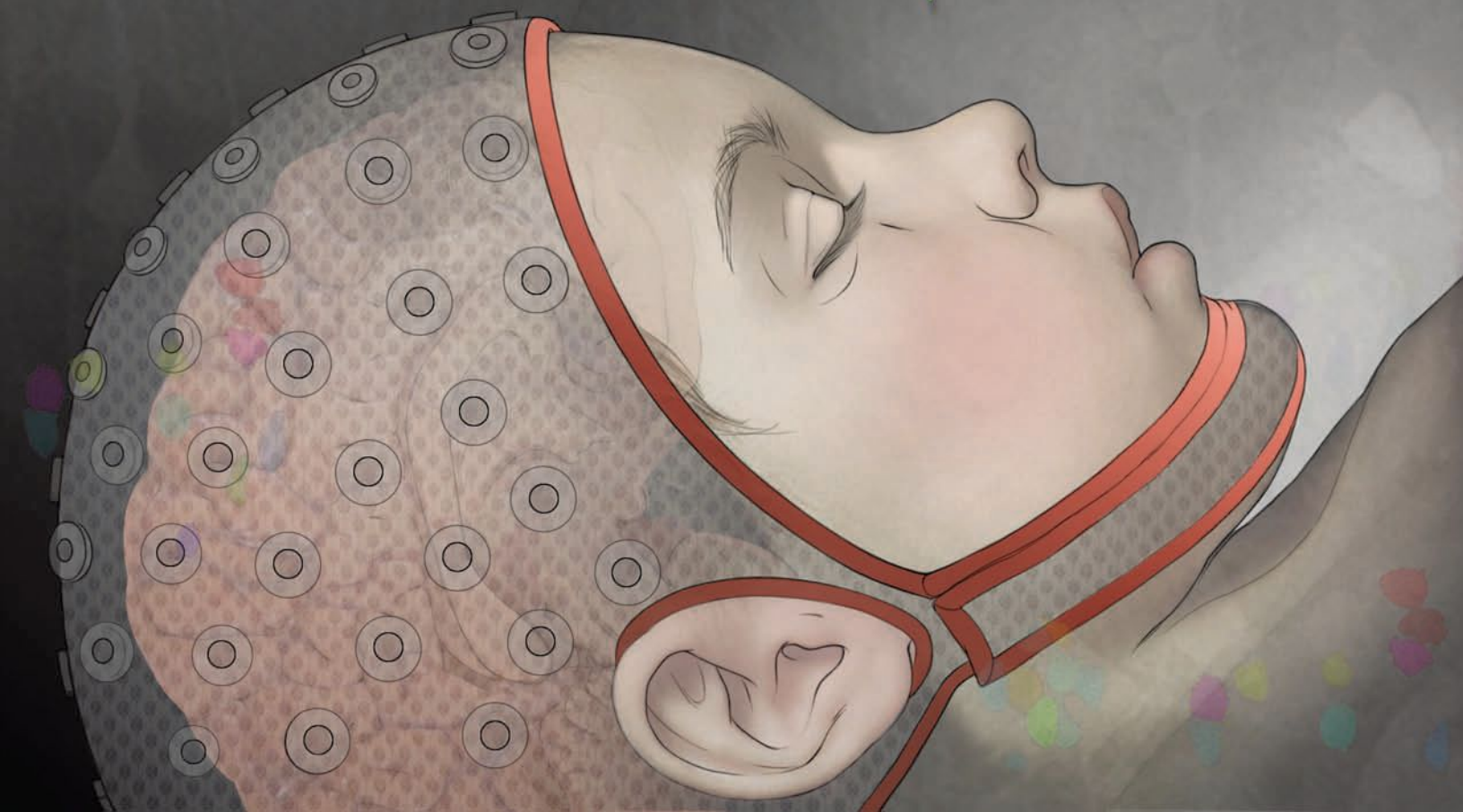
REM sleep

Slow-wave, or non-REM, sleep

Delta waves

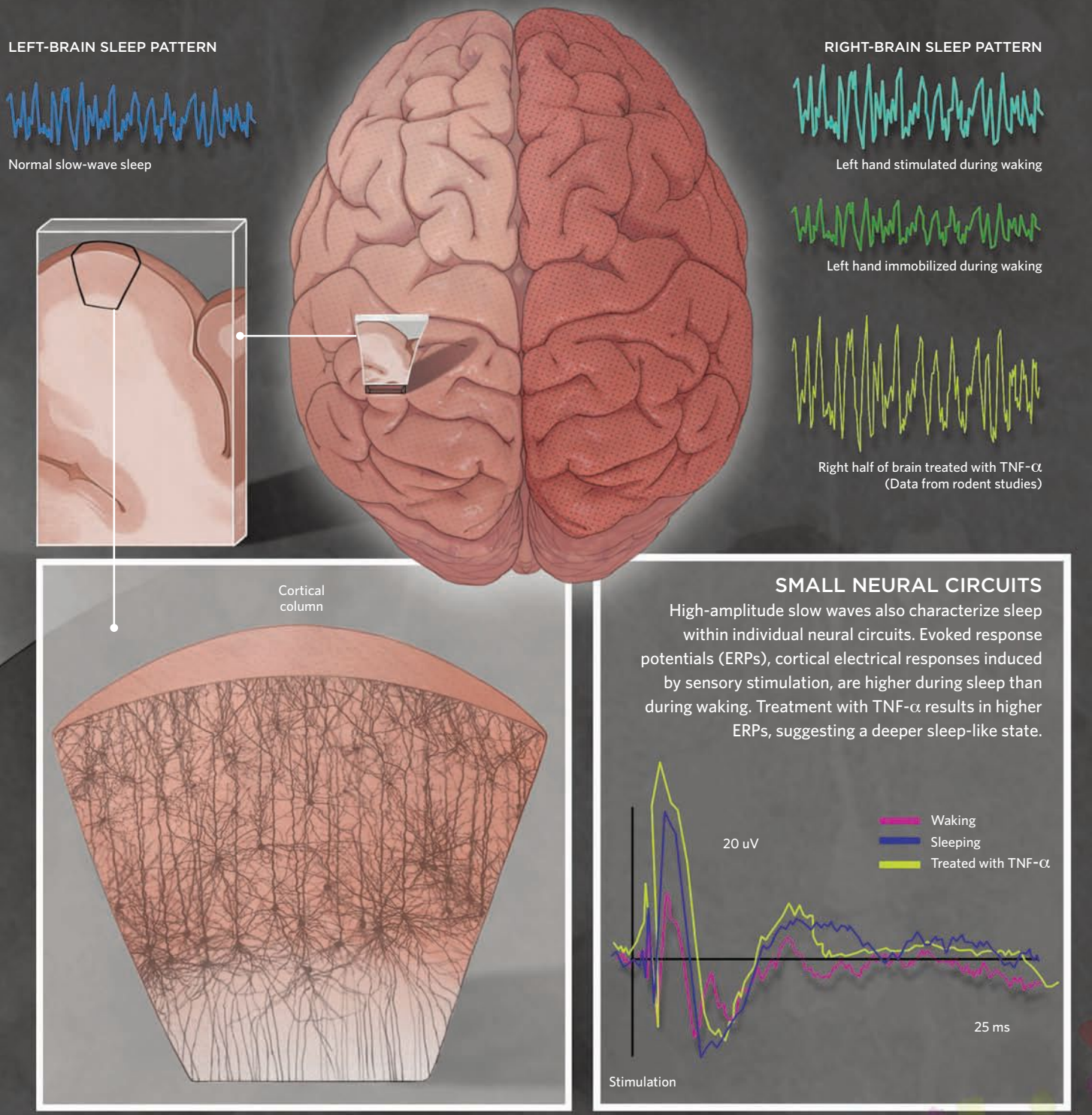
TNF-α treatment





HALF BRAIN

Research has also yielded evidence that the brain's two hemispheres can sleep somewhat independently of each other. When a person holds a vibrating wand in the left hand during waking, for example, he stimulates only the right side of the somatosensory cortex, and in subsequent sleep, the right side of the brain exhibits higher amplitude EEG slow waves than the left side, indicating greater sleep intensity. Conversely, if a person's left arm is immobilized during waking, amplitudes of EEG slow waves from the right side of the brain are lower than the left side during subsequent sleep. These half-brain measurements indicate that local sleep depth is a function of activity during waking. Moreover, rodent studies have shown that TNF-α treatment to only half of the brain will invoke higher than normal delta waves during sleep only in that hemisphere.



Sleep in a dish

Given that sleep can manifest in relatively small brain regions, perhaps it should not be too surprising that co-cultures of neurons and glia possess many of the electrophysiological sleep phenotypes that are used to define sleep in intact animal brains. During sleep, cortical and thalamic neurons display bursts of action potentials lasting about 500 ms, followed by periods of hyperpolarization lasting about the same length of time. The synchronization of this firing pattern across many neurons is thought to generate the EEG activity characteristic of delta-wave sleep, and undisturbed co-cultures of glia and neurons display periodic bursts of action potentials, suggesting that the culture default state is sleep-like. In contrast, if neuronal and glia networks are stimulated with excitatory neurotransmitters, the culture's "burstiness"—the fraction of all action potentials found within bursts—is reduced, indicating a transition to a wake-like state. Treatment of co-cultures with excitatory neurotransmitters also converts their gene expression profile from a spontaneous sleep-like pattern to a wake-like pattern.[5]

Cell cultures also respond to sleep-inducing agents similarly to whole organisms. If a neuronal and glial culture is treated with TNF-α, the synchronization and amplitudes of slow-wave electrical activity increase, indicating a deeper sleep-like state. Moreover, ERPs are of greater magnitude after cultures are treated with TNF-α than during the sleep-like default state, suggesting that the somnogen induces a deeper sleep-like state in vitro as it does in vivo.[6]

Researchers have even studied the developmental pattern of such sleep phenotypes, using multielectrode arrays to characterize network activity throughout the culture, and the emergence of network properties follows a similar time course as in intact mouse pups. Spontaneous action potentials occur during the first few days in culture, but network emergent properties are not evident until after about 10 days. Then, synchronization of electrical potentials begins to emerge, and the network's slow waves begin to increase in amplitude. If the cultures are electrically stimulated, slow-wave synchronization and amplitudes are reduced, suggesting the networks wake up. This is followed by rebound-enhanced slow-wave synchronization and amplitudes the next day, suggesting sleep homeostasis is also a characteristic of cultured networks.[6]

Clearly, even small neural networks can exhibit sleep-like behavior, in a dish or in the brain. But the question remains: What is driving the oscillations between sleep- and wake-like states?

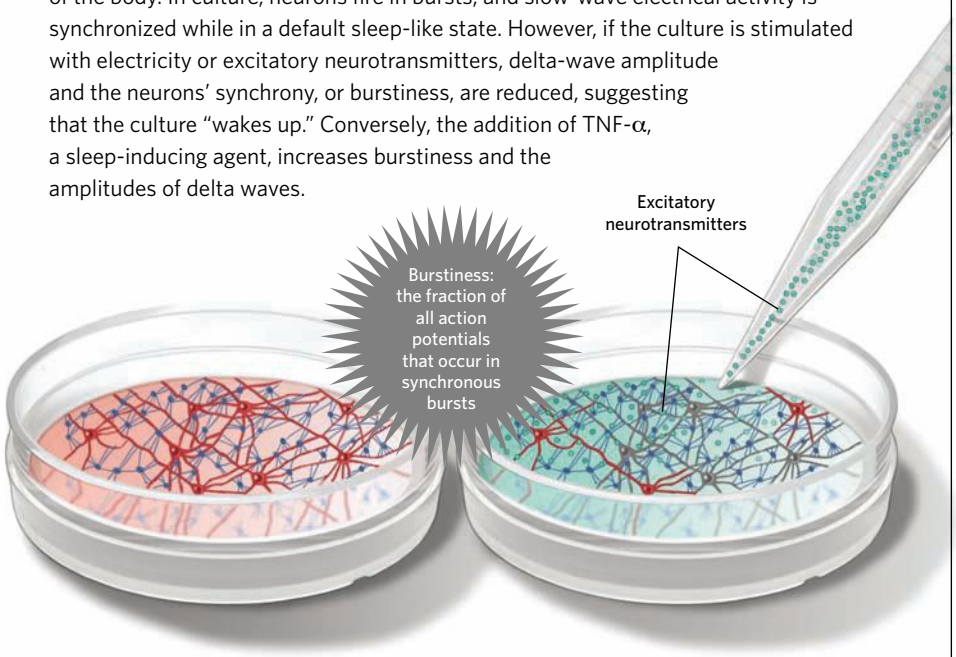
Sleep emerges

In the intact brain, communication among neurons and between neurons and other cells is ever changing. Bursts of action potentials trigger the release of multiple substances and changes in gene expression, both of which alter the efficacy of signal transmission. For instance, neural or glial activity induces the release of ATP into the local extracellular space. Extracellular ATP, in turn, induces changes in the expression of TNF-α and other somnogens known to induce a sleep-like state. Because these effects take place in the immediate vicinity of the cell activity, they target sleep to local areas that were active during prior wakefulness.

In 1993, Obál and I (J.K.) proposed that sleep is initiated within local networks as a function of prior activity.[7] The following year, Derk-Jan Dijk and Alex Borbely of the University of Zurich provided support for this idea when they had volunteers hold hand vibrators in one hand during waking to stimulate one side of the somatosensory cortex. In subsequent sleep, the side of the brain that received input from the stimulated hand exhibited greater sleep intensity, determined from amplitudes of EEG slow waves, than the opposite side of the brain. And in 2006, Reto Huber, then at the University of Wisconsin, showed that

SLEEP IN VITRO

Neurons co-cultured with glial cells display patterns of action potentials and slow (delta) waves, suggesting that small neural networks can and do sleep, even outside of the body. In culture, neurons fire in bursts, and slow-wave electrical activity is synchronized while in a default sleep-like state. However, if the culture is stimulated with electricity or excitatory neurotransmitters, delta-wave amplitude and the neurons' synchrony, or burstiness, are reduced, suggesting that the culture "wakes up." Conversely, the addition of TNF-α, a sleep-inducing agent, increases burstiness and the amplitudes of delta waves.



if an arm is immobilized during waking, amplitudes of EEG slow waves from the side of the brain receiving input from that arm are lower in subsequent sleep.

These experiments indicate that local sleep depth is a function of the activity of the local network during waking—an idea that has been confirmed by multiple human and animal studies. Moreover, local network state oscillations strongly indicate that sleep is initiated within local networks such as cortical columns. But how do the states of a population of small networks translate into whole-animal sleep?

Co-cultures of neurons and glia possess many of the electrophysiological sleep phenotypes that are used to define sleep in intact animal brains.

Small local clusters of neurons and glia are loosely connected with each other via electrophysiological and biochemical signaling, allowing for constant communication between local networks. Steven Strogatz of Cornell University showed that dynamically coupled entities, including small neuronal circuits, will synchronize with each other spontaneously without requiring direction by an external actor. Synchronization of loosely coupled entities occurs at multiple levels of complexity in nature from intact animals to molecules—for example, birds flocking, or the transition from water to ice. The patterns generated by bird flocking, or the hardness of ice, are called emergent properties.

We, Obál, and our colleagues proposed that whole-brain sleep is an emergent property resulting from the synchronization of local neuronal network states.[7,8,9] This would explain why sleep continues to occur after brain damage: because the remaining local circuits will spontaneously synchronize with each other. This view also allows one to easily envision variations in the depth or degree of sleep and waking because it allows for some parts of the brain to be in sleep-like states while other areas are in wake-like states, just as Rector observed. These independent states of local networks may account for sleep inertia, the minutes-long period upon awakening of poor cognitive performance and fuzzy-mindedness, and may also play a role in the manifestation of dissociated states such as sleepwalking. Most importantly, this paradigm frees sleep regulation from the dualism trap of mind/brain separation: top-down imposition of state is not required for the initiation of local state oscillations or for subsequent whole-organism sleep to ensue.

Our theory is also consistent with the modulation of sleep and wakefulness by sleep regulatory circuits such as those in the hypothalamus. For example, if interleukin-1, a sleep regulatory substance, is applied locally to the surface of the rat cortex, it induces local high-amplitude EEG slow waves indicative of a greater local depth of sleep.[10] The responses induced by interleukin-1 in the cortex enhanced neuronal activity in anterior hypothalamic sleep regulatory areas.[11] That hypothalamic neuronal activity likely provides information on local sleep- and wake-like states occurring in the cortex to the hypothalamus, where it can modulate the orchestration of the sleep initiated within the smaller brain units.

Finally, our ideas may inform the study of how sleep influences the formation of memories. A fundamental problem a living brain faces is the incorporation of new memories and behaviors while conserving existing ones. We know that cell activity enhances neuronal connectivity and the efficacy of neurotransmission within active circuits, a phenomenon that has been posited to be a mechanism by which memories are formed and solidified. By themselves, however, these use-dependent mechanisms would lead to unchecked growth of connectivity (in response to activity patterns) and positive feedback (since increased connectivity leads to reuse), ultimately resulting in a rigid, nonplastic network.[7] Instead, we suggest that biochemical mechanisms—specifically, the use-dependent expression of genes involved in sleep regulation and memory—induce oscillations, representing local wake- and sleep-like states, which serve to stabilize and preserve brain plasticity.[7]

For more than a century, researchers have struggled to understand how sleep works and what it does. Perhaps this lack of answers stems from a fundamental misconception about *what* sleeps. By thinking about sleep in smaller units, such as individual networks in the brain, hopefully the field will start to understand what exactly is going on during this enigmatic—but very common—phenomenon. ■

James M. Krueger is a regents professor of neuroscience and Sandip Roy is an associate professor of electrical engineering at Washington State University.

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Go To Bed!

The immediate consequences of losing out on sleep may be harbingers of long-term repercussions.

BY KERRY GRENS

On a closed-circuit television I watch Marie settle into her room, unpacking her toiletries in the bathroom and arranging her clothes for the next day. Her digs at the University of Chicago sleep lab look like an ordinary hotel room, with a bed, TV, desk, nightstand. Ordinary, except for the camera keeping watch from across the bed and the small metal door in the wall next to the headboard. The door, about one foot square, is used when researchers want to sample the study participants' blood during the night without disturbing them; an IV line passes from the person's arm through the door and into the master control room where I'm watching Marie on the screen.

She's come to the lab on a weekday evening to be screened for possible inclusion in a study on insomnia. Marie says her sleep problems started almost 20 years ago, on the first day of her job as a flight attendant. "The phone rang in the middle of the night," she recalls. It was work, scheduling her for a flight. "Something was triggered in my mind. It was the first time in my life I experienced a night with no sleep. Something clicked. Then the second night I couldn't sleep. It just went on. I lost my ability to sleep."

After a few years, Marie (not her real name—she asked to remain anonymous for privacy) stopped working. Most nights she'll sleep for a short stretch—maybe a few hours—then wake up and lie awake for hours as pain in her neck consumes her and makes her uneasy and restless. "I've seen psychologists, physical therapists, doctors. I've been prescribed medications for depression. But it didn't work," she says. "Every single day it's a struggle . . . I feel like when Job was attacked by the devil. Someone is trying to take my vitality away."

> We're not wired for sleep deprivation. Every time we sleep-deprive ourselves, things go wrong.
>
> —Eve Van Cauter, University of Chicago

A lack of sleep can do just that, sometimes with fatal consequences. Experiments have shown that keeping animals awake for days on end can kill them, for example, and in 2012, news outlets reported that a soccer fan had died after 11 sleepless nights spent watching the sport on television. "I think we forget that sleep is a basic physiological requirement," says Carol Everson, who studies the effects of sleep deprivation at the Medical College of Wisconsin.

Death is obviously an extreme consequence resulting from extreme sleep deprivation, but many of the millions of people who suffer from less-intense sleep problems do suffer myriad health burdens. In addition to emotional distress and cognitive impairments, these can include high blood pressure, obesity, and metabolic syndrome. And recent research has suggested even mild sleep loss, the kind people often subject themselves to during the work week by watching late-night TV until midnight then rising before dawn, may lead to metabolic, cardiovascular, cognitive, and neurologic dysfunction.

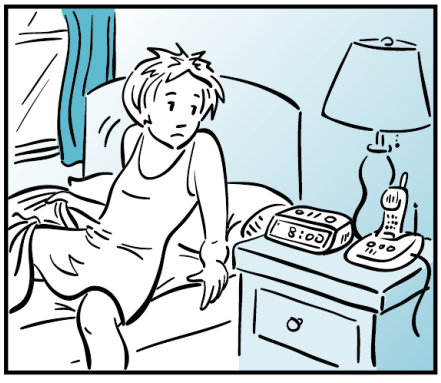
"In the studies we've done, almost every variable we measured was affected. There's not a system in the body that's not affected by sleep," says University of Chicago sleep researcher Eve Van Cauter. "We're not wired for sleep deprivation. Every time we sleep-deprive ourselves, things go wrong."

A brain without sleep

Sleep deprivation experiments go back more than a century, as researchers have sought to understand the function of sleep. A common refrain among sleep scientists until about two decades ago was that sleep was performed by the brain in the interest of the brain. That was an incomplete insight, but it was not wrong. Numerous studies have hinted at the function of sleep by confirming that neurological function and cognition are messed up during sleep loss, with reaction time, mood, and judgment suffering from being awake for too long.

Undergirding these behavioral changes are sleep deprivation's effects on basic brain function. Electroencephalogram recordings show disruptions in normal brain-activity patterns—abnormalities that are thought to result from altered neural activity in brain regions such as the cortex, basal forebrain, hippocampus, and striatum. The latter has been found to downregulate dopamine receptors, which may help maintain arousal. Hippocampal neurons, meanwhile, show a reduction in long-term potentiation, a form of synaptic plasticity that normally reinforces connections between brain cells and underlies memory formation. And the sleep-deprived basal forebrain—one of the brain's main wakefulness centers—experiences an increased release of nitric oxide leading to a buildup of adenosine, a nucleoside that can also affect neural function.

Decades-old studies found that adenosine functioned as a sedative when given to animals. For years, Bob McCarley of Harvard Medical School has probed the role of adenosine in sleep, and, in particular, sleepiness. In 1997, he and his colleagues found that when they kept cats awake by playing with them, extracellular adenosine increased in the basal forebrain as the sleepy felines stayed up longer and slowly returned to normal levels when they were later allowed to sleep.[1] McCarley's team also found that administering adenosine to the basal forebrain put animals to sleep. (It should come as no surprise, then, that caffeine—which blocks adenosine's receptors—keeps us awake.)



When people reported sleeping less than six hours per night they were almost always overweight or obese.

—David Dinges, University of Pennsylvania Perelman School of Medicine

Teaming up with Radhika Basheer and others, McCarley discovered that, as adenosine levels rise during sleep deprivation, so do the concentrations of adenosine receptors, magnifying the molecule's sleep-inducing effect.[2] "The brain has cleverly designed a two-stage defense against the consequences of sleep loss," McCarley says.

Adenosine may underlie some of the cognitive deficits that result from sleep loss. Harvard Medical School's Robert Strecker, McCarley, and colleagues found that infusing adenosine into rats' basal forebrain impaired their performance on an attention test, similar to what is seen in sleep-deprived humans and animals.[3] But adenosine levels are by no means the be-all and end-all of sleep deprivation's effects on the brain or the body.

Systemic effects

Even the earliest sleep-restriction experiments on people revealed untoward consequences for the body, not just the brain. In 1896, George Thomas White Patrick and J. Allen Gilbert of the University of Iowa kept three of their university colleagues awake for more than 88 hours and recorded the effects: visual hallucinations, decreased grip and pull strength, and slowed reaction time. Curiously, the participants also gained 0.5 to 1.5 kg by the end of the sleep deprivation period, and lost the extra kilos as soon as they resumed normal sleep.[4] "The steady increase in the subject's weight during the experiment and the sudden decrease in weight after sleep are noteworthy, and apparently not to be accounted for by accidental circumstances," Patrick and Gilbert wrote of their first volunteer.

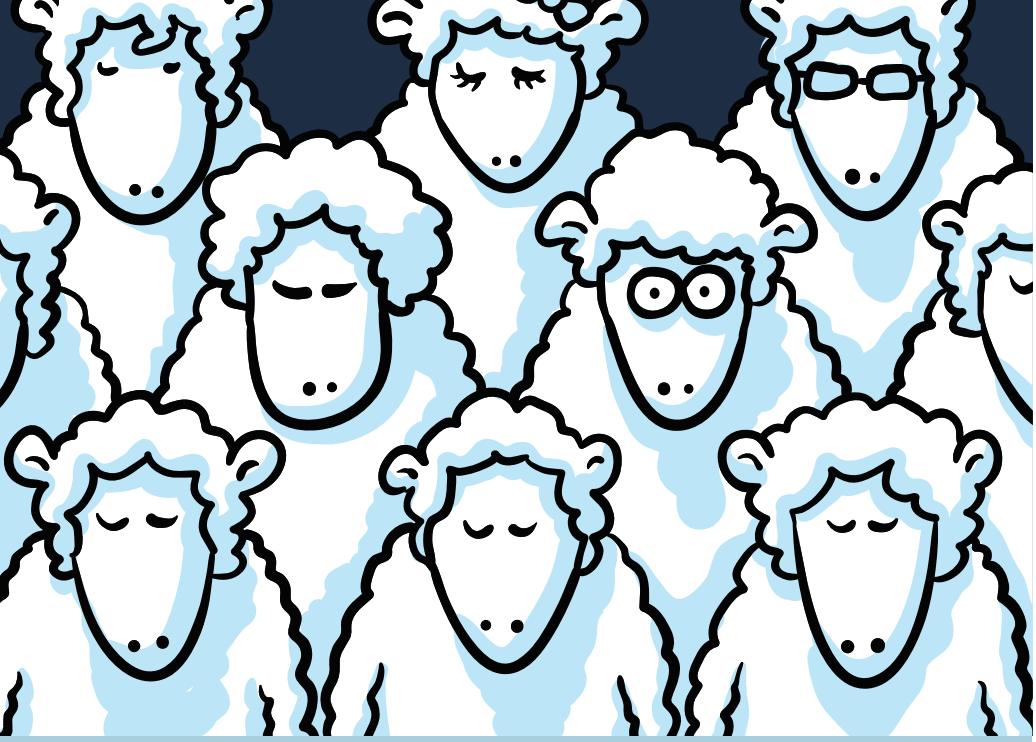
A century later, real-world observations found a similar link. "The epidemiologic studies . . . kept showing over and over again that when people reported sleeping less than six hours per night they were almost always overweight or obese," says David Dinges, who studies sleep loss at the University of Pennsylvania Perelman School of Medicine. "And it wasn't just obesity." People who slept very little (often less than six hours per night) were more likely to be diabetic or suffer a heart attack as well. "This led to an increasing medical understanding that sleep may be playing a role in things we never thought possible," says Dinges. (Interestingly, the data also revealed adverse effects of sleeping for too long, say, more than eight or nine hours every night, including an increased risk of early death.)

These were only observational studies, however. To see if there could be any causal relationship between sleep patterns and the metabolic outcomes, researchers had to bring subjects into the lab. So in 1999, Van Cauter and her colleagues had 11 men sleep in the lab at the University of Chicago. For three nights, the volunteers spent eight hours in bed, then for six nights they were allowed only four hours in bed (accruing what Van Cauter calls a sleep debt), and then for six nights the men could sleep for up to 12 hours (sleep recovery). During sleep debt and recovery, researchers gave the participants a glucose tolerance test and found striking differences. While sleep deprived, the men's glucose metabolism resembled a prediabetic state.[5] "We knew it would be affected," says Van Cauter. "The big surprise was the effect size was much greater than we thought."

Subsequent studies also found insulin resistance increased during bouts of sleep restriction. In 2012, Van Cauter's team took fat biopsies from seven adults who

UNDER THE COVER OF DARKNESS

Strange things happen at night, especially when we're not sleeping. Results from observational studies of people who don't sleep much (blue icons) and from experiments on human volunteers (pink icons) have revealed that the consequences of sleep deprivation are far-reaching, from molecules and cells to organs and behavior.



HOW DO YOU SLEEP?

Over the decades, David Dinges of the University of Pennsylvania Perelman School of Medicine has run hundreds of subjects through sleep-restriction experiments, often limiting them to just a few hours in bed each night for several days. Then, to get a sense of how the volunteers respond to losing sleep, Dinges measures how quickly and accurately they push a button after seeing a flash of light. Quite predictably, a participant's performance tends to decline with greater sleep deprivation.

But over many years of giving his subjects this so-called psychomotor vigilance test (PVT), Dinges began to notice that averaging their performance masked variability between them. "Some people were deteriorating at a much faster rate than others," he says. Nothing predicted it: age, gender, education. "And the longer you went with inadequate sleep, the larger the individual differences got."

To better understand this variation, Dinges recruited 21 volunteers to come to the lab and stay awake for 36 hours, periodically taking the PVT during the deprivation. Then, at two-week intervals, the same subjects came back to repeat the experiment two more times (*Sleep*, 27:423-33, 2004). "The day came when we repeated the exposure, and lo and behold, we got the same response," Dinges says. "If you were vulnerable at one time when we tested you, you're vulnerable again."

It turns out that people fall into one of three categories, which Dinges termed Type 1 (resilient), Type 2 (intermediate), and Type 3 (vulnerable). Interestingly, Type 1 subjects are affected by the experiment—they get sleepy, their eyelids droop, and they struggle to concentrate—but their cognitive performance stays strong, compared with that of more-vulnerable subjects.

Nearly a decade later, an independent group at Penn conducted a similar experiment on identical and fraternal twins, finding that resiliency and vulnerability to sleep loss are highly heritable (*Sleep*, 35:1223-33, 2012). And last year, Penn's Namni Goel, Dinges's collaborator, showed that it isn't just performance on the PVT that varies predictably between the sleep deprived; stable differences in eating behaviors and weight gain also appear between individuals subjected to sleep loss (*Scientific Reports*, 5:14920, 2015).

Dinges and others have looked for genes related to vulnerability phenotype, but they've yet to identify any that can explain the different responses to sleep deprivation. Dinges is also keen on finding a biomarker that can predict which type a person is. Such a test could be useful to, say, the military or to transportation agencies, jobs in which people may be operating under poor sleep conditions, he says. "We'd really like to know, how can one person tolerate this so much better than another?"

spent four-and-a-half hours in bed for four nights, and the researchers found impairments in insulin signaling in their adipocytes.[6] The participants' fat cells "were no longer responding to one of the major modulators of their function. That's what impressed me," Van Cauter says. "You could almost see that the cell was sleep deprived."

It's possible these immediate metabolic changes could explain the longer-term health impacts seen among people who don't get much shut-eye. Overeating is another possible explanation. In some research using human subjects, levels of the appetite-suppressing hormone leptin have gone down upon sleep restriction, while levels of ghrelin, a hunger promoter, have gone up, spurring a greater desire for food, especially calorie-dense snacks. One recent study showed that sleep-restricted people will add 300 calories to their daily diet.[7] "In our brain we have a system that links sleeping and fasting or being awake and eating. So when you sleep-deprive yourself, the system is thinking there must be a lack of food and therefore you begin to forage at the refrigerator," says Van Cauter.

Echoing Van Cauter's results, Basheer has found evidence that sleep deprivation sends the brain into a catabolic, or energy-consuming, state.[8] It degrades the energy molecule adenosine triphosphate (ATP) to produce adenosine monophosphate and results in the activation of AMP kinase, an enzyme that boosts fatty acid synthesis and glucose utilization. "The system sends a message there is a need for more energy," Basheer says. Whether this is indeed the mechanism underlying late-night pig-outs is still speculative.

Gene-expression analyses also highlight the metabolic mayhem wrought by sleep restriction. In 2013, the University of Surrey's Simon Archer, Derk-Jan Dijk, and colleagues monitored volunteers who slept about six hours a night for one week and eight hours each night for another. At the end of each week, they prevented the participants from sleeping for a continuous 40 hours and sampled their blood over a couple of days to look for genes that were differentially expressed. The expression of genes associated with metabolism

was all out of whack after a week of insufficient sleep. But that wasn't all. Transcriptome analysis showed that many genes linked with immune and inflammatory processes and with gene regulation were also up- or downregulated after the week of sleep deprivation.[9]

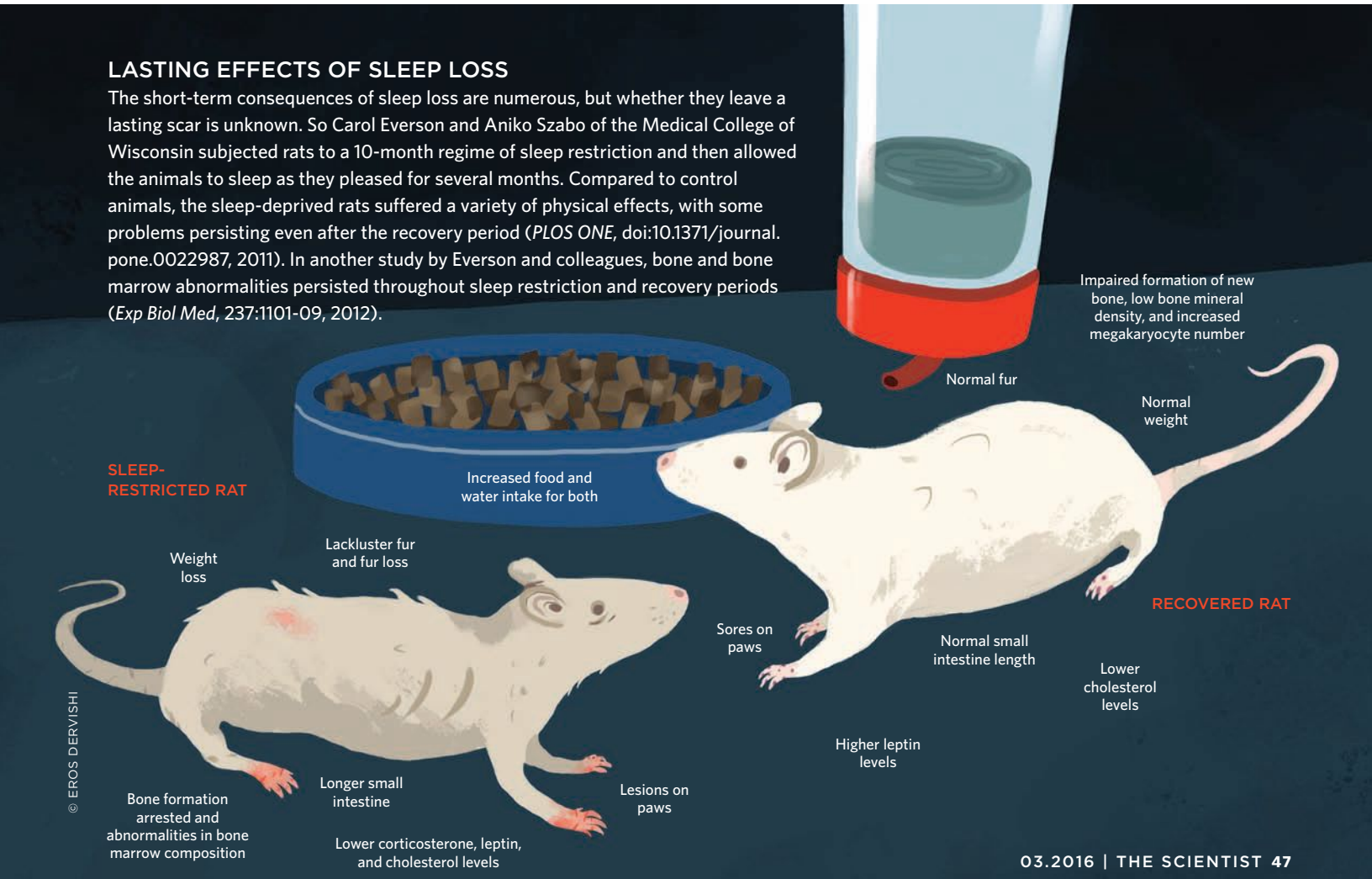
These and other studies make it clear that insufficient sleep can have profound effects on physiology. Yet most experiments have spanned fewer than two weeks. One open question is whether chronic sleep loss can produce long-term changes. Preliminary research suggests that it can.

Long-term consequences

One of the assumptions in the sleep field has been that the effects of sleep deprivation are temporary, and that after people resume a normal sleep schedule, their cognitive performance, brain chemistry, and physiology go back to baseline. In animals, researchers have found evidence that the effects of sleep deprivation can last for months. For 10 weeks, Carol Everson of the Medical College of Wisconsin subjected rats to repeated cycles of 10 days of disrupted and limited sleep followed by two days without disturbance. During the first half of the experiment, the animals seemed perfectly healthy. But after about five weeks, problems emerged. "All of a sudden their food intake took off progressively and they weren't gaining weight," Everson says.

In addition, the rodents' fur lost its luster, their adipocytes were smaller and of a different phenotype than control animals', and their small intestines were longer by 30 percent.[10] Longer intestines typically correlate with increased surface area for nutrient and water absorption, says Everson. It's not clear yet why the sleep-deprived rodents weren't gaining weight, as sleep-deprived humans do. This apparent discrepancy between the species "will become resolved when we better understand the inherent properties of the metabolic challenge posed by sleep loss and the context in which it occurs," Everson says, noting that individual differences in physical constitution or varying sleep-restriction experimental paradigms could contribute.

To test for a possible lifetime burden from the experience, Everson put another set of animals through the same 10-week regimen, then let them go back to their normal sleep behavior for four months. While some of the changes returned to baseline, metabolic abnormalities persisted—the rats were still eating more food, yet their weight was no different than control animals'.[11] And "then there



were emergent changes, things we had not seen before," says Everson. Leptin levels increased, for instance. "It's a sign that there's an energy deficit, and leptin insensitivity, which is a component of obesity."

Within the brain, scientists have glimpsed signs of physical damage from sleep loss, and the time line for recovery, if any occurs, is unknown. Chiara Cirelli's team at the University of Wisconsin–Madison School of Medicine has found structural changes in the cortical neurons of mice when the animals are kept awake for long periods of time. Specifically, Cirelli and her colleagues saw signs of mitochondrial activation—which makes sense, as "neurons need more energy to stay awake," she says—as well as unexpected changes, such as increased lysosomal activation and undigested cellular debris, signs of cellular aging that are unusual to see in the neurons of young, healthy mice.[12]

"The number [of debris granules] was small, but it's worrisome because it's only four to five days" of sleep deprivation," says Cirelli. And after 36 hours of sleep recovery—an amount of time in which she expected normalcy to resume—those changes remained.

Sigrid Veasey's team at Penn has also noticed changes in the brains of mice after several days of sleep loss—in this case, locus ceruleus neurons (LCns), which are active during waking, were found to degenerate. A short bout of sleep deprivation—three hours kept awake—sparked a protective mitochondrial response in the cells, but disrupting the animals' sleep for a few days was damaging. "While it is difficult to discern whether the loss of LCns and injury of this magnitude are sufficient to result in cognitive impairments, we propose that repeated occurrences of [extended sleep deprivation] (as is commonly observed in night-shift workers) could result in a cumulative loss of LCns . . ." the authors wrote in their 2014 paper.[13]

Veasey's results "are bizarre and incredible," says Penn's Dinges, who adds that the study raises important questions about whether there are permanent effects of sleep loss. "That has me worried about human studies, because if I can't reverse the sleep-deprivation effects, I must stop immediately." Dinges is now testing whether sleep loss can have long-lasting effects in humans by limiting volunteers to four hours in bed for five nights, then giving them 2, 4, 6, 8, or 10 hours of recovery time in bed, and then testing their cognitive performance during another bout of sleep restriction.

Shift workers, night owls, and insomniacs like Marie could also lend valuable insight into persistent changes from sleep restriction, serving as natural experiments on how the human body reacts to losing out on a basic life need for chronic periods. She ended up qualifying for the University of Chicago study, which is examining whether insomniacs have hyperactivity in their muscle sympathetic nerves. While numerous labs want to gather experimental data to probe the epidemiological data generated by such brief sleepers, Marie says she's most interested in fixing her condition. Whenever she reads about advances in sleep research in the news, it's about the consequences of insomnia, she says, "but not a huge breakthrough" in therapies to treat the effects of sleep deprivation. (See "Desperately Seeking Shut-Eye" on page 63.) With so much of our physiology affected when we lose out on sleep, an effective therapeutic—other than sleep itself—is difficult to imagine.

"People like to define a clear pathway of action [for health conditions]," says Van Cauter. "With sleep deprivation, everything you measure is affected and interacts synergistically to produce the effect." ■

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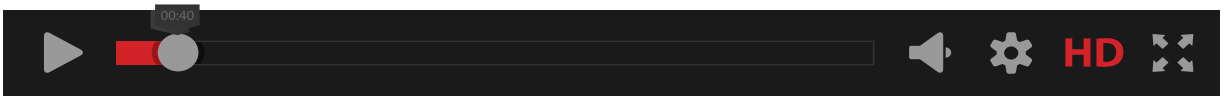

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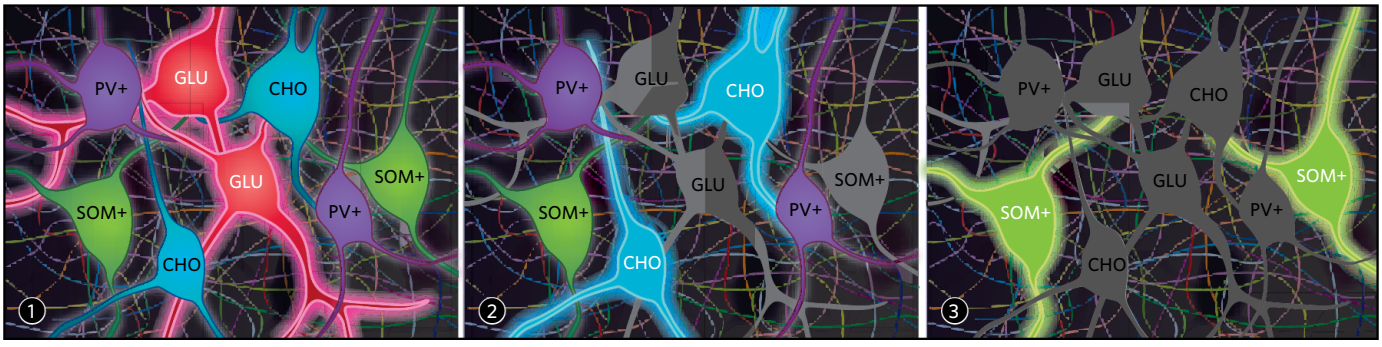
The Literature

NEUROSCIENCE

Sleep Circuit



THE PAPER
M. Xu et al., "Basal forebrain circuit for sleep-wake control," *Nature Neuroscience*, 18:1641-47, 2015.

Early studies attempting to untangle the neurological basis of sleep typically removed or injured part of an animal's brain to measure the effects. The results implicated a region called the basal forebrain in inducing sleep, yet some studies indicated that it was important for arousal. "The impression is that maybe in that region there's a mixture of mechanisms," says Yang Dan, a neurobiologist at the University of California, Berkeley. "But that's not a very satisfactory answer."

Dan sought to identify which cells in the basal forebrain promote which brain state. The region contains three main types of neurons: cholinergic, glutamatergic, and GABAergic. Dan and her colleagues further classified the GABAergic neurons into those containing somatostatin (SOM+) or parvalbumin (PV+).

The researchers optogenetically activated each of these four cell types in mice to locate them and track their activity. The cholinergic, glutamatergic, and PV+ GABAergic neurons typically fired multiple times per second when the mice were awake or in REM sleep, but less often during non-REM sleep, a sleep stage in which the brain is less aroused overall. In contrast, non-REM sleep was when the SOM+ GABAergic neurons were most active.

Dan's team then fired a laser pulse to stimulate the different cell types. If the mice were already awake when the laser activated the cholinergic, glutamatergic, or PV+ GABAergic neurons, the animals' arousal increased further—and if the animals were in non-REM sleep, they woke up. But when the researchers activated the SOM+ GABAergic neurons, the mice were more likely to go to sleep. "To us, that's the most exciting," says Dan. "Everywhere in the brain, it's easy to find neurons that promote wakefulness," she explains, but very difficult to find neurons that promote sleep.

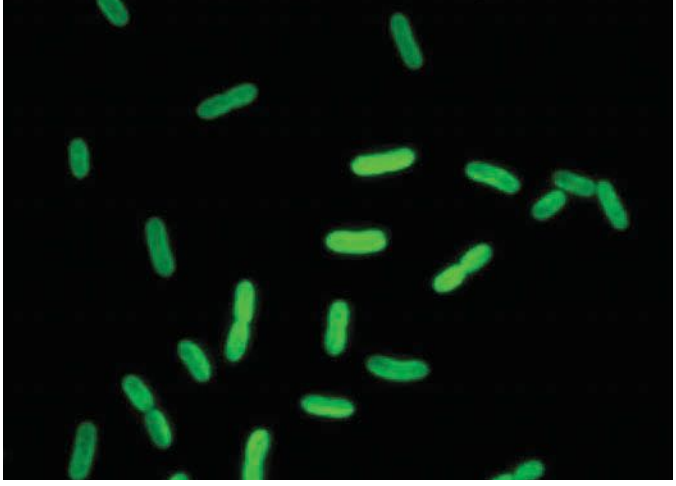
To map the connections between each cell type, Dan's team examined slices of the mouse basal forebrain. In each sample, one type of neuron expressed channelrhodopsin-2 and another a fluorescent identifier. By flashing a light onto the brain tissue, the researchers could activate the light-sensitive neurons and track the synaptic inputs, or lack thereof, to those that were fluorescently labeled.

The different neuron types interacted in a complex hierarchy. Activating glutamatergic neurons excited the three other cell types. Cholinergic neurons activated some neurons, but suppressed others, while PV+ GABAergic neurons had little impact on the labeled cells. Activating the SOM+ neurons, on the other hand, inhibited all three types of wake-promoting neurons. "We think that this is one mechanism for how some neurons can promote sleep," says Dan. "But, of course, it's not the only mechanism."

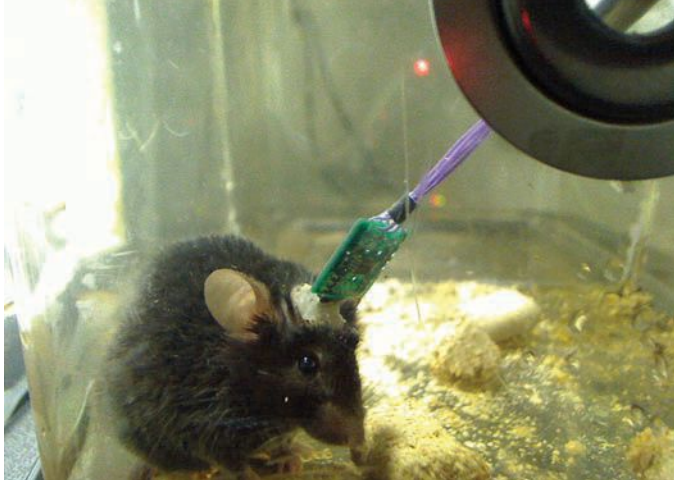
"There's an important knowledge gap in the neurosciences as far as how these subpopulations individually contribute to the processes of cortical arousal and wakefulness," says Patrick Fuller, a neurologist at Harvard Medical School. "The system is wide open for additional empirical testing," he adds. "That's the next step."

—Karen Zusi

INTERWEBS: Activating glutamatergic (GLU) neurons optogenetically excites all other labeled cells types in the basal forebrain of a mouse ❶. When cholinergic (CHO) neurons are turned on, they inhibit GLU neurons and excite a subtype of GABAergic neurons containing parvalbumin (PV+). They also excite and inhibit somatostatin-containing (SOM+) GABAergic neurons ❷. SOM+ neurons, the only cell type less active during REM sleep and waking, inhibit other neuron types ❸. PV+ neurons have minimal or no effects on the other cell types (not shown).



SWEET TIMEKEEPERS: *S. elongatus* cyanobacteria (chlorophyll visible here) set their circadian clocks by metabolism rather than light.



BRAINWAVES: A serotonin-depleted mouse with an electroencephalography headmount implanted to record sleep activity

CELL & MOLECULAR BIOLOGY

Sugar Time

THE PAPER

G.K. Pattanayak et al., "Controlling the cyanobacterial clock by synthetically rewiring metabolism," *Cell Reports*, 13:2362-67, 2015.

TINY TICKERS

Like many organisms, the photosynthetic cyanobacterium *Synechococcus elongatus* has a circadian clock that cycles with light/dark rhythms. The timekeeper has just three core proteins—KaiA, KaiB, and KaiC—making it the simplest circadian clock known to science. Given that researchers have failed to identify light-sensitive clock components in *S. elongatus*, but have demonstrated that Kai proteins respond to metabolic activity, some scientists suggest that cyanobacteria are synchronizing to the downstream metabolites of photosynthesis, as opposed to light itself.

DECOUPLING METABOLISM

To tease apart light-dark cycles and metabolism, researchers led by Michael Rust at the University of Chicago engineered a strain of *S. elongatus* that could grow without photosynthesis by metabolizing externally supplied glucose.

SUGAR DEPENDENCY

The team found that these cyanobacteria's clock phases were essentially unresponsive to light cues in the presence of glucose, and that in constant darkness, they could be entrained by a periodic glucose supply. The results establish metabolic activity as the primary clock driver in *S. elongatus*. "All you need is a metabolic cycle and the [clock] proteins will follow it," says Rust.

A GENERAL MECHANISM?

The discovery of this role for metabolism in some of the world's oldest organisms indicates that "probably the most ancient thing that led to the creation of circadian clocks was the presence of daily metabolic cycling," Rust explains. As for gut bacteria, which also show daily rhythms in the absence of light-dark cycles, the Weizmann Institute's Eran Elinav, who was not involved in the work, says the study "brings the very attractive hypothesis that metabolic activity or different combinations of metabolites may drive periodicity in this system as well." —Catherine Offord

NEUROSCIENCE

Out in the Cold

THE PAPER

N.M. Murray et al., "Insomnia caused by serotonin depletion is due to hypothermia," *Sleep,* 38:1985-93, 2015.

SLEEPLESS NIGHTS

Early research into serotonin's functions suggested that the neurotransmitter promotes sleep: lab animals deprived of the chemical often developed insomnia. More recent evidence indicated that serotonin plays a part in wakefulness instead, a theory that has gained significant traction. But explanations of the initial experimental data were scarce—so Nick Murray, then a research fellow at the University of Iowa Carver College of Medicine, started digging.

FAULTY FURNACE?

"Over the past 5 or 10 years, we've found that serotonin is a key neurotransmitter for generating body heat," says Murray. To investigate whether this role was related to serotonin's impact on sleep, he and his colleagues injected para-chlorophenylalanine into mice to inhibit serotonin synthesis.

ON ICE

When kept at room temperature (20 °C), the mice with depleted serotonin slept less and developed a lower body temperature compared with their control counterparts. However, when housed at 33 °C—a thermoneutral temperature for mice—the sleep and body temperature of the treated mice stayed normal. "Serotonin isn't a sleep-promoting neurotransmitter," concludes Murray, now a resident at California Pacific Medical Center. He suggests that mice lacking serotonin had a tough time sleeping under early experimental conditions simply because the animals were cold, and that at higher temperatures other neurotransmitter systems in the brain would function to allow them a normal sleep-wake cycle.

CASE CLOSED

The study "solves a long-standing mystery" in the field, says Clifford Saper of Harvard University. "Not very many labs measure sleep and body temperature at the same time," he adds. "It just basically escaped everybody's notice for all these years." —Karen Zusi

YI LIAO, UNIVERSITY OF CHICAGO; NICK MURRAY

PROFILE

In Dogged Pursuit of Sleep

Unearthing the root causes of narcolepsy keeps Emmanuel Mignot tackling one of sleep science's toughest questions.

BY ANNA AZVOLINSKY

In November 1986, Emmanuel Mignot arrived at Stanford University's Center for Sleep Sciences and Medicine for a 16-month stint as a research associate. His goal was to find effective drugs to treat narcolepsy; his study subjects belonged to a colony of canines that suffered from the malady. "[When I got there], the dogs were being maintained, but not much was being done with them other than some chemistry studies on known neurotransmitters," says Mignot, a professor of psychiatry and behavioral sciences at Stanford University and now director of the center. "As a pharmacologist, I wanted to study potential treatments for narcolepsy and understand the molecular biology to improve treatment in humans."

The first narcoleptic dog, a French poodle named Monique, was brought to Stanford in 1974 by William Dement, the so-called "father of sleep medicine," who had founded the center in 1970, the first in the world dedicated to the study of sleep. Dement and other researchers there established a full breeding colony in 1977 when dogs with a genetic form of the neurological disorder were discovered—initially, some puppies from a litter of Dobermans and, later, some Labradors. Narcoleptic dogs and humans both exhibit a combination of symptoms: perpetual sleepiness, cataplexy—muscle paralysis attacks triggered by emotions—and abnormal rapid eye movement (REM) sleep. While the condition in humans and dogs is treatable, there is no cure.

To study which narcolepsy drugs increased wakefulness and decreased cataplexy in the dogs, Mignot and psychiatry professor Seiji Nishino used a food-elicited cataplexy test: administration of the drug followed by release into a room with pieces of food on the floor and careful observation. "The dog would rush into the room and be so happy to eat the treats, and then would have an attack and collapse on the floor." The researchers counted the number and duration of the attacks after treatment with a drug at various doses. In humans, cataplexy episodes are triggered by a positive emotion such as laughter at a joke or pleasant surprise. "For the dogs, it is food or the joy of playing. That is what is great about dogs as a model for this condition. When you give a treatment to a rat or mouse and they stop having cataplexy, you really don't know if it is because they don't feel good or if it is a genuine effect. But the dogs show you emotions like humans. I knew all of these dogs by name. They were my friends. I could see if they were worried or didn't feel well."

Mignot worked mostly with the Dobermans and Labs, but there were also dogs donated to the colony that seemed to have a sporadic form of narcolepsy, "There was Vern, a miniature poodle; Wally, a big poodle; Tucker, a mutt; and Beau, my beloved dachshund." Using the cataplexy test in animals along with in vitro studies of the drugs' chemical properties, Mignot and Nishino found that antidepressants suppress cataplexy by inhibiting adrenergic reuptake, and that amphetamine-like stimulants promote wakefulness in narcoleptics by increasing the availability of dopamine. "We improved the then-current treatments and started to understand the kinds of chemicals important to regulate narcolepsy symptoms."

But Mignot wanted to understand the molecular mechanism of narcolepsy, so he turned his focus to the genetic basis of the disorder. A lack of genetics training and no map of the dog genome to guide him did not deter Mignot. He has tirelessly pursued this previously little-studied and, so far, only known neurological disorder that fundamentally perturbs the nature of sleep states.

Here, Mignot talks about pursuing a master's, PhD, and MD simultaneously, the paper retraction that has been the most difficult episode in his career so far, and his unexpected devotion to a Chihuahua.

MIGNOT MOTIVATED

Sir Mix-a-Lot. The youngest of six siblings, Mignot had a penchant for collecting fossils and for conducting chemistry experiments in the bathroom of his family's home in Paris. "I bought chemicals sold by a Chinese shopkeeper on Rue Saint-Dominique to do all kinds of experiments, mixed them, and occasionally made mistakes. There were burn marks and projections on the walls of my bathroom." In high school, the self-proclaimed "nerd with glasses" became interested in biology, and, after graduation in 1977, went to study for a medical degree at the René Descartes University Faculty of Medicine in Paris.

Collecting degrees. "In the second year of medical school, I got bored from all of the memorization." He took the entrance exam for the prestigious École Normale Supérieure (ENS), which gives students freedom to pursue their academic interests at other institutions while providing a stipend, housing, and the support of professor mentors. He passed, and entered the ENS in 1979. Mignot worked towards a master's in biochemistry, and then a PhD in molecular pharmacology while still continuing his medical studies. "Nothing was set up for MD-PhD programs at the time. It was all in parallel, which was crazy. I had an exam every few weeks," says Mignot. In 1984, he received both his medical degree and, later, a PhD from Pierre and Marie Curie University.

New to narcolepsy. Mignot became interested in the effects of drugs on the brains of psychiatric patients, studying how different compounds affected the metabolism of neurotransmitters in the brains of rats, and pursued a residency in psychiatry to complement

COURTESY OF STANFORD UNIVERSITY

EMMANUEL MIGNOT

Professor, Department of Psychiatry and Behavioral Sciences
Stanford University School of Medicine
Director, Stanford Center for Sleep Sciences and Medicine

Greatest Hits

- Identified the gene for hypocretin receptor 2, which, when mutated, causes an inherited form of narcolepsy in Dobermans and Labradors
- Identified how antidepressant and stimulant drugs work as treatments for narcolepsy
- Identified *DQB1*0602* as the main human gene associated with narcolepsy
- By genome-wide association, found immune polymorphisms, such as one in the T-cell receptor alpha, that also predispose people to the disease, further suggesting the disease is autoimmune
- Found that human narcolepsy, unlike canine narcolepsy, is not caused by mutations in the *hypocretin receptor 2* gene but is due to an immune-mediated destruction of hypocretin-producing neurons in the brain

his laboratory research. In 1986, he was offered a professorship in pharmacology at the Paris V University School of Medicine. But first, Mignot needed to complete the mandatory military service that he had deferred. "Instead of going to a former French colony to practice medicine, I convinced the French government to send me to Stanford to study modafinil, a wakefulness-promoting drug created by a French pharmaceutical company called Lafon Laboratories for the treatment of narcolepsy. I had never heard about [narcolepsy] during medical school—it must have been a single line in my textbooks. I discovered that Stanford was doing work on sleep and that Dement had started a colony of narcoleptic dogs there. I thought I could study these animals and figure out how modafinil worked."

So Mignot came to Stanford for 16 months as part of his military service with financial support from Lafon Laboratories. "The company had claimed modafinil worked by a novel mechanism, unrelated to how stimulants work," says Mignot. But Mignot found that modafinil bound the dopamine transporter, inhibiting the reuptake of the neurotransmitter, boosting wakefulness. "This is a similar mode of action as Ritalin, but the company was claiming otherwise. It took 10 years for my results to be validated, finally, by Nora D. Volkow, now director of the National Institute on Drug Abuse, who showed . . . that indeed the drug displaces the dopamine transporter at doses that increase wakefulness in humans."

MIGNOT MOVES

Going to the dogs. At Stanford, Mignot immersed himself in his work with the dog colony. "I worked all the time and came home just to sleep. I was definitely not very successful with girls then, because I smelled like dog all the time. I spent all day with the dogs, going to the facility, hugging, playing, and working with them. When we bred them, sometimes the mothers rejected their puppies so we had to come in every few hours, even in the middle of the night, to bottle-feed the puppies. Even after I took a shower, you could still smell the dogs. It was a strange part of my life."

From pharmacology to genetics. Mignot kept extending his stay at Stanford. "After a few years I realized our pharmacology studies were never going to lead to narcolepsy's cause. We needed to find the genetic cause in the dog." In 1988, he resigned a faculty position in Paris—which was being held for him even as he continued to extend his time at Stanford—deciding to search for the mutated gene responsible for narcolepsy in dogs. In 1993, Mignot became the head of the Center for Narcolepsy at Stanford. A connection between an immune gene, the human leukocyte antigen (HLA)

allele *HLA-DR2*, and narcolepsy in humans had already been identified by Yutaka Honda at the University of Tokyo, so Mignot's lab tried to ascertain whether the same connection was true in the dogs or if the immune gene was simply a genetic linkage marker. These were the days before the dog or human genome had been sequenced, so the work took Mignot's lab 10 years, and almost 200 narcoleptic Dobermans and Labradors: years of painstaking chromosome walking experiments, DNA fingerprinting, and the construction of a bacterial artificial chromosome library of dog genomic pieces. "What helped us a lot was that we knew the Dobermans and Labs had the same genetic defect because we interbred and got narcoleptic puppies—what's called a complementation test." In 1999, Mignot's team identified the mutated gene as *hypocretin receptor 2*, whose protein binds hypocretin (also called orexin), a neuropeptide that regulates arousal and wakefulness. Several weeks later, after seeing these findings, Masashi Yanagisawa's lab independently published a confirmation, showing that hypocretin knockout mice also have narcolepsy.

In parallel narcolepsy studies across ethnic groups, Mignot's lab found that it was not the initial *HLA-DR2* allele that predisposed humans to narcolepsy, but another, nearby *HLA* gene, *DQB1*0602*.

Humans are not like dogs. "After we found the gene, the research went fast. We decided to look at hypocretin itself and see if it's abnormal in humans." Mignot's lab sequenced the genes for the hypocretin receptor and its ligand in narcoleptic patients, expecting mutations in either to be rare because of the known HLA-narcolepsy linkage and the fact that most cases in humans, unlike in dogs, are not familial. Only one documented case, a child who had narcolepsy onset at six months of age, has been found to harbor a hypocretin gene mutation. "I think you need to knock out both *receptor 1* and *2* in humans to get the full narcoleptic phenotype," says Mignot. "Those with just one mutation may be more prone to tiredness but not full narcolepsy."

In 2000, Mignot's and Nishino's groups reported that hypocretin was not present in narcoleptic patients' cerebrospinal fluid—a test still used diagnostically today. The same year, independent studies from Mignot's laboratory and that of Jerome Siegel at the University of California, Los Angeles, found that the lack of hypocretin was not due to gene mutations but to the fact hypocretin cells were missing in the brains of narcoleptic patients. *HLA* genes were well known to be associated with many autoimmune diseases, and Mignot hypothesized that hypocretin was missing due to an autoimmune attack against hypocretin-secreting neurons. What the abnormality is in those narcolepsy patients with normal hypocretin levels remains a mystery.

MIGNOT MOVES FORWARD

Still a missing link. "I have been working on this [autoimmunity] hypothesis for 10 years, and we see that this hypothesis is more and more likely, but we cannot find any direct proof. It's frustrating, but that kind of struggle is the story of my life." All known autoimmune diseases result in the generation of antibodies in patients, but antibodies against hypocretin or the hypocretin cells have never been detected. So Mignot's lab tested whether T-cells were the immune component attacking hypocretin. In 2013, his lab published a study identifying the T-cell culprits. But the study was retracted by Mignot himself one year later, when Mignot's group couldn't reproduce the results after the scientist who did most of the experiments had left the lab. "It was really painful and the worst time in my career."

> "I want to study the genetics of 40,000 people with sleep issues. . . . I think this will help us crack open the mysteries of sleep."

A new lead. "In 2010, a lot of people suddenly started to develop narcolepsy after receiving the Pandemrix vaccine against swine flu. It's very odd. We still don't understand why this particular vaccine increased the risk of narcolepsy." Mignot thinks that a component of the vaccine or the virus itself triggers the immune system to attack hypocretin-producing neurons. "So now I am doing a lot of studies comparing the different vaccines and the wild-type virus to try to understand what could be common to produce this response. I think the vaccine will give us a final clue to isolate the immune T-cells involved in narcolepsy."

Genetics of sleep. Mignot's lab is working on a genome-wide association study, which shows that the genetic variants linked to narcolepsy are mostly immune-related, similar to Type 1 diabetes, celiac disease and other autoimmune diseases, further supporting the autoimmune hypothesis. Mignot is also getting a large human study off the ground. "I want to study the genetics of 40,000 people with sleep issues to see if there are genetic traits that cause people to sleep well or not sleep well, to need more sleep or less sleep. This hasn't been done yet. I think this will help us crack open the mysteries of sleep."

A new companion. "The dog colony was officially dismantled in 2000 after we found the canine narcolepsy gene. The dogs were adopted and we got Bear, a narcoleptic Schipperke. He passed away over a year ago. I loved that dog and miss him a lot. He was an unusually kind soul. Three months later, a breeder from Vermont called and said he had a narcoleptic Chihuahua. I flew to Vermont and adopted Watson and he's been with us ever since. I never would have thought to adopt a Chihuahua, but now I can't think of life without Watson. He is faithful and cuddly. I really think you can bond with any dog."

The journey continues. "This story of narcolepsy, it's a difficult story. Finding the gene was very difficult, and finding the autoimmune connection should have been trivial, but it has been an ordeal because there is absolutely no collateral damage. As [Stanford neurologist] Larry Steinman said to me, it's like a 'hit and run'—it looks like it was cleaned up and the players disappear. It's hard, but by learning about this disease, we may discover other diseases where a similar autoimmune destruction happens in the brain but we have never realized it. I wouldn't be surprised if some forms of depression and schizophrenia have an autoimmune basis in the brain. By experience, the more difficult it is, the most interesting the answer will be." ■

Christina Schmidt: Chronobiology Crusader

Research Fellow, Cyclotron Research Center, University of Liège. Age: 35

BY KAREN ZUSI

Christina Schmidt was catapulted into sleep research by an ad in a Belgian newspaper. She had earned her first degree in psychological sciences from the University of Liège in 2004 and completed an internship in a neuropsychology research lab under Philippe Peigneux as an undergrad. But at graduation, Schmidt didn't have a concrete plan for her future.

Her grandfather saw the newspaper ad, placed by Switzerland's Federal Commission for Scholarships for Foreign Students to announce open applications for research fellowships, and encouraged her to apply. On the advice of Peigneux, Schmidt wrote to a chronobiology lab run by Christian Cajochen at the University of Basel and secured the scholarship.

Inviting Schmidt to his lab "was a very good decision," says Cajochen. While working in the Swiss lab, Schmidt studied the relationship between memorizing a difficult or easy list of words and subsequent brain activity during a nap. Subjects who memorized the challenging words displayed more sleep spindles, or quick bursts of brain activity in their EEG recordings—and more sleep spindles meant better post-nap recall of the word list.[1] "If you're doing a nap during daytime, this might help you to consolidate your memories," explains Schmidt.

Two PhD students in the lab had already turned down this project by the time Schmidt arrived, but she tackled it head-on, Cajochen says. "She took the idea and thought about it, improved it, and excelled," he recalls. "This you would never expect from a master's student, but she's special."

While in Switzerland, Schmidt realized that she needed to look at sleep-wake cycles, not just sleep, if she wanted to gain a better understanding of the field. In 2005, she returned to Peigneux's lab at the University of Liège to begin her PhD. "We already knew her as a very discreet and modest student, but very serious and clever," says Peigneux, now a professor at the Université libre de Bruxelles.

Using fMRI, Schmidt compared people with extremely different chronotypes—either night owls or early risers whose circadian cycles were naturally offset from each other by about four hours—at times corresponding to the individual subjects' early and late hours. During fMRI sessions performed a few hours before participants settled in for their preferred bedtime, night owls were less sleepy, performed faster on an assigned task, and had more activity in arousal-promoting brain regions compared to the morning chronotypes, indicating a relationship between preferred circadian patterns and times of optimal alertness.[2]

In 2009, Schmidt moved on to a postdoc, working again under Cajochen. "I gave her a lot of freedom because I trusted her, and she did a great job," he says. For her research project, Schmidt demonstrated that individuals' responses to sleep deprivation at different times of the day are modulated by a polymorphism in a gene called *PERIOD3*.[3]

Schmidt came full circle in 2013, moving back to Belgium and taking a position as a research associate at the University of Liège's Cyclotron Research Center. She's currently writing up the remainder of her postdoc results and beginning to explore how sleep regulation changes with age. Of her now-deceased grandfather, Schmidt reflects, "He just said 'Look, try this out,' and now I'm here. He would be happy to hear about it, I think." ■

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Spoiler Alert

How to store microbiome samples without losing or altering diversity

BY WUDAN YAN

In the last decade or so, researchers have spent billions of dollars categorizing the microbes that populate sites as disparate as the human body, soil, and the oceans. Much of what we know about these different microbiomes has been determined using increasingly sophisticated next-generation sequencing technologies. For instance, scientists typically gauge a sample's microbial diversity by performing high-throughput sequencing on the gene coding for 16S rRNA, a component of the small subunit of bacterial ribosomes. Shotgun metagenomics is a complementary technique used for microbiome studies when a researcher aims to sample *all* the genes in all organisms in a sample. These DNA sequencing technologies are quite sensitive, and can pick up fine-scale changes caused by contamination or by the hazards of sample processing.

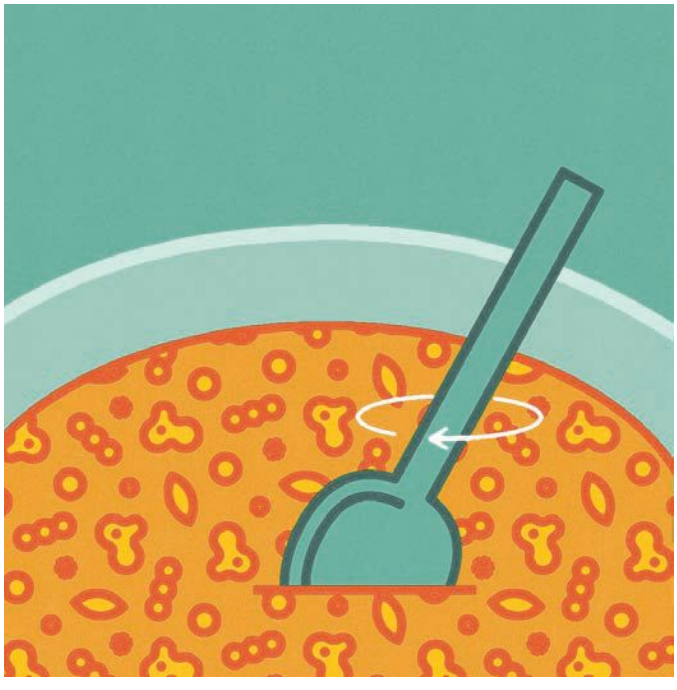
Because variations in handling and storage of samples can impact a study's results, maintaining the integrity of samples collected in the field is a major challenge in microbiome research. Vanessa Hale, a microbial ecologist and postdoctoral fellow at the Mayo Clinic in Rochester, Minnesota, says the gold-standard procedure is to extract DNA or RNA from a fresh sample immediately. When immediate processing is not feasible, it's best to store samples at –80 °C. The microbial composition of a fecal sample starts to shift after one to two days at room temperature (*PLOS ONE*, 7: e4695, 2012; *Open Microbiol J*, 3:40-46, 2009); other samples, such as soils, are similarly temperature-sensitive. However, having access to an ultra-low-temperature freezer may not always be possible. Study subjects may collect fecal samples at home, for example, or researchers might sample soil at remote sites. In some scenarios, samples must be stored and preserved for days or weeks before DNA and RNA can be extracted and analyzed.

"The microbiome is so horrendously diverse and complex," says Jack Gilbert, a microbial ecologist at the University of Chicago. "Any particular thing you do will change some part of [it]." As technology allows for more-in-depth investigations of microbial environments, even the smallest changes in the presence or relative abundance of bacterial populations can interfere significantly with community comparisons. The fewer variations a storage method allows, the better.

The Scientist spoke with microbiome experts to bring you the low-down on how to store samples in order to preserve their bacterial diversity.

GIVE IT A GOOD MIX OR TWO

Maintaining the fidelity of the original sample starts from the moment of collection. Step one is ensuring that you have a homogenous sample. "If a sample is not homogenized, homogenize it," says Kim Heylen, a microbiologist at Ghent University



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Even the smallest changes in the presence or relative abundance of bacterial populations can interfere significantly with community comparisons. The fewer variations a storage method allows, the better.

in Belgium. "[Doing so] is crucial if you want to have reproducible subsamples."

Holly Ganz, a microbiologist and postdoctoral fellow at University of California, Davis, started KittyBiome, a nonprofit that investigates the microbiomes of both wild and domestic cats, generally using samples of their feces. "Poop is not homogenous," Ganz says. "And that's an issue: which part of the poop you sample will influence your results." Ganz homogenizes her sample by first removing the exterior layer, which could contain environmental contaminants, and putting the rest into a plastic bag to manually mush and mix together. Next, she spreads the sample out and collects subsamples from across the sheet. "It's really difficult to homogenize poop, so I just increase how many subsamples I take," she says.

In a recent study of the human gut microbiome, researchers used a mortar and pestle to mash stool samples in liquid nitrogen before partitioning the resulting powder into smaller samples, transferring to test tubes, and freezing at –80 °C. Samples prepared by this method showed reduced variability in microbial composition compared with subsampling from unhomogenized fresh samples (*PLOS ONE*, 10:e0134802, 2015).

Soil has an even more complex matrix. Gilbert suggests putting your samples in a blender to break down the particles as much as possible. "It's impossible to homogenize completely," he says.

Researchers also suggest making multiple aliquot replicates after collecting the initial sample, because freeze-thaw cycles degrade the quality of nucleic acids. For instance, scientists have used sterile, disposable biopsy punches to collect frozen stool pellets of approximately the same size from a larger, original sample. The pellets are then put directly into extraction tubes containing a nucleic acid lysis buffer. This method saves scientists a full freeze-thaw cycle just for aliquoting the original sample.

SHOULD I USE A CRYOPRESERVANT? IF SO, WHICH?

Cryopreservants—buffers that help stabilize the nucleic acids in a given sample—offer a low-tech, cold-storage solution. Cryopreservation protects cells from mechanical injuries caused by ice formation and fluctuations in freeze temperature.

Which cryopreservant to use depends largely on your plans for the sample. If you intend to culture the microbes or otherwise need to keep them alive, you might use glycerol, which prevents ice crystals from forming at temperatures as low as –20 °C to –80 °C. OpenBiome, a nonprofit stool bank based in Medford, Massachusetts, collects fecal material from healthy subjects to treat patients with *Clostridium difficile* infections. Because the success of the fecal transplant treatment depends on having viable microbes, the samples are mixed with a saline solution containing 12.5 percent glycerol, says Daniel Blackler, OpenBiome's quality manager, and stored at –20 °C for up to six months and at –80 °C for up to two years.

Researchers have more choices if they only plan to investigate nucleic acid composition. For this type of study, the samples are added to a lysis buffer prior to storage. Although few studies have compared the effects of different cryoprotectants on maintaining the diversity of the original sample, available evidence suggests that cryopreservants can affect the presence of different species of bacteria to varying degrees (*J Microbiol Methods*, 113:16-26, 2015). "The key is in choosing a method that is feasible and appropriate for your study while also being aware of [its] potential biases," Hale advises.

Overall, researchers have found that adding a cryopreservant is helpful for human samples. One quick way to evaluate cryopreservation success before comparing the microbial composition of your stored and fresh samples is to look at your yield of DNA, Ganz notes. "If you don't get enough DNA, you're not getting a good enough representation of the microbiome," she says.

DMSO + Trehalose & Tryptic Soy Broth

Heylen uses a solution of dimethyl sulfoxide (DMSO) with trehalose and tryptic soy broth that helps maintain fidelity of frozen samples in three different microbiome systems (*PLOS ONE*, 9:e99517, 2015). "Trehalose protects against cell drying, and tryptic soy broth is an additional cryopreservant," says

Heylen. She advises bringing the solution to a temperature of around 4 °C and adding it right before the sample is placed in an ultra-low-temperature freezer. "If you don't have access to a –80 °C freezer, you can use a household freezer. But definitely freeze your sample as soon as possible, and don't freeze without a cryopreservant."

RNA*later*

Originally designed as an RNA preservant, RNA*later* is an aqueous, nontoxic solution sold by manufacturers such as Qiagen (50 mL for $78; 250 mL for $324) and Thermo Fisher (100 mL for $125; 500 mL for $386) that can also be used to preserve DNA. In their work with the Human Microbiome Project, computational biologist Curtis Huttenhower of the Harvard T.H. Chan School of Public Health and his colleagues found that adding the preservant after sample collection can help if a freezer is not available.

But RNA*later* comes with some user-reported downsides. While the fact that it is not flammable makes the preservant a suitable choice for shipping samples, RNA*later* significantly reduces the population of *Bacteroidetes* and *Ruminococcaceae* compared with freshly extracted samples. Another challenge is that it must be removed before the sample is processed to get a good RNA yield. "There's something about RNA*later* that prevents the sample from pelleting normally, so after you remove the RNA*later*, you have to wash the pellet a second time with phosphate-buffered saline," Ganz advises.

Another potential problem with RNA*later* is its high salt content, which can make sample clean-up a pain for some protocols, according to Mak Saito, a marine biochemist at the Woods Hole Oceanographic Institution in Massachusetts. Saito has used the solution to help preserve protein composition in marine microbiomes (*Front Microbiol*, 2:215, 2011).

Ethanol

Ethanol is a cheaper alternative to RNA*later* and has been shown to stably preserve the community structure of fecal samples for more than eight weeks, even at room temperature (*J Microbiol Meth*, 113:16-26, 2015). However, ethanol is flammable, so it can't be used in samples that will be shipped. What's more, cryopreservation with ethanol leads to an increase in *Cyanobacteria* compared with freshly collected and analyzed samples. "All these storage methods introduce bias, so it's important to [always] use the same method in your study," says Ganz, who keeps her cat feces samples in 70 percent ethanol upon the recommendation of colleagues who store spider monkey feces.

No cryopreservant

For some types of samples, adding a cryopreservant changes the composition too much to be worthwhile. That's the case for soil, says Gilbert. He suggests finding a way to freeze soil samples at –80 °C within 48 hours. "We want to look at the community composition and structure at the time the sample was collected," he says. In situations where soil samples are very moist, Gilbert recommends freezing them immediately, because high moisture content can cause certain types of microbes to proliferate.

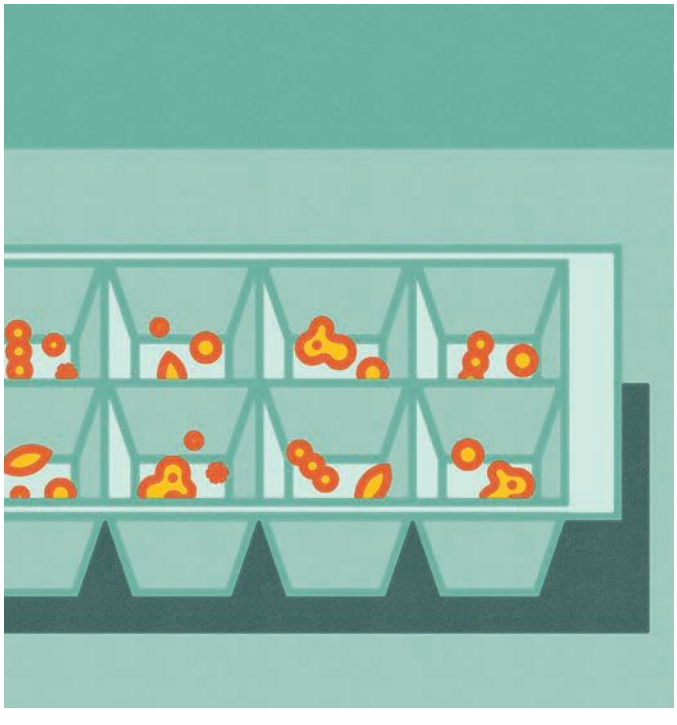
With fecal samples, too, there are times when cryopreservation is not appropriate. Samples that will be used to treat *C. difficile* infections, for example, can sit at room temperature for up to six hours without significant changes in the quality of treatment, Blackler says.

OTHER TEMPERATURE CONSIDERATIONS

Microbiome samples are very sensitive to freeze/thaw cycles. "Thawing can present serious biases for microbiome samples," Hale says. "DNA degrades with each freeze/thaw cycle, the opportunity for contamination and microbial blooms can occur when samples thaw, and volatile compounds are lost and cannot be recovered for potential metabolomics analyses."

If only a –20 °C freezer is available, then it is best to use one that is dedicated solely to storing microbiome samples. "If a –20° freezer is continually being opened, then the temperatures can become so variable that the success of storage decreases significantly," Heylen says.

At OpenBiome, researchers keep frozen samples at –20 °C for up to six months before transferring them to longer-term storage at –80°. Switching the samples to the ultra-low temp freezer should not negatively affect the microbes, says Blackler. However, "a sudden rise in the temperature should probably be avoided as this could impact the microbial community." ■



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Dial It Up, Dial It Down

Newer CRISPR tools for manipulating transcription will help unlock noncoding RNA's many roles.

BY KELLY RAE CHI

One of the biggest surprises to come from sequencing efforts of the past 15 years is how little of the human genome is translated into proteins. We have about as many protein-coding genes, 20,000, as the roundworm *Caenorhabditis elegans*. And yet, roughly 80 percent of our genome is transcribed into RNA. Long or short, looping or straight, rigid or not, most of this rabble of transcripts never crosses what was once thought of as molecular biology's finish line by being translated into proteins. Those RNAs may well harbor some explanations for why we differ from worms, and they often turn up in genome-wide studies as being associated with disease. But most of these so-called noncoding RNAs have no known function.

That's where CRISPR/Cas9 serves an important role. Soon after scientists developed the system as a gene-editing method, they went to work on versions they could use to dial gene expression up or down, not by cutting genes and inserting new genetic material, but by having Cas9 take up residence on predetermined sites on the genome to initiate or stop transcription. These innovations, known as CRISPR activation (CRISPRa) and CRISPR inhibition (CRISPRi), are allowing users to tweak the expression not only of protein-coding genes but also of genes for noncoding RNAs to probe the functions of those transcripts.

Although more researchers are beginning to use CRISPRa and CRISPRi, the methods are—like anything CRISPR—still new. "When it comes to these technologies, we are all beta testers," says Jacob Corn, scientific director of the University of California, Berkeley's Innovative Genomics Initiative.

The Scientist talked to developers and users to bring you this basic primer on employing CRISPRa and CRISPRi to study and tweak the expression of both coding and noncoding regions of the genome.

CRISPRi

CRISPRi refers to a set of techniques that inhibit transcription of a given target, whether that's a stretch of DNA that controls the transcription of a protein-coding mRNA or the transcription of a noncoding RNA gene. The conventional Cas9 nuclease is a large protein that complexes with a guide RNA strand that directs Cas9 to a chosen spot on the genome, where the enzyme cleaves the DNA. CRISPRi uses a catalytically inactive or "dead" version of Cas9 (dCas9), which still arrives at its programmed destination but is unable to cut it (*Cell*, 152:1173-83, 2013). While the dCas9 is latched onto the genome, it prevents transcription by physically blocking binding of the necessary machinery.

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An improved version of CRISPRi attaches a domain from a transcription silencer called the Krüppel-associated box (KRAB) to dCas9. This add-on of about 50 amino acids prevents DNA from uncoiling for transcription. "Right now, using the KRAB domain seems to be working really well," Corn says. Andrew Bassett of the University of Oxford agrees, noting that if you're just starting to use CRISPRi you should use KRAB. Although it may act over a larger site than the dCas9 alone, it is more likely to give you an observable effect, he adds.

The reversibility of CRISPRi is either a feature or a bug, depending on your goals. Using the KRAB domain attachment, some researchers are working to make dCas9's binding more stable, opening the possibility of permanently inhibiting transcription at specific places on the genome to study if and how man-made epigenetic marks are passed along to future generations, Corn says.

RNAi or CRISPRi?

Whereas RNA interference (RNAi) targets mature RNA, CRISPRi prevents transcription from occurring in the first

place. That has advantages in situations where the act of transcription, rather than a specific RNA product, might be at the heart of the function you're studying. Another plus of CRISPRi is that it can be used to target transcripts within the nucleus, which has been difficult to target with RNAi reagents.

Off-target effects have long been a concern with RNAi. And although CRISPR machinery has its own off-target effects, they are far fewer, and for them to make a difference in CRISPRi, they would have to act near transcription start sites. Researchers are more knowledgeable now about RNAi problems, Corn says. For that reason, his team still uses it, though he can imagine it playing a smaller role in the future.

CRISPRa

Researchers have also attached transcriptional activators—various proteins that work in concert to boost the reading of DNA—to the dead Cas9, creating CRISPRa tools. In contrast to cDNA overexpression, which is commonly used to dial up expression of individual genes, CRISPRa more easily and inexpensively activates single genes as well as multiple genes at the same time. This fact could lead to CRISPRa's becoming a gold standard in the study of RNA, says Mitchell O'Connell, a postdoctoral researcher in the lab of CRISPR pioneer Jennifer Doudna at the University of California, Berkeley.

Several next-generation CRISPRa systems are already available. One of them, designed by CRISPR developer Feng Zhang of MIT, optimizes the recruitment of the activation machinery by modifying both dCas9 (by attaching a VP64 domain, a transcription activator derived from a herpes simplex virus protein) and the single-guide RNA (by incorporating two looping stretches of RNA that in turn recruit several transcriptional activators). Those tweaks have allowed the scientists to boost RNA expression by more than an order of magnitude (*Nature,* 517:583-88, 2015).

Zhang's lab has also figured out a way to control a gene's level of transcription. By positioning the system closer to the transcription start site you get stronger activation. However, if you target upstream of the site, just outside a 200-base-pair window, you can trigger more-modest—and perhaps, depending on the gene, more physiologically relevant—activation, says Silvana Konermann, a postdoctoral researcher in Zhang's lab who developed the system. In unpublished work, she has used this trick to see a linear correlation between the amount of gene expression and the strength of her phenotype.

Also developing activators and specifying their rules for use is Jonathan Weissman of the University of California, San Francisco. His team's CRISPRa system is a string of up to 10 short peptides (called the SunTag array) fused to dCas9 that works by recruiting transcriptional activators within the cell (*Cell*, 159:

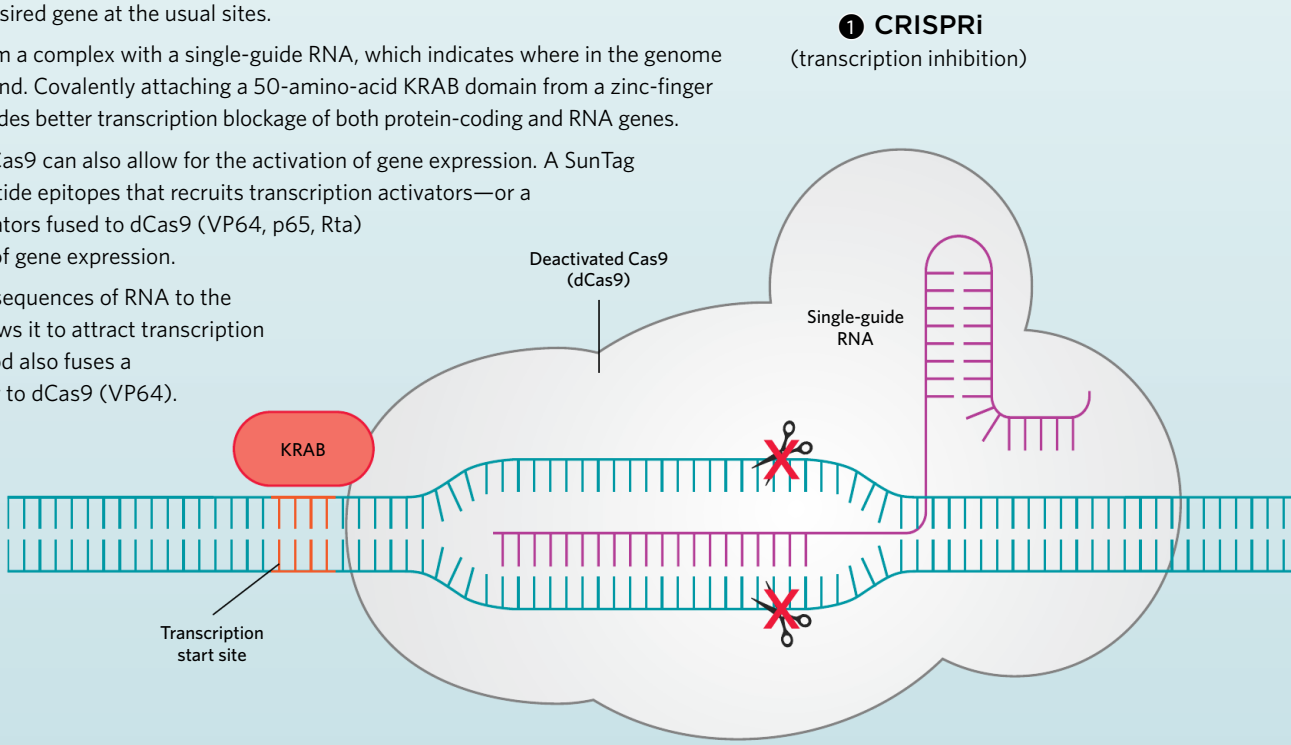
NEW CRISPR TOOLS FOR STUDYING TRANSCRIPTION

Both CRISPRi and CRISPRa rely on a mutated version of the Cas9 endonuclease (dCas9) that cannot cut the desired gene at the usual sites.

❶ dCas9 can still form a complex with a single-guide RNA, which indicates where in the genome the complex should bind. Covalently attaching a 50-amino-acid KRAB domain from a zinc-finger protein to dCas9 provides better transcription blockage of both protein-coding and RNA genes.

❷ Attachments to dCas9 can also allow for the activation of gene expression. A SunTag array—a string of peptide epitopes that recruits transcription activators—or a tandem array of activators fused to dCas9 (VP64, p65, Rta) enhance the amount of gene expression.

❸ Adding particular sequences of RNA to the single-guide RNA allows it to attract transcription activators. This method also fuses a transcription activator to dCas9 (VP64).



647-61, 2014). "That's the one we're using right now, and that's because it's the whole package," Corn says. "You get the activator; you know the rules for using that activator really well."

Yet another improved CRISPRa, reported by CRISPR pioneer George Church of Harvard University, includes three activation domains (VP64, p65, Rta) in tandem that are fused to the tail end of dCas9 (*Nature Methods*, 12:326-28, 2015). Of the newer transcription activation systems, Bassett says he has had the most success with this one (though, unlike Weissman's and Zhang's, it has not yet been used for large-scale gain-of-function screens).

But experts caution that it's too soon to tell which of these activation techniques will come out ahead, so the advice is to just give one or more of them a try. Besides, something newer and better is probably in the pipeline. "The onus is on users of the technology to find out if it works in their particular system for their particular question," Corn says. "There are no guarantees." One of those users, Uttiya Basu of Columbia University, agrees. He plans to use CRISPRi and CRISPRa in mixed populations of primary immune cells, which vary in their ability to take up the machinery. In contrast, most work to date has employed CRISPR in established cell lines. "Different cells have different abilities to take up things," Basu says. "Primary cells die after a period," which gives you a limited time frame in which introduce CRISPR systems.

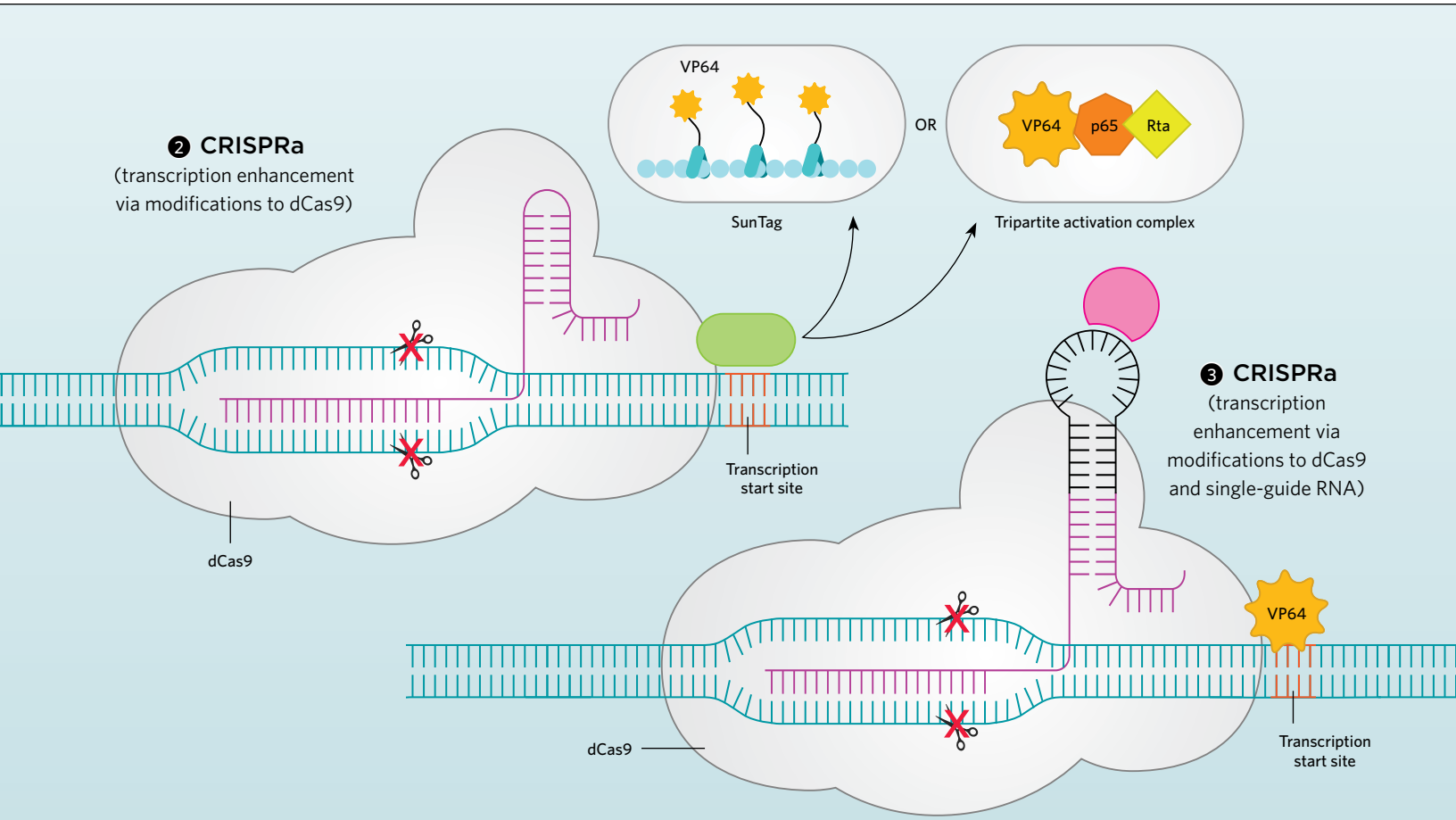
Getting these new Cas9s into cells

It's still a challenge to deliver any Cas9 protein into cells, especially those modified for CRISPRi and CRISPRa. One way to get around that, at least for CRISPRa, is to use CRISPR-Cas9 mutant mice that express Cas9 all the time or after being induced. Konermann found that she can deliver a shortened guide RNA into the mouse: 14 or 15 base pairs instead of 20, packaged in a virus (preferably adeno-associated virus, AAV) and delivered via injection. This pared-down guide no longer allows Cas9 to cleave DNA, but, in the case of Zhang's system, it includes the loops that recruit transcriptional activators.

There is also a smaller version of Cas9, from *Staphylococcus aureus* rather than *Streptococcus pyogenes*, that can be packaged into AAV for delivery into cells. Because of some design differences, the *S. aureus* Cas9 targets fewer sites. But, for an activator, that's not a major issue, Konermann says.

How many guides should you test?

If you are studying noncoding RNAs, expect to make more single guide RNAs (sgRNAs)—each about 20 nucleotides long—than the number you would make to edit a protein-coding gene. Bassett suggests anywhere from 5 to 10 for CRISPRi. (For gene editing he normally makes three guides.) "I would go for almost as many as you can in the appropriate window, which is usually around 100–200 bases before the transcription start site," he



says. Using computational tools to predict off-target effects can help you narrow down guide selection.

For CRISPRa, Konermann had to make more sgRNAs to suss out ones that would activate the transcription of genes for noncoding RNAs compared with coding RNAs. "It might just be that our annotation of the transcription start sites are not as good for noncoding RNAs," she says. "Or it could be that they're somehow still more difficult to activate." But the technique works, and it's something the group is actively pursuing, she adds.

Special considerations for investigating noncoding RNAs

CRISPRa and CRISPRi are a boon to the study of noncoding RNAs. One challenge of using these technologies to study noncoding RNAs, especially the lengthy ones, is that the noncoding RNAs often share a regulatory region with protein-coding genes. It's hard to get around this problem. But you can at least account for it: check what genes are nearby and do a round of polymerase chain reaction (PCR) or a Western blot to see whether the expression of those genes has also changed along with your intended target.

If the inhibition or activation of an RNA does affect a neighboring gene, it may be unclear what it is about your setup that is causing the change. "The good news is that by using all those tools together in a smart way it starts to become possible to sort out these questions," Konermann says.

And, although they are powerful, CRISPRa and CRISPRi should be part of a much larger set of experiments for deciphering the functions of a given noncoding RNA. Disabling the transcript using CRISPR-based gene editing is another important strategy to help validate your results, for example. "If you really think there is a function for a long noncoding RNA, you have to prove it with multiple assays and, ideally, coming from different directions," says Jens Durruthy-Durruthy, a postdoctoral researcher in the Stanford University laboratory of Vittorio Sebastiano who employs Zhang's CRISPRa method with traditional CRISPR editing to study the role of long noncoding RNAs in human development and cancer.

What's next?

Weissman's group is working on another round of guide RNA library designs that will allow researchers to more efficiently target transcription start sites on the genome.

O'Connell is repurposing a version of the Cas9 protein, called RCas9, that recognizes and cleaves RNA rather than DNA. Although in theory it shows promise for functional studies of noncoding RNAs, the main challenge is to get it working efficiently in cells (as opposed to cell lysates).

Meanwhile, the search is on for naturally occurring CRISPR systems that edit RNA directly, a simpler version that will be easier to introduce into cells. "I'm sure it exists," Bassett says. ■

Desperately Seeking Shut-Eye

New insomnia drugs are coming on the market, but drug-free therapy remains the most durable treatment.

BY ANNA AZVOLINSKY

In the early 1970s, a colleague of Stanford University's William Dement remarked on the resemblance of a narcolepsy patient's symptoms to those of a recent canine patient he had read about. The similarity of the symptoms—excessive daytime sleepiness, sudden switch from an awake state to rapid eye movement (REM) sleep, sleep paralysis, and muscle weakness called cataplexy—prompted psychiatrists at the center to track down a narcoleptic dog of their own to study, and then to gather a kennel full of such dogs to figure out what caused the disease. When Dement bred two affected Doberman pinschers in 1976, he found that their narcolepsy was genetic; many of the puppies had episodes of muscle cataplexy and would collapse into sudden sleep, especially when excited.

In 1986, Emmanuel Mignot came to Stanford to work with the narcoleptic dogs, first to evaluate the effects of different narcolepsy drugs and then to tease out the molecular basis of the disorder. More than a decade later he discovered an autosomal recessive mutation in the orexin receptor in the dogs' brains that was responsible for the disorder (*Cell*, 98:365-76, 1999). (See "In Dogged Pursuit of Sleep" on page 52.) Although orexin receptor mutations have not been found in humans with narcolepsy, patients with the disorder do have reduced levels of orexin (also called hypocretin), a neuropeptide that regulates wakefulness (*The Lancet*, 355:39-40, 2000).

"The biological basis for orexin mediating wakefulness was pretty strong," says Joseph Herring, neuroscientist and executive director of the clinical neuroscience program at Merck Research Laboratories. And if lower levels of orexin were associated with less shut-eye, perhaps stimulating the pathway could restore normal sleep, he adds. "If you antagonize the downstream [orexin] receptors with small molecules, you may have an effective sleep therapy by dampening the wakefulness."

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In 2014, the Food and Drug Administration (FDA) approved the first orexin receptor antagonist, Merck's suvorexant (Belsomra), and at least two more drugs of this class are currently in clinical trials. Previous sleep medications acted as sedatives, targeting GABA receptors to facilitate brain inactivity. Suvorexant, on the other hand, decreases wakefulness by blocking the brain's orexin receptors. "The orexin system is a super candidate as a target for insomnia treatment," says Michael Perlis, director of the Behavioral Sleep Medicine Program at the University of Pennsylvania. "There are lots of reasons to believe some people with insomnia are hypersecretors of orexin, which is why they can't sleep. Toning down orexin is a brilliant idea."

Treating insomnia

As many as 40 million people in the U.S. experience some form of insomnia, making it the most common sleep disorder. Sufferers can have trouble falling asleep, staying asleep, or returning to sleep after waking in the middle of the night, and can experience persistent drowsiness, irrita-

bility, anxiety, and difficulty learning and remembering the next day. When chronic, insomnia can impede daily functions and increase the risk of cardiovascular disease.

Insomnia used to be divided into primary insomnia, which is not linked to any other medical condition, and secondary insomnia, trouble sleeping due to an underlying condition such as depression or chronic pain. But in 2013, the American Psychiatric Association eliminated the two subtypes, hoping to bring clinical attention to the sleep disorder regardless of other clinical issues. "There is increasing evidence that insomnia can be a cause or consequence of depression and other issues," says Charles Morin, a clinical psychologist who studies sleep and insomnia at Laval University in Quebec City.

But researchers and doctors still distinguish between acute insomnia—short-term bouts of sleeplessness triggered by a stressful event such as an important exam or a job loss—and chronic insomnia, when someone does not sleep well at least three nights a week for three months. To treat acute insomnia, doctors often turn to drugs, such as zolpidem (Ambien). For chronic insomnia, sleep experts recommend combining a drug, to take the edge off initially, with cognitive behavioral therapy for insomnia (CBT-I), which focuses on relaxation training, sleep hygiene (e.g., limiting caffeine before bed), stimulus control, cognitive therapy (changing beliefs and habits to promote sleep), and limiting the amount of time spent in bed not sleeping. Most physicians treating chronic insomnia aim to phase out the drug as the patient begins to have a more regular sleep pattern (*JAMA*, 301:2005-15, 2009). "There is an ongoing evolution of therapy for insomnia," says David Neubauer, an associate professor of psychiatry and behavioral sciences at Johns Hopkins School of Medicine and associate director of Johns Hopkins Sleep Disorders Center. "The use of hypnotic drugs [is] never more than an adjunct therapy."

David Cunnington, a physician and director of the Melbourne Sleep Disorders Centre in Australia, and colleagues found in a recent meta-analysis that CBT-I is effective; patients treated with CBT-I took an average of 19 fewer minutes to fall asleep and slept for an extra 26 minutes (*Annals of Internal Medicine,* 163:191-204, 2015). But CBT-I can be expensive and difficult to access, and can require as many as eight sessions with a certified physician. To broaden insomnia patients' access to CBT-I, researchers have created at least two commercial online programs backed by scientific evidence—SHUT*i* (Sleep Healthy Using the Internet), developed in collaboration with Morin, and Sleepio. "These may not be as effective as therapist-delivered CBT-I but are more effective than self-help," says Cunnington.

None of the available sleeping pills are curative, and therapy with hypnotics is considered a form of palliative care.

—Michael Perlis, University of Pennsylvania

Still, the easiest form of insomnia treatment, at least in the short term, is a drug. Benzodiazepines (also known as "benzos" or "bennies"), discovered in 1955, were the first class of drugs used as sleeping pills. These agents, of which temazepam (Restoril) is now the most commonly prescribed for insomnia, bind to GABA receptors to enhance the sedative effects of the neurotransmitter. By the 1970s they had replaced the use of barbiturates, which also target the GABA system. Although effective at increasing total sleep time in some, benzos—also used to treat anxiety, panic disorder, and other psychiatric disorders—decrease the amount of time in those stages of sleep that are associated with cognitive restoration. "Often people with insomnia are desperate for more minutes of sleep, thinking that is what will result in them feeling better," says Cunnington. "Yet no study of benzos has shown improvements in daytime functioning, even though they increase sleep length." The drugs can also be abused.

A newer class of GABA receptor agonists, the so-called Z-drugs, was approved for insomnia in the 1990s. These agents, including zolpidem, zaleplon, and eszopiclone, are chemically different from benzos: they tend to be more readily absorbed for faster sleep onset, and they have a shorter half-life that decreases next-day grogginess. The range of half-lives of these newer hypnotics gives clinicians good choices to customize the sleep aid based on sleep-disturbance pattern and other patient characteristics, says Neubauer. But, while Z-drugs "appear to improve sleep quantity without impairing sleep quality, they can also cause memory impairment, and some clinicians remain concerned about the drugs' safety profiles," adds Perlis (*BMJ Open,* 2:e000850, 2012).

Like benzos, Z-drugs are effective for some insomniacs, but for others they decrease the amount of certain stages of sleep and can lead to a lack of concentration as well as memory impairment. Other agents approved as sleep medications include melatonin receptor agonists, antihistamines, and antidepressants, but these have limited efficacy, according to most sleep experts.

"We've come to understand the physiology of insomnia better. For some it has to do with overactivation of the alertness system rather than a problem with the brain's sleep-promoting system," says Cunnington. "Something that blocks the alerting system, as suvorexant does, may be a better fit than [GABA-targeting drugs] that promote components of the brain's sleep system."

Reinventing the sleeping pill

Suvorexant, approved by the FDA in August 2014, is a dual orexin receptor antagonist (DORA), meaning it binds to orexin receptors 1 and 2. In a long-term study, 521 patients who received 40 mg of the drug nightly (30 mg for those older than 65) for 12 months took less time to

fall asleep and stayed asleep throughout the night (*Lancet Neurol,* 13:461-71, 2014). And while suvorexant can slightly increase the duration of REM sleep, the drug maintains the sequence and cycling of the REM/non-REM stages typical of normal sleep.

Initially, there were concerns that drug doses that dampened most orexin activity could lead to cataplexy or narcolepsy-like symptoms, says Matthew Ebben, a psychologist at the Weill Cornell Medical College in New York City. But according to the company's clinical trial data, there were no signs of these side effects in the trial participants, though some did experience next-day sleepiness and a few rare cases of sleep paralysis not linked to narcolepsy. As a result, the FDA approved administration of the drug only at the two lowest (15 milligrams and 20 milligrams) of the four doses tested in the Phase 3 trials, and, subsequently, at two doses that are even lower (5 mg and 10 mg).

At least two other orexin receptor–targeting drugs are now in development. Tokyo-based Eisai and Connecticut-based Purdue Pharma are set to begin a global Phase 3 trial with a DORA called lemborexant. And Belgium-based Janssen Pharmaceutica NV, a Johnson & Johnson company, is teaming up with Massachusetts-based Minerva Neurosciences to test JNJ-42847922, an orexin-2 single receptor antagonist (2-SORA). Also known as MIN-202, the drug is being evaluated for insomnia in a Phase 1 trial of patients who also have major depressive disorder as well as in a Phase 2 study of insomnia patients without comorbidities.

Whether it's better to target the two orexin receptors or just one remains to be determined. While DORAs typically result in faster REM sleep onset and slightly increase the total amount of REM sleep, rodent studies have shown that orexin receptor 2 mediates most of orexin's sleep effects. Targeting orexin receptor 1, for example, does not appear to improve animals' ability to fall asleep (*Neuron,* 38:715-730, 2003; *JPET,* 330:142-151, 2009). But 2-SORAs may not increase total REM sleep time to the same extent as suvorexant, says Neubauer. "There is a lot of debate right now on the potential risks and benefits of DORAs versus 2-SORAs."

Rethinking insomnia

Even as more orexin-targeting sleep drugs make it to market, a fundamental challenge to treating insomnia remains: the risk of psychological addiction. Most clinicians prescribe insomnia pills to be used sparingly, only a few times per week. "But this is a disaster because on the nights the patient doesn't take the pill, he expects to sleep poorly, and learns that he can't sleep without a pill," says the University of Pennsylvania's Perlis. In a recent pilot study of 55 individuals, Perlis and his colleagues found that those who took a placebo on nights they would have skipped a dose maintained the effect of the sleeping drug and felt more rested (*Sleep Medicine*, 16:1160-68, 2015). On nights without a pill, those who followed the standard intermittent dosing slept more poorly and had next-day insomnia symptoms.

The results point to the need for smarter approaches to insomnia treatments that tap into what is known about human behavior and psychology. "Insomnia is complex and heterogeneous, and patients have different degrees of response to interventions," says Herring.

Even with the new class of orexin-targeting drugs, doctors who work with sleep-disorder patients still see medication as playing a supportive role to CBT (and possibly its more readily accessible self-administered versions). "None of the available sleeping pills are curative, and therapy with hypnotics is considered a form of palliative care," says Perlis. "Only CBT-I appears to confer durable results lasting months and years after treatment is discontinued."

Most importantly, there is still a need for recognition of insomnia as a clinical condition. "We need to stop trivializing insomnia," says Morin. "There is clear evidence that it's a significant health problem—a risk factor for depression, hypertension, and other medical conditions. Doctors need to pay attention to it, and there are effective treatments available." ■

In Your Dreams

Understanding the sleeping brain may be the key to unlocking the secrets of the human mind.

BY DAVID GELERNTER

Many scientists who study the mind live in fantasyland. They ought to move back to reality: neuroscientists, psychologists, computer scientists pursuing artificial intelligence, and the philosophers of mind who are, in many cases, the sharpest thinkers in the room.

The mind makes us rational. *That* mind is the one we choose to study. When we study sleep or dreaming, we isolate them first—as the specialized topics they are. But, as I argue in my new book *The Tides of Mind*, we will never reach a deep understanding of mind unless we start with an integrated view, stretching from rational, methodical thought to nightmares.

Integrating dreaming with the rest of mind is something like being asked to assemble a car from a large pile of metal, plastic, rubber, glass, and an ocelot. Dreaming is hallucination, centering on a radically different self from our waking selves, within unreal settings and stories. Dreams can please or scare us far more vividly than our ordinary thoughts. And they are so slippery, so hard to grasp, that we start losing them the moment we wake up.

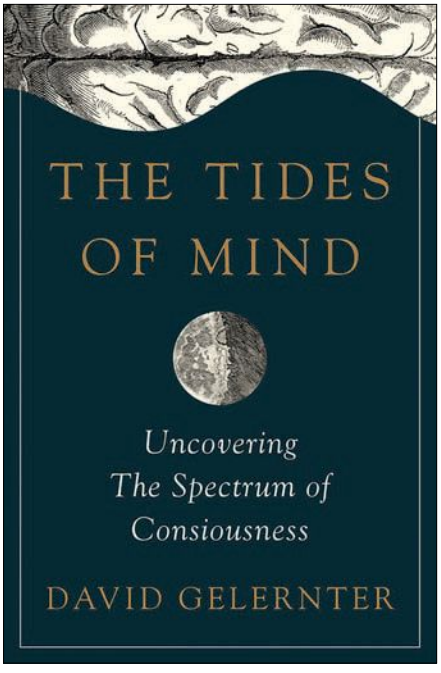
But dreaming fits easily into the big picture of mind; and we will make no basic progress on understanding the mind until we see how. Dreaming is the endpoint of the spectrum of consciousness, the smooth progression from one type of consciousness to the next, that we each experience daily.

The simplest approach to the spectrum centers on mental focus. The quality of our attention goes from concentrated to diffuse over the course of a normal day; from a state in which we can concentrate—we can think and remember in a relatively disciplined way—to one in which, with our minds wandering and memory growing increasingly vibrant and distracting, we approach sleep. Then our thinking becomes hallucinatory (as we pass through "sleep-onset thought"); and finally, we are asleep and dreaming. Usually, we oscillate down and up more than once during the day. We move partway down, come partway back, then finally slide slowly to the bottom, when we sleep and dream.

We can also describe the spectrum as a steady shift from a mind dominated by action to one dominated by passive mental experience; from mental doing to mental *being*. In the upper spectrum, we tend to ignore emotion as we pursue some mental object by means of reasoning or analysis. But the daydreams and fantasies that occupy us as we move down-spectrum are often emotional. And in dreaming we encounter the most saturated emotions, good and bad, that the mind can generate.

The spectrum clarifies important aspects of the mind. "Intentionality," the quality of aboutness ("I believe that bird is a sparrow" is about "that bird"), is sometimes called "the mark of the mental"—the distinguishing attribute of mental states. But intentionality belongs strictly to the upper spectrum, and disappears gradually as we descend. At the bottom, our minds are dominated by experience, pure being. Happiness or pain or "the experience of seeing purple" are states that have causes but are about nothing.

Software simulations of the upper spectrum, of thinking-about, have grown steadily stronger over the years. That trend will continue. Being, however, is not computable. Software can no more reproduce "being happy" than it can reproduce "being rusty." Such states depend on physical properties of particular objects. A digital computer resembles only the upper-spectrum mind. Software will never come close to reproducing the mind as a whole.

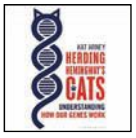


Liveright, March 2016

Leaving sleep outside our investigation is a good way not to see any of this. Arbitrarily hacking off one end of any natural spectrum is an invitation to conceptual chaos. There has been plenty of that in the science of mind. We must start by understanding sleep and dreaming, and go from there. ■

David Gelernter is a professor of computer science at Yale University. Read an excerpt from his latest book, The Tides of Mind: Uncovering the Spectrum of Consciousness *at the-scientist.com.*

Herding Hemingway's Cats: Understanding How Our Genes Work
Kat Arney
Bloomsbury Sigma, March 2016



Mendel had his peas, Darwin his finches. Geneticist Kat Arney (perhaps unsurprisingly) chooses cats as her biological muse. But the U.K.-based science writer doesn't pick just any felines to illustrate science's emerging understanding of gene regulation—she leans on the six-toed cats popularized by Ernest Hemingway, whose Florida estate still teems with the polydactyl felines. "Learning about Hemingway cats and their broken [genetic] switches got me thinking about my own understanding of how genes work," Arney writes in the introduction to *Herding Hemingway's Cats*.

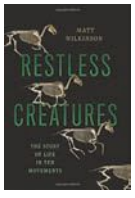
The author explains that it's not faulty DNA that leads to polydactyl cats or humans, but rather mistakes in the molecular machinery that ushers genes through the processes of expressing their proteins at the right times and places during development. What follows is an engaging journey through the science, both historical and cutting-edge, of the complexities of genetic functioning. Arney wants the reader to understand that we have a lot to learn about how DNA works—moving away from the concept of DNA as a blueprint and closer to that of an orchestral score, where a conductor and several musicians interpret, modulate, and bring the tune to life.

Hair: A Human History
Kurt Stenn
Pegasus Books, February 2016

Virtually everyone who has hair fusses over it (Bernie Sanders types notwithstanding). But few of us amateur *coiffeurs et coiffeuses* understand the stuff to the extent that biologist Kurt Stenn does. The former Yale dermatology professor and current hair-follicle biotech CSO explores the history, biology, and cultural significance of tresses in his book *Hair: A Human History*.

Chapters on the evolution of hair-like structures in long-extinct mammalian ancestors, on the somewhat mysterious cellular biology of hair follicles, and on pathologies of hair set the stage for Stenn's forays into the cultural impacts and oddities surrounding human pelage. Stenn peppers these later chapters with factoids and a cast of characters that will intrigue readers of all stripes: in 2007, a book dealer bought a single strand of hair plucked from the corpse of South American revolutionary Che Guevara for more than $119,000; a lock of 10,000 hairs has the tensile strength to support the weight of more than one adult person; etc.

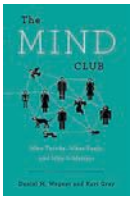
Restless Creatures: The Story of Life in Ten Movements
Matt Wilkinson
Basic Books, February 2016



Students of evolution are well aware of the beauty inherent in the process. Matt Wilkinson captures this splendor in *Restless Creatures*, a book that views evolution through the lens of locomotion. "Life, you see, despite its overwhelming diversity, has a single overriding theme—one that has dominated evolutionary possibility from the very outset," the University of Cambridge zoologist writes in the introduction to the book. "That theme is locomotion—the apparently simple act of moving from one place to another."

Wilkinson puts forth examples of adaptive solutions to problems, shifts in the locomotory strategies of life. From tree-dwelling hominins who eventually adapted to life on the ground to the incredible leap from earthbound organisms to airborne life forms, from how the forces of underwater living shaped the vertebrate backbone to the spinning of a falling maple fruit, *Restless Creatures* traces the movements of life through eons of evolution. And in the book's closing pages, Wilkinson opines that studying the importance of movement in our past just might deliver us from our all-too-sedentary, fossil-fuel-fouled present to a brighter future. "Our 4-billion-year history of self-generated movement has given us everything we hold dear, down to the very awareness and curiosity that's enabled us to piece that history together," he writes. "It's now up to us to decide whether that long, wondrous story ends here."

The Mind Club: Who Thinks, What Feels, and Why It Matters
Daniel M. Wegner and Kurt Gray
Viking, March 2016



It's an alliance we all perceive ourselves to be a part of: the group of beings who can sense, think, feel, and experience the world. Most humans are relatively sure of their membership in this "mind club," but disagreements over what other entities—animals, computers, spiritual beings, etc.—deserve inclusion are where the conversation gets interesting. The philosophical problem, which has occupied thinkers for centuries, serves as fodder for *The Mind Club*, a book by two psychologists, Harvard University's Daniel Wegner (who died in 2013) and Kurt Gray of the University of North Carolina at Chapel Hill.

Bubbling beneath Gray and Wegner's exploration of who or what belongs in the mind club from historical, sociological, philosophical, and biological viewpoints is the remarkable story of how the book itself was written. Wegner, Gray's graduate advisor, was stricken with ALS in 2010. As the disease slowly claimed the ability of his muscles to move, Wegner enlisted Gray to help him finish a book that he had only just started to write prior to his diagnosis. This arrangement is perhaps the single most crystalline illustration of one of the authors' main points: "By understanding that we *perceive* the world instead of understanding it directly, we can realize not only that the self is fragile and that free will is an illusion but also that other minds can be both more and less than they appear." —Bob Grant

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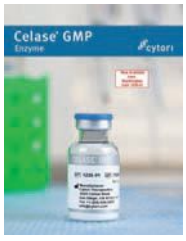

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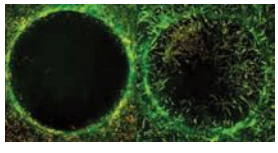

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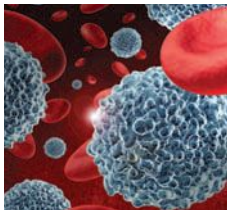

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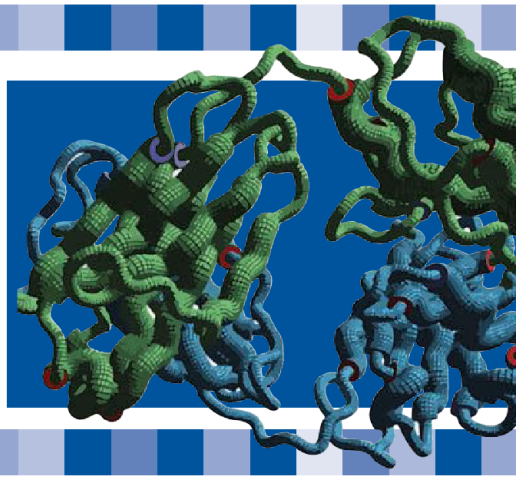

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Cave Dwellers, 1938

BY ANNA AZVOLINSKY

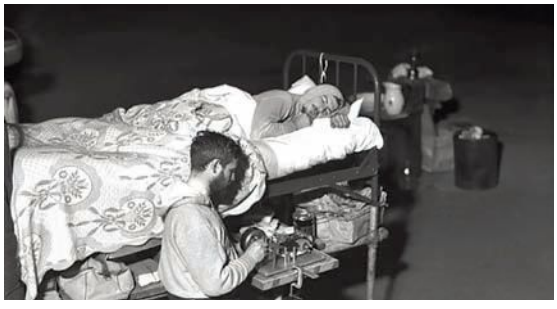
Even though we spend about one-third of our lives sleeping, probing how sleep works seemed a trivial, soft science to most researchers in the early 20th century. But the nightly habit intrigued Nathaniel Kleitman, who launched the world's first sleep laboratory at the University of Chicago after joining the physiology department in 1925.

"When he began, I think he was the only man on the planet devoting his full time to the study of sleep," says William Dement, a sleep researcher at Stanford University and one of Kleitman's students. "Kleitman always said his interest in sleep was sparked by the question of what was required for us to stay awake."

Kleitman often used himself as a subject in his sleep research, particularly on sleeplessness—whether in a pitch-black cave in Kentucky, in an underwater submarine during World War II, or on a trek above the Arctic Circle. To test one of his hypotheses—that the 24-hour clock could be modified—Kleitman and a student spent 32 days living in a deep cave in Kentucky, where the two imposed a 28-hour day on themselves. "The idea was to see how sleep could be generated in the absence of the normal environmental cues, especially light and temperature," says Jerome Siegel, a sleep researcher at the University of California, Los Angeles.

The Mammoth Cave experiment demonstrated that human bodies maintain a roughly 24-hour temperature cycle even in the absence of external cues, and that our sleepiness ebbs and flows in synch with this temperature cycle. "This study was important, [a] step towards identifying the [human circadian rhythm]," says Siegel.

Kleitman was also interested in the sleep cycle and the depth of sleep. After reading studies on eye movements and respiratory cycles in sleep, Kleitman wanted to test whether eye movement could be used as a measure of sleep cycles. In the early 1950s, he instructed his graduate student, Eugene Aserinsky, to monitor eye and body movements, first of sleeping infants and then of older adults.



SPELUNKER SCIENTISTS: From June 4 to July 6, 1938, Nathaniel Kleitman (at left in both photos) and his student Bruce Richardson camped out in Mammoth Cave in Kentucky to study the body's ability to conform to a non–24-hour cycle. The cave, 140 feet underground with no natural light, provided a location free from visual cues of day and night or temperature fluctuations—it was a constant and chilly 54 °F. The men brought lanterns to regulate their exposure to light, a table, and a bunk bed, and staked out a spot in a rock chamber about 26 feet high by 65 feet wide inside the cave. Each "day" they slept for 9 hours, worked for 10, and rested for another 9 hours. The researchers measured the daily rhythm of body temperature, finding an endogenously generated 24-hour body temperature cycle, despite the self-imposed 28-hour cycle. Still, Richardson, who was 20 years old at the time, felt he was better able to adjust to the new pattern after just a week in the cave. In contrast, Kleitman, then 43, found that his body clock did not move towards a non–24-hour schedule—he would get tired at 10 o'clock at night and feel awake and alert eight hours later.

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Aserinsky's observations of adults led to his and Kleitman's discovery of the rapid eye movement (REM) phase of sleep (*Science*, 118:273-74, 1953), although Aserinsky had been tempted to call the observed phenomenon "jerky eye movement" or "paradoxical" sleep. "It's such a beautiful paper. There is nothing in that paper that is not absolutely true in the context of all the sleep research that followed," says Siegel.

Kleitman died in 1999 at the impressive age of 104. Emmanuel Mignot, director of the Stanford Center for Sleep Sciences and Medicine, met him at a sleep meeting when Kleitman was being honored on the occasion of his 100th birthday. Although he made seminal contributions to understanding sleep, Kleitman left behind an as-yet-unexplained phenomenon in the field, says Mignot. "Considering [him] and the other founders of sleep research . . . Dement, who is still an active professor at age 88 without ever exercising, and Michel Jouvet, who is 90, it is very clear that studying sleep is associated with increased longevity!" ■

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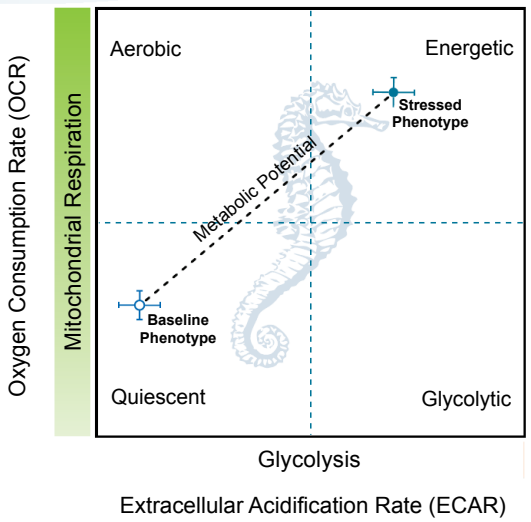

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