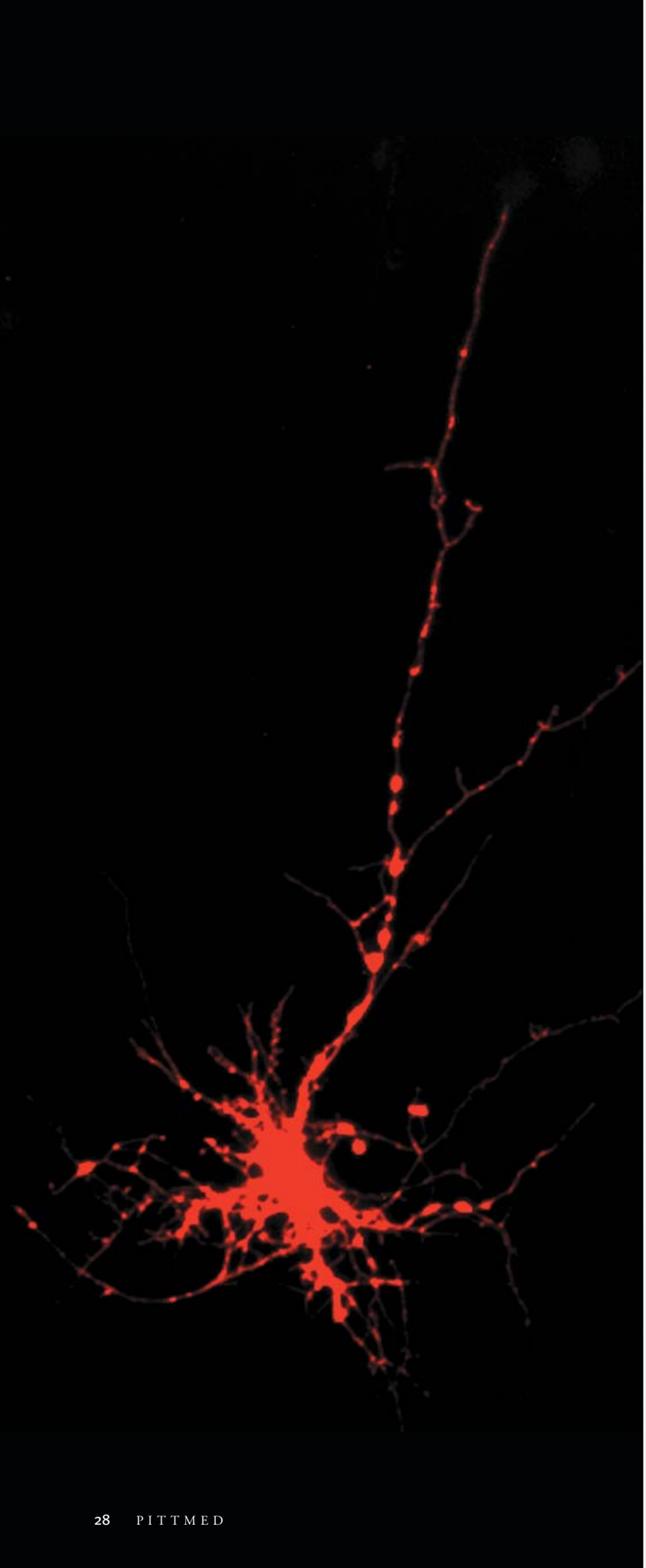
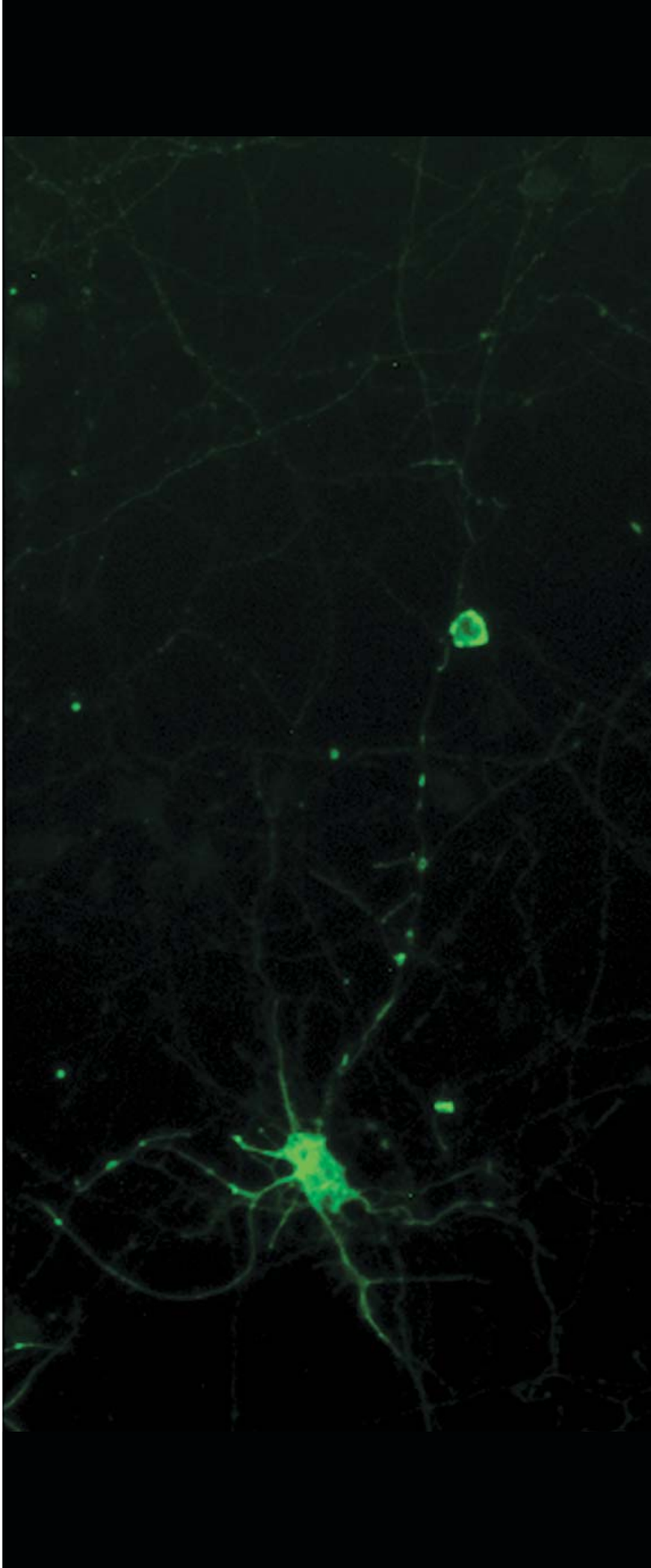




A MENTOR AND STUDENT TRY TO KEEP DYING CELLS ALIVE | BY JOE MIKSCH

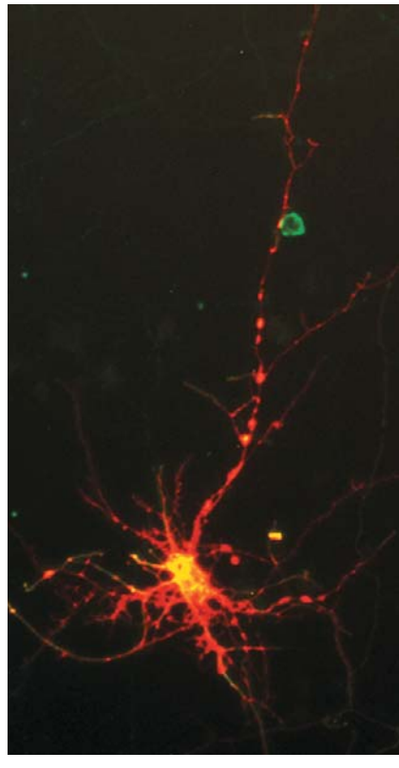
IN THE SHADOW OF A STROKE

About five years ago, 59-year-old Randy Zotter was having some trouble with his knee. So much so, that he arranged to have an X-ray taken at UPMC Passavant in Pittsburgh's North Hills. No big deal, he thought, just a small diversion ahead of his plans to take his wife, Leslie, out that night for their 25th wedding anniversary.

The X-ray completed, Zotter got into his car. "My left hand goes like this," he says, flopping it off a table. "It wouldn't move. Immediately, I went into a state of denial." He started the car and drove off. His left arm still wouldn't move, and recalling that his father had a stroke when he was 39 prompted Zotter to turn around and head back to Passavant.

He parked about 100 yards from the emergency room—"I didn't want to block the entrance," he says, laughing—and hobbled to the door. "I told a woman out there smoking that I was having a stroke. She ran away," Zotter recalls.

Once inside, he was taken in for a CT scan. Doctors confirmed Zotter's self-diagnosis. A cerebral artery had collapsed, they told him. For the next two weeks, Zotter rested in the hospital. For months afterward, he went through round after round of physical and occupational therapy.



Neural cells not immediately killed by a stroke can suffer from a deadly inability to synthesize proteins. Jun Chen and Peter Vosler have found that a protein called eIF4G, vital to synthesis, is torn apart by calpain, a protease, in the wake of a stroke. By inhibiting calpain, the two think they may have found a way to save such cells and perhaps have acquired the tools for an exciting new therapy. (The first image, in red, is a neural cell stained to show the expression of eIF4G. The second, in green, is a normal neuron. The third, at right, is an overlay of the two images.)

COURTESY P. VOSLER

However, Zotter says, his doctors told him there was nothing to be done to save the brain cells injured by the stroke.

Jun Chen and Peter Vosler, of the University of Pittsburgh School of Medicine, are trying to build a brighter future for patients like Zotter who make it to the hospital within a few hours of experiencing a stroke. They have found a way to halt neuronal cell death caused by lack of blood flow, or ischemia.

Chen is an MD professor of neurology and pharmacology and chemical biology. He holds an endowed chair and directs the Cerebrovascular Research Center at Pitt. Vosler is an MD/PhD student who recently completed his PhD work in neuroscience and begins his third year of medical school in the fall.

Stroke is the third-leading cause of death in the United States and the second worldwide. About 80 percent of those cases are considered ischemic—brought on by a lack of blood flow. When a stroke strikes, the halted blood flow decidedly and immediately kills cells at its epicenter. The damage eventually spreads to the penumbra, the area surrounding the dead cells.

According to Chen and Vosler, stopping the death of neuronal cells in the penumbra of a stroke can curtail the long-term physical and emotional damage caused by this serious insult to the brain. These cells don't die immediately in a stroke's wake. But when they do die, the area of the brain affected is greatly expanded, leading to more problems for a stroke survivor.

Zotter regained use of his arm—only after months of physical therapy. He recalls trying to refine his motor skills by attempting to screw a nut onto a bolt. The frustration, he says, was overwhelming. "But I told myself, 'Yes I can, and yes I will.'"

Though he suffers no speech impediment, Zotter says that he has to concentrate much more when speaking. And he's more emotional now: "I tear up during 'chick flicks.' I never did that before," he says.

Although Zotter has recovered well overall—thanks, he thinks, to the fact that he was very close to a hospital when he suffered his stroke—few treatment options were available to him when he entered the emergency room.

One of the most exciting advances in stroke therapy in recent history came in the early 1990s, when doctors began using a clot-buster called tissue plasminogen activator, or tPA. If delivered within three hours of a stroke, tPA breaks up the clot that limits blood flow in the brain. After that window closes, tPA isn't effective and increases the risk of brain bleeding.

"Only about five percent of the 800,000 stroke patients a year in the U.S. can get tPA and benefit from it," says Vosler.

That's because of the small time window and a host of other factors excluding patients from tPA therapy.

The therapy wasn't an option for Zotter, for instance, who didn't have a blood clot but did suffer from what's known as an ischemic infarction.

Chen and Vosler anticipate they've hit upon a molecular mechanism that, if translatable into a drug, could limit the damage done in such cases. Their work is predicated on a discovery European researchers Paul Kleihues and Konstantin-Alexander Hossmann made in the early 1970s. Using animal models of stroke, Kleihues and Hossmann determined that the brain uniformly shuts down protein synthesis in all ischemia-affected areas. Hossmann later found that brain regions that recovered protein synthesis lived, whereas regions where protein synthesis inhibition persisted died.

His doctors told him there was nothing to be done to save the brain cells injured by the stroke.

Chen and Vosler posit that the neuronal cells in the penumbra of a stroke might be salvageable if the disruption of protein synthesis can be remedied.

"[Neuronal cells affected by ischemia] lose over 90 percent of their capacity to synthesize proteins, and if protein synthesis is inefficient and persists, the neuron will die," says Chen. That situation is not remedied by tPA or any other current stroke therapy, including cerebral angioplasty and surgery.

"The question is," adds Chen, "'What causes the persistent protein synthesis deficiency?' That question has been pestering the field for years."

He adds: "We believe that if we are able to identify the molecular mechanism underlying persistent protein synthesis deficiency, we may be able to develop a new therapeutic strategy to prevent cell death after stroke."

Perhaps, Chen says, they've completed the first step and are now on their way to achieving the second.

The effort started with Vosler looking for a PhD project and mentor. In Chen's Starzl Biomedical Science Tower office, the pair recount the history of their nascent partnership.

"When Peter came to my lab, and we sat down to discuss what he wanted to do, I said, 'Peter, we have a major mystery in the [stroke] field, and it's a big challenge,'" Chen recalls. 'Do you want to take it on?' He said, 'Yes.'"

"I was a young, naïve student," Vosler adds, before both he and Chen dissolve into laughter. Chen leans back and lets his student tell the story.

Regaining a bit of composure, Vosler says seriously, "I thought that this work might be risky, but I was sure I was going to learn under Dr. Chen no matter what I did."

The reason for Chen and Vosler's laughing fit is that researchers have been poking around the question of how to rescue stroke-damaged cells for decades. And though Chen discovered that protein synthesis disruption is probably responsible for the death of neuronal cells present in the penumbra of a stroke, getting into the nitty-gritty of protein synthesis could be considered a bit much for a "young, naïve student."

Vosler's charge? First, create a credible model of protein synthesis in the lab. The process of studying the causes and effects of stroke is complicated enough that the additional vagaries introduced by studying it in vivo are excessive and confounding. So Vosler took the petri dish path.

Vosler took primary cortical neurons from rats, cultured them, and was able to mimic ischemic conditions in culture dishes. He also succeeded in reproducing persistent protein synthesis inhibition. "What he did was establish a model system to replicate the condition seen in vivo," Chen says proudly. "Now we can study the mechanism in a very tightly controlled situation."

Chen adds that he's also impressed with Vosler's feat because though the steps by which cells make proteins are understood, there is less familiarity with how the whole business gets started. Thanks to advances in molecular biology, neuroscientists now know that there are 36—read: a whole lot of—proteins involved with initiating protein synthesis. "That's before you get any translation, any elongation, or any amino acids being added," Vosler says.

Earlier in vivo studies indicated that during ischemia there is a decrease in the presence of a scaffolding protein called eukaryotic growth factor 4G (eIF4G), which is responsible for transporting messenger RNA (mRNA)—protein-making instructions—to a cell's ribosome. Ribosomes are cells' protein factories, the seat of protein synthesis. If there's a problem with eIF4g, mRNA messages cannot get to the ribosome, dooming protein synthesis. With that, the cell dies.

Enter calpain. Calpain is a protease that cleaves, or breaks down, eIF4G. As Vosler explains, calpain depends upon calcium in order to be active. In the wake of ischemia, calcium rushes into neurons and stirs up a calpain storm, with the calpain cutting up eIF4G and blocking normal mRNA activity. Chen and Vosler thought, if calpain-mediated eIF4G cleavage and the resultant inhibition of synthesis are a direct consequence of ischemia, perhaps inhibiting calpain could restore the process.

Experiments conducted by Vosler inspire confidence that this hypothesis is correct. Vosler tried to calm down calpain by introducing a load of its inhibitor, calpastatin. Doing so, he found, stopped eIF4G cleavage, restored protein synthesis, and increased the viability of his cultivated rat neurons. And by maintaining the proper level of eIF4G, Vosler was able to sustain these gains.

Don DeGracia, a PhD associate professor of physiology in Wayne State University School of Medicine in Detroit, calls Chen and Vosler's work "impressive." DeGracia is an author of a 1994 paper that implicated calpain as an enemy of eIF4G.

"What Pete and Jun have done is prove beyond a shadow of a doubt that calpain degrades eIF4G," DeGracia says. "There were some possible scientific doubts with our original methods. Pete and Jun have used foolproof methods to prove it."

Also, DeGracia adds, "They've shown that by preventing calpain from degrading eIF4G, they not only recover the cell's ability to make protein, the cells don't die, either."

The process leading to these discoveries wasn't particularly simple, Vosler says.

The eIF4G protein, at 220 kilo daltons (that's equivalent to the mass of 220,000 hydrogen atoms), is pretty huge as these things go. The size, Vosler says, makes it difficult to synthesize its DNA—a process that becomes harder to do without errors the longer a sequence is. Neuronal cells, he adds, are notoriously difficult to transfect—to introduce DNA into a cell.

Volser says he tried a new reagent that was supposed to ease the transfection process. "It was touted to work in primary neurons," he says. But it didn't. Eventually he settled on using a lentiviral vector, a kind of "neutered" virus that can't reproduce but can carry and insert cargo into cells. Vosler feared the size of the cargo might gum up the works. (Lentiviruses can typically carry a 10 kilobase load; Vosler had 12 kilobases of stuff he wanted to put into them.)

"At this size, we expected that either there would be no expression of eIF4G or there would be very little," he says. "Much to our surprise, the virus was able to transfect primary neurons with approximately 75 to 90 percent efficiency."

So with the technical issues solved and after collecting and interpreting their data, Chen and Vosler felt confident that they had established a direct relationship between ischemia, eIF4G, calpain, and the untimely end of protein synthesis in neuronal cells. This, Chen says, is a very big deal: "This is the first time it has been shown that protein synthesis inhibition and cell death are directly related."

With publication of the work pending—Chen hopes the results will see print in the *Proceedings of the National Academy of Sciences* this spring—Vosler has been on a bit of a speaking tour, presenting the findings at the American Heart Association's Fellows Research Day and at a conference at Cold Spring Harbor Laboratory on Long Island, N.Y.

"That I was able to get a platform presentation [at Cold Spring Harbor] in front of all these big-time protein synthesis researchers was great," Vosler says. "Postdocs through senior scientists said it was very interesting work. And the fact that it's related to disease directly and has the potential to really help means a lot."

"Their work is one thread in a tapestry that is being woven by many labs right now recognizing how important it is that brain cells recover their ability to make their own proteins," Wayne State's DeGracia says. "It's the overall picture coming out of this tapestry that will offer a new way to understand stroke and may help us to prevent neurons from dying."

And that's what will mean the most to Chen and Vosler—translating lab-generated knowledge into a clinical application.

"We can develop a way to deliver the [calpain] inhibitor to the brain, and that's the way to attack it," Chen says.

They envision attaching the inhibitor to a segment of HIV protein that can pass the blood-brain barrier. The process uses an 11-amino acid sequence of HIV that does not contain the pathogenic domain of the virus. Earlier this decade, Chen was the first to provide neuroprotecion in an animal model of stroke using a protein connected to this HIV amino acid sequence.

A nanoparticle might also serve as a delivery vehicle for the therapy. Nanoparticles are tiny, inert bubbles that, like the HIV protein segment, can cross the blood-brain barrier. They're particularly attractive for drug delivery because they can be directed toward specific neuronal receptors. Kind of like a GPS system for stroke therapy.

The Randy Zotters of the world, Chen and Vosler hope, will someday be able to receive an injection in the wake of a stroke that will halt and reverse the slow cell death caused by ischemia-related protein synthesis cessation.

The researchers are far from monitoring safety trials, yet they are hopeful that they can avoid the risks related to conventional tPA therapy, such as brain bleeding. Chen imagines a prospective new therapy coming out of their studies that—unlike with tPA and other treatments—wouldn't just restore blood flow to the brain, it would redeem neural cells in the penumbra of a stroke, limiting damage and making a patient's recovery easier.

Time will still be a factor in the treatment, however. "The median time of arrival [to a hospital after a stroke] is six hours," Vosler says. At this point, Vosler and Chen are unable to pinpoint a required time frame for any therapy arising from their work.

Vosler is unsure whether he'll be travelling along with Chen through the intensive lab work and lengthy clinical trials necessary to bring a calpain inhibitor–based therapy to stroke patients. Come fall, he'll resume the pursuit of his MD and, after that, the future is unwritten.

"I want to figure out how I can do clinical and lab work at the same time," Vosler says. "I really enjoy research, but I haven't decided on a field. Surgery is enticing but would limit the amount of time I could have in the lab. I really haven't figured out what I want to do."

In a way, Chen says, it doesn't matter much what discipline Vosler ultimately chooses to pursue.

"The purpose of the MD/PhD program is to train scholars, to train physician-scientists," Chen says. "Regardless of the area he goes into, he is going to be outstanding. What he's doing is very rare, to be honest with you." ■