Addicted to Nicotine

The first pull on a cigarette should send you into convulsions. But instead, smoking can mellow you out and sharpen your mind. The series of unfortunate events by which nicotine works its magic in your brain is now becoming clear.

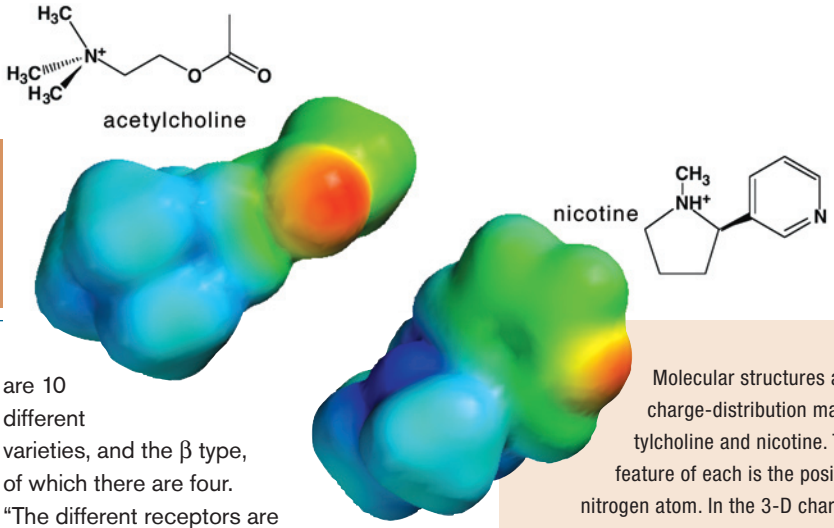
Despite his best efforts to quit, President Obama may still sneak a smoke from time to time. But can you blame him? He's got two wars, a sagging economy, and a cranky Congress to contend with; throw in a colossal gusher a mile deep in the Gulf of Mexico, and most people would be up to two packs a day. More than one billion people worldwide smoke regularly to enjoy its calming qualities and its mind-sharpening benefits; about five million people die from smoking-related diseases each year. But the fact that we can smoke at all without seizing up at each puff is a case of unlucky chemistry.

Nicotine—the relaxing yet addictive drug in tobacco—works its magic at the connections between the brain's nerve cells, where chemicals do the talking. At the heart of each connection is a gap called a synapse, where the electrical current traveling down a nerve fiber must somehow make the leap to the next cell. The neuron forwards its message by releasing molecules called neurotransmitters that spread within the void and bump into proteins on the surface of the receiving cell. There the neurotransmitters slip into pockets called binding sites, triggering a new electrical current that continues on its way. Nicotine sneaks into the synapses, usurping the binding sites and, in effect, sending its own messages.

The brain proteins that nicotine affects are nearly identical to a receptor protein on muscle cells that tells them to contract, but nicotine is essentially impotent at your muscle cells. "If you think about it, it must be true that these muscle proteins wouldn't be very sensitive to nicotine," says chemist Dennis Dougherty. "Because if they were, smoking would be intolerable—every puff would activate every muscle in your body." So Dougherty and biologist Henry Lester set out to discover why nicotine prefers brains over brawn.

Dougherty and Lester have been studying the chemistry of nerve signaling for almost

By Michael Torrice



Molecular structures and surface charge-distribution maps for acetylcholine and nicotine. The important feature of each is the positively charged nitrogen atom. In the 3-D charge maps, red signifies negative charge and blue is positive. The same color scheme applies to the drawing on the opposite page, which shows a nicotine molecule being attracted to a molecule in the brain.

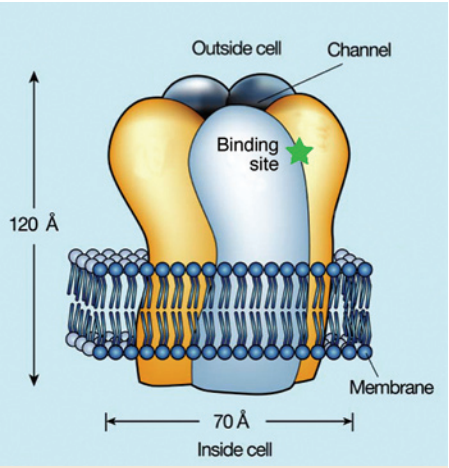
two decades. (See "Smoke Gets in Your Brain," *E&S* 2002, No. 4.) Their work may help explain why smoking is addictive, and could enable the design of drugs to help you quit. Surprisingly, it might also lead to treatments for neurological diseases including Parkinson's and schizophrenia. There is no medical justification for smoking, but people who have smoked for 30 or more years are almost 50 percent less likely to develop Parkinson's disease than nonsmokers, and about 90 percent of schizophrenics smoke compared to 20 percent of the general population. It may be that nicotine helps counteract schizophrenia's attention and memory losses.

Nicotine hijacks a family of proteins called the nicotinic acetylcholine receptors, or nAChRs. Acetylcholine is a neurotransmitter-of-all-trades. In the brain, acetylcholine is involved in learning and memory, in maintaining alertness, and in the sensation of pleasure. Out in the rest of you, it's the intermediary between your nerve cells and your muscle cells, carrying commands across the synapse that separates them and setting your body in motion. So when you flex your pecs in the mirror and think to yourself, "Dang, I look good!" that's acetylcholine at work.

The nAChRs loosely resemble molars, with five roots and a crown, and sit embedded in a cell wall like teeth in a jawbone. Each tooth has a cavity on one side of the crown—the binding site, into which the acetylcholine molecule fits perfectly. The act of binding opens a pore that runs down the center of the tooth like a root canal, allowing ions to flow and create an electrical current.

There are more than 20 known types of nAChRs, each with a different assortment of five parts called subunits. Each subunit runs from a root up to the corresponding cusp, and together they surround the root-canal pore. The subunits, in turn, come in various kinds, including the α type, of which there are 10 different varieties, and the β type, of which there are four. "The different receptors are siblings—more closely related than cousins—but not identical twins," Dougherty says. "They all do the same thing—bind acetylcholine and then open the pore." But while binding acetylcholine brings nAChRs together as a family, their subtly different structures cause them to have distinct preferences when it comes to other molecules, such as nicotine. "It's a big family and each sibling has its unique personalities," Dougherty says. The brain's versions all consist of two or more α subunits, with β subunits filling out the remaining slots.

Adapted from Dougherty & Lester, *Nature* 411: 252 (2001) © Macmillan Publishers Ltd.



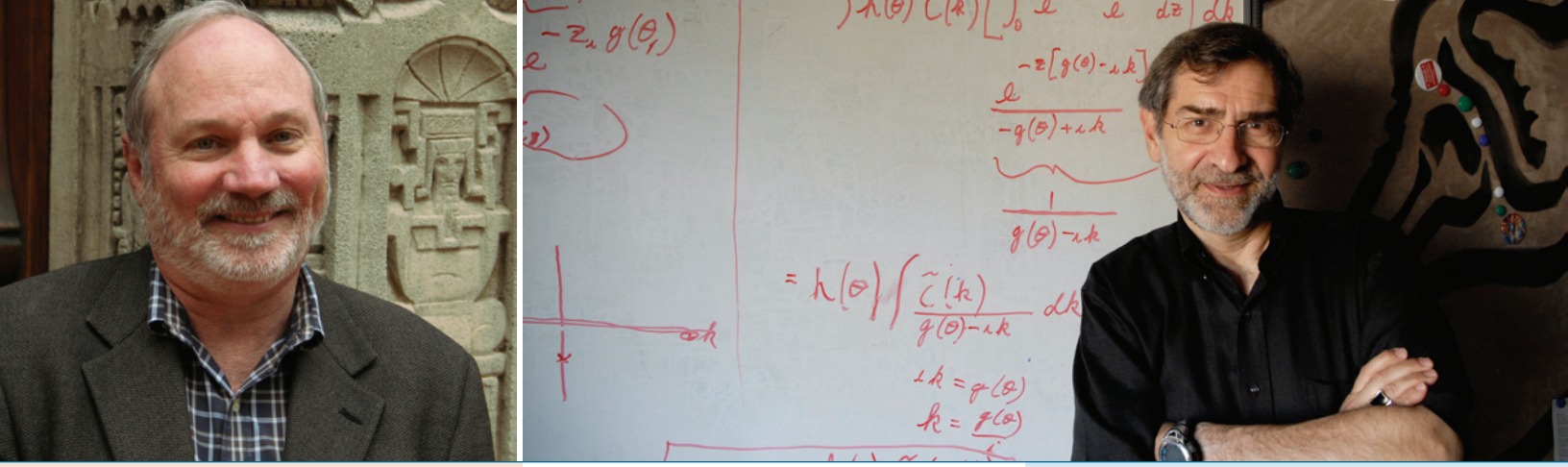
A nicotinic acetylcholine receptor sticks out of the cell membrane like a molar protruding from a jawbone. This one has two α units (yellow) and three β units (blue). The binding site is marked by the green star.

SO A CHEMIST WALKS INTO A BIOLOGY LAB . . .

Since nicotine and acetylcholine both fit into the same pocket, you'd think that they'd look pretty similar. They don't. Acetylcholine is a slender chain of carbon atoms, while nicotine is a stout fellow made of two bulky rings linked like a pair of handcuffs. But—and this is the key—both molecules have a nitrogen atom that can take on a positive charge.

A positively charged atom might seem like an unlikely key, since the protein molecule as a whole has no net charge. Normally, charged and uncharged molecules don't fraternize, avoiding one another like oil and water. But Dougherty's lab had spent years studying greasy molecules containing swirling clouds of electrons called π systems that impart regions of negative charge to otherwise neutral molecules. Opposites attract, and these π systems can bind to positively charged molecules through what's known as a cation-π interaction. (Chemists call positive charges "CAT-ions," pronouncing the first syllable like the house pet. For the full cation-π story, see "Sing a Song of Benzene, A Pocket Full of π," in the fall '94 issue of *E&S*.)

Meanwhile, neurobiologists at the Pasteur Institute, Columbia University, and elsewhere had found that the muscle receptor's



Above: Dennis Dougherty (left), the Hoag Professor of Chemistry, and Henry Lester, the Bren Professor of Biology.

nAChR binding site sits in the seam between two of the protein's subunits, both of which contribute amino acids to the pocket. Furthermore, five of these amino acids—three tyrosines and two tryptophans—were fingered as crucial for binding acetylcholine. The cation-π interaction was largely unknown in neurobiological circles, so it had been presumed that the crucial amino acids would be negatively charged in order to attract the acetylcholine molecule's positive charge. Tyrosine and tryptophan have no charge but they do have π systems, and in 1990 Dougherty and David Stauffer (PhD '89) proposed that a cation-π interaction might be at work.

However, studying simple substances in a lab flask is child's play compared to probing the workings of a large molecular machine such as the nAChR, whose 70,000 or so atoms make the job of trying to figure out which ones are the important ones nearly impossible. (By comparison, the previous molecules Dougherty had been working on contained about 100 atoms.) Chemists like to methodically alter their quarry an atom or two at a time and see how the molecule's behavior changes. "If there's a chlorine atom in a molecule, we want to know what it's there for," Dougherty says. Biologists, too, like to swap out parts and observe the effects. But since proteins are long chains of amino acids strung together, the biologists' unit of change is the amino acid, which can contain up to 27 atoms. To a chemist, this is like using a hatchet to dissect a stopwatch. And for someone accustomed to having the entire periodic table at their disposal, the 20 naturally occurring amino acids make for a seriously understocked parts inventory.

But there is a trick to making proteins more chemist-friendly—a neat bit of biological sleight-of-hand (see sidebar) devised by Peter Schultz (BS '79, PhD '84) in the late 1980s when he was a professor at UC Berkeley. The stratagem essentially inserts a new word into the DNA code book that commands the cell's protein-making machinery, allowing scientists to splice any molecule they like into a protein. The interloper, called an unnatural amino acid, merely has to have the standard amino-acid backbone in order to get strung into the chain.

In order to test Dougherty's hunch, graduate student Wenge Zhong (PhD '98) attempted to use this method to plant molecular informants on the muscle receptor. These amino-acid stoolies looked like ordinary tyrosines and tryptophans, but they were wearing wires—anywhere from one to four electron-hungry fluorine atoms that progressively siphoned the negative charge

Chemists and biologists both swap out parts of molecules to see how they work. But the biologists' unit of change is the amino acid, which can contain up to 27 atoms. To a chemist, this is like using a hatchet to dissect a stopwatch.

AN UNNATURAL TRICK

To sneak unnatural amino acids into proteins, you have to pull a fast one on the cell's protein-making machine. This machine, called the ribosome, synthesizes proteins by stringing amino acids together according to the instructions encoded in that protein's gene. The genetic alphabet has four letters, A, C, G, and T, that are combined into three-letter words, such as AAA for lysine and TCT for serine. As the ribosome reads the gene one word at a time, it decodes the words into amino acids with the help of adapter molecules called tRNAs. Each tRNA molecule recognizes one specific word, and hands the ribosome the corresponding amino acid to link onto the growing protein chain. But not every possible word in the genetic code translates into an amino acid, so an adapter molecule can be constructed to recognize a meaningless word and hand over an unnatural amino acid designed by the scientist.

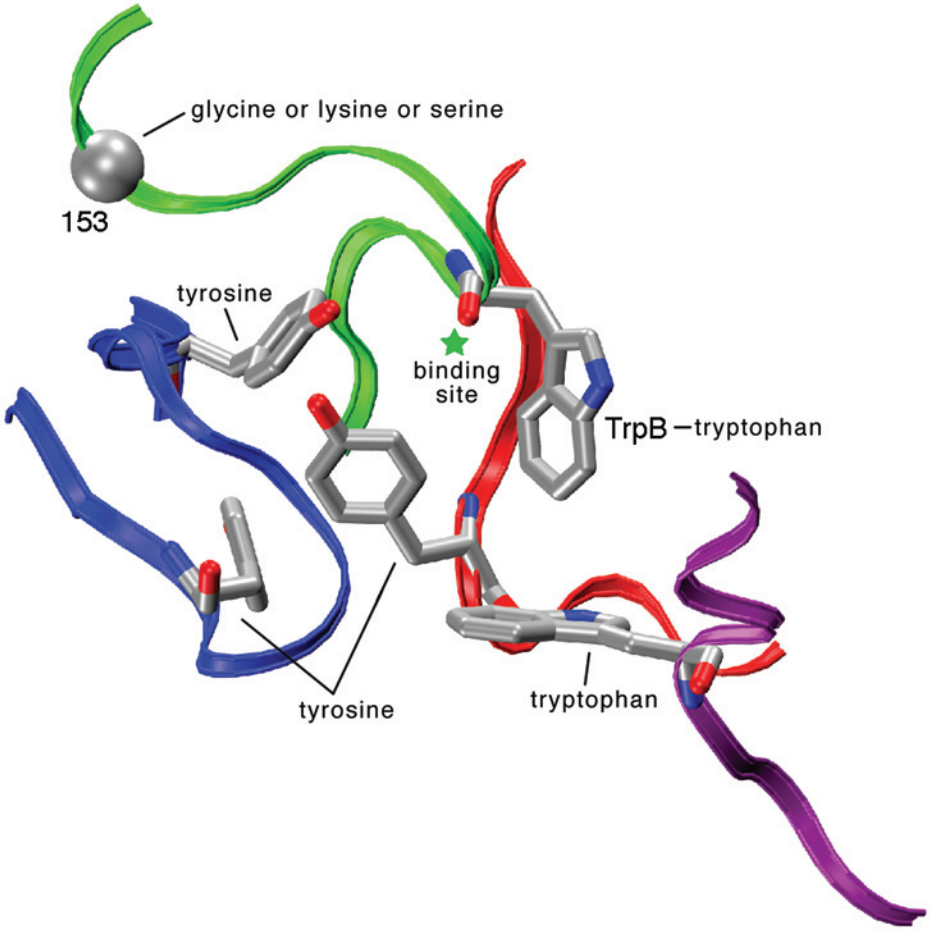
In order to insert a custom-built amino acid into a protein chain, the researcher rewrites the protein's gene to include the new code word in the correct spot. This designer gene and the new tRNA adaptor molecule linked to the ersatz amino acid are injected into the cell in overwhelming quantities. Responding to the flood of work orders, as it were, the cell's ribosome starts executing its new instructions. When it reaches the magic word, the man-made adapter presents it with the unnatural amino acid. The imposter is dutifully inserted, and the ribosome happily continues translating the gene, unaware that it has just been conned. E&S

out of the π systems. With each loss of charge, that amino acid's π system would become less attractive to acetylcholine, making the neurotransmitter less likely to bind and the pore harder to open. So if the flow of ions through the pore tapered off as more fluorine atoms were crammed on to one of the five suspects, the cation-π interaction would be betrayed.

There was one small hitch in the plan, however. The receptor proteins need to be in their native environment in order to work properly, and Schultz's system for sneaking unnatural amino acids into proteins didn't work in nerve or muscle cells; it only worked in test tubes. So Lester and his biologists showed the chemists the next best thing: frog eggs. The South African clawed frog, *Xenopus laevis*, has been a neurobiology workhorse for decades because its unfertilized egg cells can be persuaded to sprout a crop of receptor proteins on their surfaces. And at one millimeter in diameter, the half-brown-half-white spheres are easy to inject with the receptor gene and Schultz's magical ingredients, and to gently impale on thin glass electrodes. Then all you have to do is spritz the cell with an acetylcholine solution and measure the current flow as the pores spring open. At least, that was how it was supposed to go—in practice, the Dougherty and Lester groups had to substantially modify Schultz's procedure to get it to work in frog eggs. This proved to be time well spent, however, as the technique they came up with is now a standard tool.

It took about two years to get results, but by 1998 Zhong had found that he couldn't pin anything on four of the five alleged perps. However, a tryptophan called TrpB—so named because it sits on the α subunit's "B" loop—was caught red-handed. With each fluorine atom added to the TrpB, the receptors grew steadily less responsive to acetylcholine. Surprisingly, though, the same set of experiments with nicotine showed

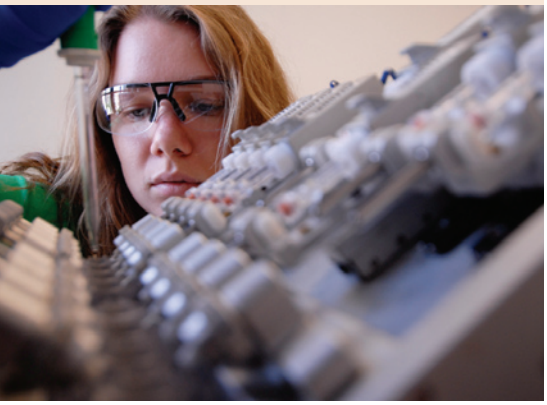
Adapted from Xiu, et al., *Nature* 458: 534 (2009) © Macmillan Publishers Ltd.

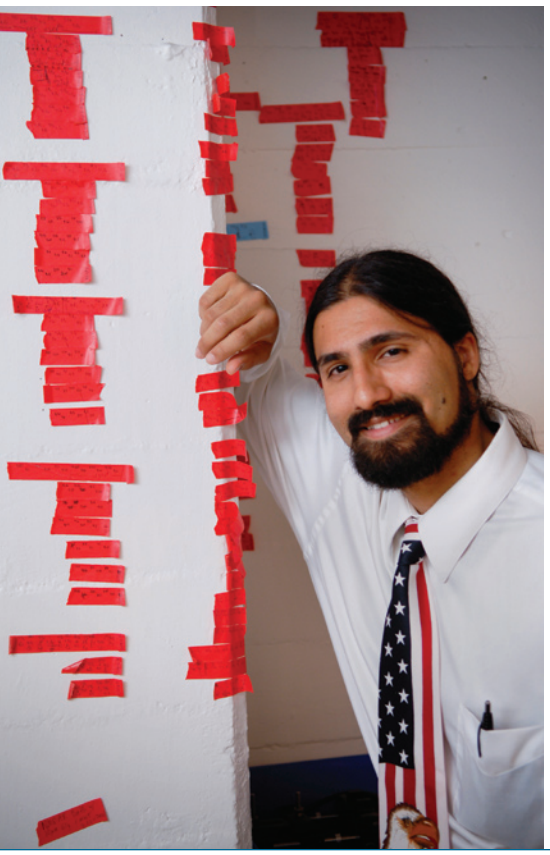


The nicotinic acetylcholine receptor's binding site is made up of four loops of protein. Five amino acids are critical for binding—a tyrosine in the "A" loop (the red ribbon), a tryptophan in the "B" loop (green), two tyrosines in the "C" loop (blue) and a tryptophan in the "D" loop (purple). When the acetylcholine neurotransmitter or the nicotine interloper slips into the binding site (green star), the molecule is surrounded by five π systems—the hexagons or hexagon-pentagon combos shown in the gray tube renderings of the amino acids.

The gray sphere marks the location of the 153rd amino acid in the "B" loop. Different amino acids are found in this position, depending on which receptor protein is being examined. This difference would provide a critical clue to solving the mystery of nicotine's selectivity, as we shall see.

Below: Grad student Nyssa Puskar and the OpusXpress, a refrigerator-sized robot biologist that can test eight egg cells at once.
Bottom: On each piece of red tape is electrical-current data from nicotine receptors in the egg cells. Grad student Jai Shanata poses with his pelts, as it were.





no loss of function, no matter how many fluorine atoms the chemists sent in. No amino acid in the lineup seemed to have any affinity for nicotine, which would explain why it doesn't turn on the muscle nAChR.

GETTING INSIDE THE BRAIN

Having flexed his muscles, as it were, Dougherty was ready to hunt for a cation-π interaction in the brain. But there the trail went cold for seven years—although the frog eggs had willingly churned out the muscle nAChR, they refused to make brain receptors. This biological brush-off shouldn't be too surprising, because egg cells don't naturally produce such proteins. "We were grateful that *any* nicotinic receptor can be grown this way," Lester says. "It was only a minor misfortune that the most studyable was not the most interesting one."

It eventually turned out that a simple mutation along the root-canal pore—originally discovered in 2001 in unrelated studies by Cesar Labarca, a Member of the Professional Staff in Lester's lab—coaxed the egg cells to work. So in 2006, Dougherty's grad students Xinan Xiu (PhD '08), Nyssa Puskar, and Jai Shanata began work on a protein that Lester and others had shown to be nicotine's main target in the brain—a receptor called α4β2 because of the two α4 and three β2 subunits that sit around its central pore.

The grad students rounded up the usual suspects, as all members of the nAChR family have that same cluster of three tyrosines and two tryptophans in their binding sites. But now there were crime-scene photos: molecular snapshots of a related protein found in snails (taken by Titia Sixma of the Netherlands Cancer Institute and others) had revealed that the five amino acids are arranged so that their π systems act as the four sides and bottom of what Dougherty calls the "binding box."

Technology had marched on, too. Zhong had measured the electrical currents in his muscle-receptor experiments one egg cell at a time. Getting the goods on a single fluorine substitution in one amino acid meant grilling several cells—a process that took hours. But in 2003, Dougherty and Lester jointly bought a machine about the size of a refrigerator laid on its side that automates this tedious process by sweating eight cells at once. Each cell sits in solitary confinement in its own well, impaled on its glass electrode. A robotic arm with eight nozzles sucks up a solution of acetylcholine or nicotine, zooms over to the line of wells, hovers, and squirts the liquid onto the eggs, repeating the process for increasing concentrations of the drug. "In 30 minutes, you can tell whether you have created either a gain-of-function mutation or a loss-of-function mutation," Puskar says.

Not surprisingly, acetylcholine latched onto the π system belonging to the brain protein's TrpB. But now the nicotine data mirrored the acetylcholine data: each additional fluorine produced a harder-to-activate receptor. This newfound cation-π interaction presumably explains nicotine's hundredfold greater potency at brain nAChRs compared to the muscle receptor. "It just makes sense," Puskar says. "The brain receptor has to have an interaction that doesn't exist in the muscle receptor. If smokers had this cation-π interaction in their muscles, they'd all be paralyzed."

SIBLING DIFFERENCES

But the five amino acids in the binding box are exactly the same in the muscle receptor, the α4β2 receptor, and every other nAChR sibling. "At this level, they're identical twins," Dougherty says. "So this raises a fascinating question. We have two dozen different acetylcholine receptors with noticeably different pharmacologies. What's

"The receptor has 3,000 amino acids, but by changing just one—the right one—we can make the muscle receptor look like a brain receptor."

happening?" He adds drily, "We had to think outside the box to find a solution to this puzzle." The binding box is held in shape by four loops of protein. Would changing amino acids elsewhere on these loops make a difference?

The chemists started scouring the neurobiology literature and found a spot four amino acids away from the critical TrpB that sparked their curiosity. In the muscle receptor, a small, simple amino acid called glycine sits in this position. But in α4β2 and other nAChR receptors sensitive to nicotine, the glycine's spot is occupied by a much bigger amino acid called lysine.

The final clue came from work at the Mayo Clinic in Rochester, Minnesota. In 1982, Andrew Engel published a study of a group of patients with a rare genetic disorder that caused their muscles to waste away, leading to labored breathing, progressive clumsiness, and other problems. Engel suggested that their muscle nAChR was hypersensitive to acetylcholine: the receptor had a hair trigger and stayed active for much longer than normal. In 1995, his colleague Steven Sine discovered that the hypersensitivity was due to a mutation that replaced that glycine with a medium-sized amino acid named serine. Furthermore, the neuron's α4β2 receptor is more sensitive to acetylcholine than the muscle receptor, so, in some ways, this mutation caused the patients' muscle receptor to become more "brainlike."

When Puskar, Xiu, and Shanata swapped in a lysine for that glycine in the muscle receptor and retried the experiments that had flopped seven years earlier, they hit the jackpot—a muscle receptor with an affinity for nicotine. "The receptor has 3,000 amino acids, but by changing just one—the right one—we can make the muscle receptor look like a brain receptor," Dougherty says.

It's not clear exactly how the muscle receptor changes, but Dougherty thinks that the mutation reshapes the binding box. The slender acetylcholine molecule fits into both the muscle and the α4β2 boxes, but the bulkier nicotine can't. Somehow, putting a lysine at this critical spot in the muscle receptor pries open the box enough so that nicotine can squeeze in.

NICOTINE'S UNLUCKY BIOLOGY

While Dougherty and his chemists have focused on what nicotine does on the cell surface, Lester and his biologists have peered inside the cell to pinpoint what makes the drug so addictive. Nicotine, like many drugs, is adept at slipping through cell membranes. The cation-π interaction that lures nicotine to the receptor depends on the nicotine molecule having a positive charge. But nicotine as a neutral molecule is oily, and by shedding its charge it can then easily infiltrate the palisade of greasy molecules in the cell membrane. Once safely inside, the nicotine molecule can snag a passing proton and become positively charged again, ready to bind to receptors in the cell's protein nursery and seize control. "The binding that the cation-π experiments discovered takes over," Lester says. "This very strong interaction allows nicotine to play three roles in a story that my lab is just starting to understand."

In a 2007 collaboration with researchers at the University of Pennsylvania and the University of Colorado at Boulder, Lester postdocs Raad Nashmi and Cheng Xiao and staff biologists Purnima Deshpande and Sheri McKinney created mice with fluorescent α4β2 receptors, and watched the results as the rodents received nicotine doses equivalent to a person smoking two to three packs per day. Over the course of a week or two, the mice sprouted significantly more α4β2 receptors in the midbrain, which processes rewards and is the seat of addiction. (Interestingly, Parkinson's disease causes some dopamine-producing nerve

From top: Postdoc Cheng Xiao, lab manager Purnima Deshpande, and Sheri McKinney work in Lester's lab.

cells within the midbrain to slowly die off.) When these cells were sprayed with nicotine, they fired about twice as often as cells from "nonsmoking" mice. "We're essentially taking movies of events inside the neurons during the first minutes, hours, and days of nicotine addiction," Lester says.

It appears that nicotine acts like a chaperone, a matchmaker, and a traffic cop inside the cell—a combination of roles that maximizes the odds that each nAChR the cell produces will actually reach the cell's surface. As a chaperone, nicotine binds to nascent receptors' subunits as they are being synthesized, preventing them from being chewed up by the cell. The details are still being worked out, but "the simple idea is that nicotine stabilizes the receptor in a conformation that does not appeal to the cell's mechanisms for eliminating poorly folded proteins," says Lester. And, because the receptor's binding box is made from amino acids on two of the five subunits, nicotine the matchmaker expedites their assembly by binding to the two free-floating halves of the box and holding them in the correct orientation. This gives the remaining three subunits something firm to latch onto, helping them fall into place. And finally, as the cell transports the newly assembled nAChRs to the neuron's surface, the nicotine molecules bound to the receptors could act like a police escort, once again protecting them from the cell's protein-digesting machinery. "Scientists don't understand how chronic drug use leads to addiction—in any type of addiction," Lester says. "But the hypothesis that chaperoning, matchmaking, and traffic direction are necessary and sufficient is our lab's best bet at the moment."

Lester and colleagues at the University of Colorado at Boulder, and the drug company Targacept, have a grant from the National Institutes of Health to find drugs to help people quit smoking. The collaboration, which also includes Lester's postdoc Ryan Drenan, has set its sights on another nAChR family member that seems to consist of an $\alpha4$, an $\alpha6$, a $\beta3$, and two $\beta2$ subunits. "These $\alpha4\alpha6\beta2\beta3$ receptors seem to be as strongly activated by nicotine as the $\alpha4\beta2$ receptors, but they seem less susceptible to nicotine's roles as a chaperone, matchmaker, or traffic cop," Lester says. He thinks that if smokers took an $\alpha4\alpha6\beta2\beta3$-specific drug, they'd experience some of nicotine's benefits—soothed nerves or focused minds—without the addiction. Popping that pill might slowly wean them off cigarettes.

Lester's lab is also looking for branches on the nAChR family tree that might make good targets for drugs to treat neurological diseases such as Parkinson's and schizophrenia. The experiments are beginning to show how chaperoning and matchmaking might underlie the protective effects against Parkinson's disease.

In addition, another collaboration with the University of Colorado at Denver is working on the $\alpha7$ receptor, which nicotine also hits. Schizophrenics' neurons don't produce as many $\alpha7$ receptors as healthy people do, and some scientists think that when these patients smoke to assuage their chaotic minds, they get relief by sparking the few $\alpha7$ receptors that they do have into overdrive. So a drug that acted like nicotine, without its addictive properties, could make a better schizophrenia treatment.

Dougherty and Lester plan to continue to explore the nAChRs' sibling differences. But Dougherty points out that this portfolio of pharmacological preferences is, in itself, a side effect: "Remember all of these proteins evolved to respond to acetylcholine, not ever to nicotine or to any other drug." Evolution had some reason to tweak the muscle receptor's binding site, but it definitely wasn't to block nicotine. So, when you take a drag on a Camel and feel your scattered mind come into focus, the fact that you might be worrying—if you're so inclined—about lung cancer or heart disease rather than about instant paralysis is, as Dougherty says, "just bad luck." E&S

Dennis Dougherty, the Hoag Professor of Chemistry, started his academic career as a physical organic chemist after receiving his PhD in chemistry at Princeton University in 1978. He's been on the faculty at Caltech since 1979—first studying small organic molecules in flasks, and now investigating large brain proteins in frog eggs.

Henry Lester, the Bren Professor of Biology, studied biophysics at Rockefeller University and received his PhD in 1971. After spending two years as a researcher at the Pasteur Institute in France, he joined Caltech in 1973. He and Dougherty have been collaborating since 1992 on projects involving unnatural amino acids. The current work is supported by grants from the National Institutes of Health, the California Tobacco-Related Disease Research Program, the Michael J. Fox Foundation, the Gordon and Betty Moore Foundation, and Louis Fletcher (BS '56, MS '57).

***Michael Torrice (PhD '09) was a graduate student of Dougherty's. He is now an assistant editor at* Chemical & Engineering News.**

This article was edited by Douglas L. Smith.