Although the idea is still controversial, evidence is mounting that triggers for the disease kill neurons by activating their internal cell-death programs

New Leads on the 'How' of Alzheimer's

A medical examiner investigating a suspicious death wants to know more than just the fact that someone died. Equally important is *how* that person died, especially when it comes to getting the evidence needed to put away a murderer. Neurobiologists seeking to understand the brain's degeneration in Alzheimer's disease find themselves with a similar problem. Although they know that patients' brains suffer a massive loss of nerve cells, exactly how those neurons meet their end has been much less clear.

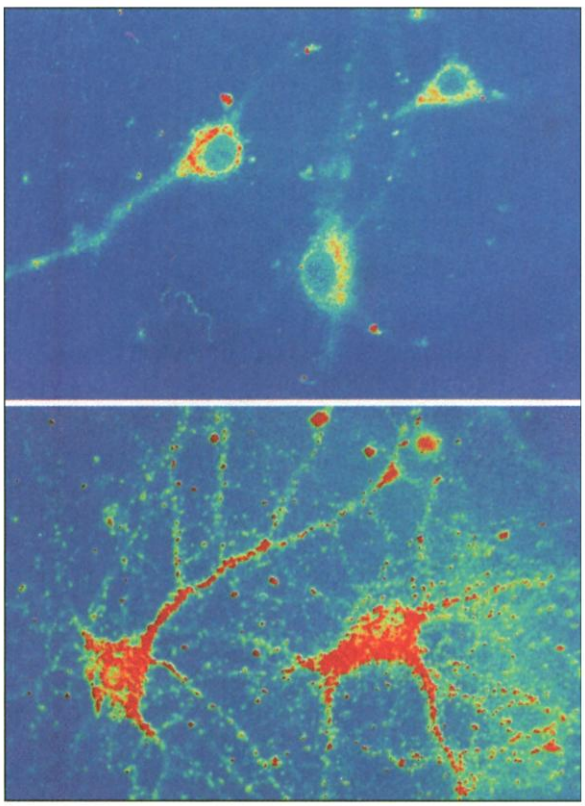
Within the past few years, however, work by several labs has indicated that the neurons ultimately die by apoptosis, also known as programmed cell death, because cells undergoing it typically show certain characteristic signs such as cell shrinkage and DNA fragmentation. Working with a variety of experimental systems, including brain samples taken from patients at autopsy, researchers have found several signs that the cellular machinery involved in apoptosis has been activated in Alzheimer's. In particular, they've shown that several caspases—protein-digesting enzymes instrumental in bringing about the destruction of cells undergoing apoptosis—are active.

What's more, this work indicates that the caspases can be activated by a small protein already well known in the field—β amyloid (Aβ)—which many researchers think is a trigger of neuronal loss in Alzheimer's. Other stresses that afflict aging brains, such as decreased metabolism and long-term bombardment with highly reactive free radicals, may lead to caspase activation as well. "The risk factors that are known to occur [in the aging brain] push neurons into apoptosis," says neuroscientist Carl Cotman of the University of California, Irvine.

Not everyone agrees that nerve cells die this way in Alzheimer's, but if the findings are confirmed, they could provide new targets for drugs aimed at slowing the progression of the disease or even preventing it. Such drugs are badly needed. Currently, some 4 million people in the United States alone are afflicted by Alzheimer's, and that number is expected to grow to 25 million by 2025 unless effective treatments can be found.

Biochemical suspects

Hints that apoptosis might be involved in Alzheimer's began emerging in the early to mid-1990s. Cotman's team, and also that of Gianluigi Forloni at the Institute for Pharmacological Research in Milan, Italy, showed that Aβ causes neurons in culture to die by apoptosis. Cotman and his colleagues, Hans Lassmann's group at the University of Vienna, Austria, and other researchers then proceeded to look for signs of apoptosis in brain samples taken at autopsy from Alzheimer's victims.



Trigger. Aβ causes caspase activation (brown staining) in both the dendrites and cell bodies of these neurons from rat brains (bottom); controls are at top.

CREDIT: M. MATTSON *ET AL.*, *BRAIN RESEARCH* **807**, 167 (1998)

Those signs include fragmentation of the nuclear DNA, which can be detected using the so-called TUNEL stain; researchers found that the stain picked up many more dying neurons in Alzheimer's patients' brains than in those from people who had died of other causes. The problem, however, was that they found too *many* TUNEL-stained nerve cells. The number was so large, in fact, "that it was not consistent with the time course of the disease," says Mark Smith of Case Western Reserve University School of Medicine in Cleveland, Ohio, a critic of the idea that brain neurons succumb to apoptosis in Alzheimer's.

Cells undergoing classical apoptosis, he notes, die within 15 hours or so, and given the high numbers seen in Alzheimer's brains, he calculates that the disease would run its course in the range of 10 weeks—not the many years it is known to take. Other insults, such as free radicals, which can also fragment DNA, or the cellular breakdown that occurs after death, may have accounted for the numerous TUNEL-stained cells in Alzheimer's brains, Smith suggests.

Cotman concedes that "people were really disturbed by how many cells were affected" by TUNEL staining. So more recently his group and others have taken a different tack, looking for more specific biochemical evidence that in Alzheimer's disease, the apoptotic machinery has been activated.

Several teams have looked at one or another of the 14 caspases so far identified. Like many other dangerous, protein-splitting enzymes, the caspases are made in inactive form and have to be turned on by removal of part of the enzyme molecule. Because of this structural change, it's possible to produce antibodies that recognize only the active caspases. Using such an antibody, Lassmann and his colleagues showed in 1999 that brain tissue from people who died of Alzheimer's contained more neurons with activated caspase-3 than did samples from age-matched controls who died of other causes.

Some of the activated caspase was encapsulated in granules where it couldn't damage the cells—a possible sign, Lassmann says, that the neurons were trying to resist apoptosis by taking the enzyme out of action. But the active enzyme was present in the cytoplasm as well, and in those neurons the classic morphological signs of apoptosis were apparent. And the number was small enough—only about one in 1100 to 5000 neurons was affected in this way—to be consistent with the slow course of Alzheimer's.

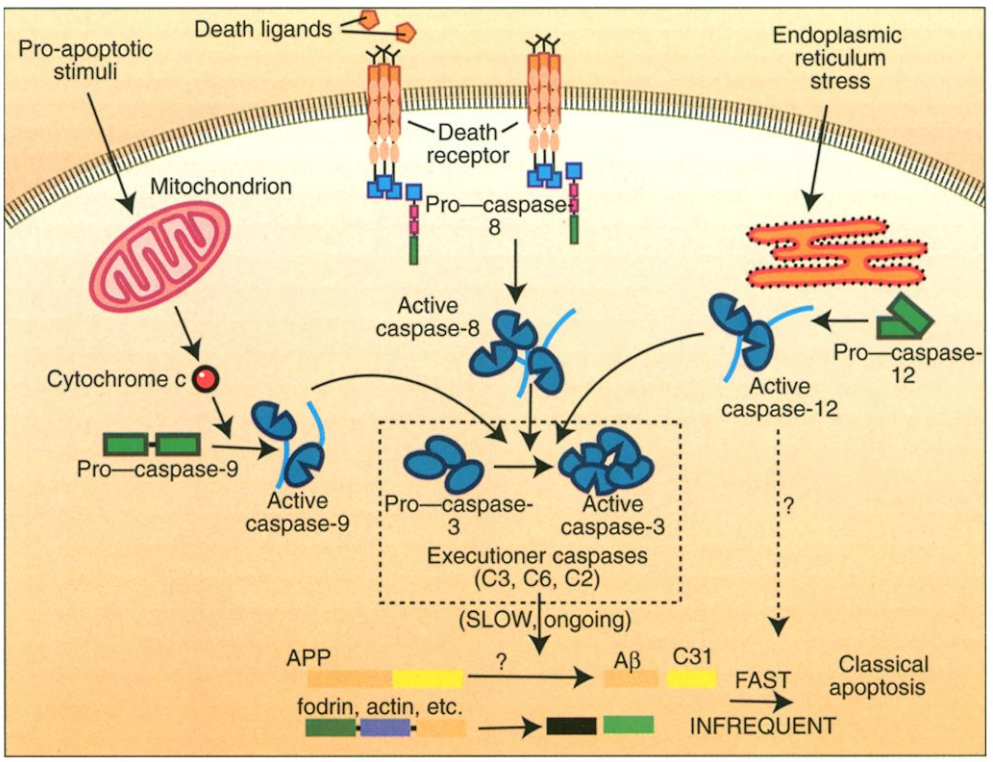
This year, Cotman's team reported similar percentages of neurons with active caspase-3 in Alzheimer's brains. In addition, they showed that the enzyme tends to be located in and around the abnormal amyloid-containing plaques and neurofibrillary tangles that are characteristic features of Alzheimer's brains—an indication that the enzyme is somehow linked to that pathology.

But caspase-3 is not the only caspase that may contribute to neurodegeneration in Alzheimer's, although much of the evidence for the others comes from studies of mouse models of Alzheimer's or nerve cells in lab culture. Caspase-12 is among those implicated by that work. Last year, Junying Yuan, Bruce Yankner, and their colleagues at Harvard Medical School in Boston showed that this enzyme is located in the membranous intracellular compartment called the endoplasmic reticulum (ER), which is important for protein synthesis and folding. The ER also regulates cellular responses to stresses such as protein misfolding and aggregation, free radicals, and the high concentrations of calcium ions and chemical toxins that may build up with age.

Prolonged exposure to those stresses can result in cell death through apoptosis, and the Harvard team's results indicate that caspase-12 activation is needed for that cell death to occur. Although everyone's brain is exposed to these stresses, those of people destined to develop Alzheimer's may be more susceptible. Indeed, the ER is a potential site of Aβ's toxic effects: Yuan, Yankner, and their colleagues also showed that cortical neurons taken from the brains of mice in which the caspase-12 gene had been knocked out resist the protein's apoptosis-inducing effects, thus possibly linking caspase-12 to Aβ toxicity.

The list doesn't stop with caspase-12, however. In experiments reminiscent of those of the Harvard team, Michael Shelanski and his colleagues at Columbia University College of Physicians and Surgeons in New York City looked at how Aβ affects hippocampal neurons from mice with an inactivated caspase-2 gene. They found that the neurons were completely resistant to apoptosis when exposed to this peptide. "When you treat neurons with Aβ, you have a cell-death mechanism mediated by caspase-2," Shelanski concludes. And other investigators have implicated activation of caspase-6, -8, and -9 in Alzheimer's.

At least one caspase may even trigger a nonapoptotic form of cell death. In work reported late last year, Dale Bredesen of the Buck Institute for Age Research in Novato, California, and his colleagues found that activation of caspase-9 in cultured neurons can lead to cell death that does not show many of the typical morphological and biochemical signs of apoptosis. Nevertheless, Bredesen says, this death was "programmed," because it could be prevented by inhibitors of gene transcription, an indication that the cells had to turn on genes in order to die.



Routes to apoptosis. Triggers acting through various paths may lead to caspase activation in the brain neurons of Alzheimer's patients. This in turn may lead to Aβ production and the breakdown of various neuronal proteins, thus causing neuronal malfunction. Ultimately, the neurons die.

ILLUSTRATION: C. SLAYDEN (ADAPTED FROM C. W. COTMAN)

Caspase cascades

Although these various results are confusing at present, they are not necessarily contradictory. "The fact that all these caspases are involved implicates apoptosis as contributing to Alzheimer's," Yankner says, "but the fact that more than one is involved is not problematic." He points out that caspases often act in "cascades"—in which one caspase, when triggered, activates another. So some of the enzymes may be working together. Or, because the cell has more than one pathway for triggering apoptosis, they may be working in different pathways.

Still, Smith isn't ready to buy the idea that apoptosis is going on in Alzheimer's brains. He says that work by his team, which includes Case Western Reserve pathologist George Perry, shows that although some caspases are activated in the brains of the patients, they are "upstream" in the caspase cascade—they are enzymes, such as caspase-8 and -9, that are turned on early. Contrary to what other researchers have found, Smith, Perry, and their colleagues don't see activation of downstream caspases such as caspase-3. This leads them to propose that although apoptosis may be initiated in the neurons, it is aborted before it can kill them.

The pro-apoptotic camp has a few more arguments of its own, however. These scientists point out that the activated caspases not only break down important neuronal proteins such as actin and fodrin, but they may also be responsible for releasing Aβ itself. Donald Nicholson of Merck Research Laboratories in Kirkland, Quebec, and his colleagues have evidence, both from cultured cells and examination of Alzheimer's brains, that caspases cut APP, releasing Aβ. They also find that an APP mutation known to predispose carriers to Alzheimer's creates a site for caspase cleavage action, thus fostering Aβ release.

"It's very clear to us that APP is cleaved by caspases in aging cells of all types and in Alzheimer's brains," Nicholson says.

In addition, Bredesen's team has found that caspase cleavage of APP releases a second apoptosis-promoting peptide, which the researchers call C31 because it contains 31 amino acids from APP's carboxyl end. If brain caspases do in fact attack APP to release these toxic products, then there may be a vicious cycle in which Aβ, by triggering caspase activation, fosters its own production—and thus further caspase activation and cell death.

There is a potential flaw in this picture, however. Edward Koo's team at the University of California, San Diego, found that caspase cleavage of APP actually *decreases* Aβ secretion by cells because it removes a so-called "signal sequence" that would direct the peptide into the cell's secretory pathway. "This is contrary to what we got," Nicholson says, "and we don't know how to reconcile the two sets of results."

"Synaptosis"

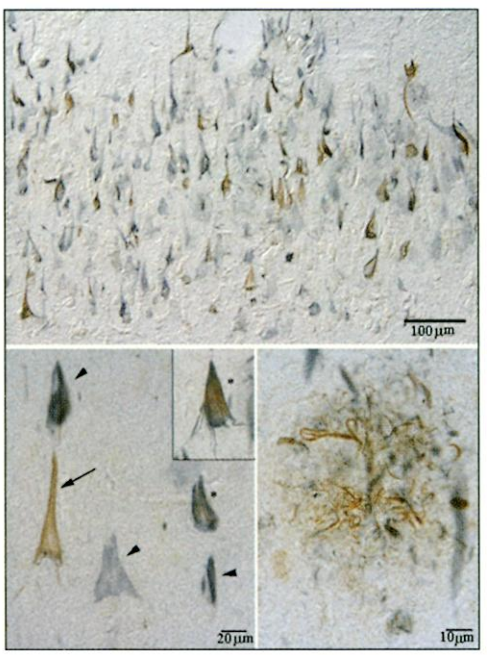
Whether or not caspases increase Aβ production, there is evidence that they break down other cell proteins whose destruction could contribute to neuronal malfunction in Alzheimer's. For example, Cotman and his colleagues produced an antibody that specifically recognizes a caspase-3–cleaved fragment of fodrin—a protein they chose because it is a major component of the fibers that form the cell skeleton of neurons and is a known caspase target.

The researchers found that this antibody stained many more neurons in brains from Alzheimer's patients, notably in the hippocampus—a region hit particularly hard by the disease—than in control brains. The difference was so marked, Cotman says, that he describes it as "fairly astonishing." Many of the stained neurons also contained Alzheimer's characteristic neurofibrillary tangles, which consist of abnormally twisted cytoskeletal filaments. What's more, Cotman adds, "as the disease progressed, the brains got more of the fodrin staining and the tangles."

Similarly, Greg Cole's group at the Sepulveda Veterans Administration Medical Center in North Hills, California, has used an antibody to show that actin, another prominent protein of the cytoskeleton, undergoes caspase cleavage in Alzheimer's brains. The researchers localized the cleaved actin to the degenerating nerve terminals.

Other evidence also suggests that the nerve terminals may be a prime target for caspase activation. Work with cultured brain neurons by Mark Mattson's team at the National Institute on Aging's Gerontology Research Center in Baltimore, Maryland, shows that staining for caspases activated by Aβ can be found in the dendrites, the neuronal projections that receive incoming signals from other nerve cells. There, the caspases appear to localize to the synapses, the actual points where the incoming neurons make contact with their target cells.

In addition, Cotman and his colleagues cultured brain neurons in chambers that allowed them to apply Aβ to the terminals without having it come in contact with the cell body, a situation that may resemble what happens in Alzheimer's brains. The Irvine team found that the terminals degenerated. They underwent membrane changes much like those seen in apoptotic cells, and the whole process was caspase-dependent. "You could have focal activation of the caspase cleavage pathways," Cole suggests. This could lead to elimination of individual terminals without necessarily killing the entire nerve cell, at least not immediately.



Partners in crime? Alzheimer's brain tissue is stained for cleaved fodrin (brown), a caspase product, and for pathological tangles (blue). Some neurons (bottom) show both stains.

Several of the researchers have suggested that Alzheimer's begins with such nerve-terminal degeneration—or "synaptosis," as Cole calls it. This would be consistent with findings over the years that patients' degree of dementia is more highly correlated with the loss of nerve terminals in their brains than with other pathological features, such as plaque formation. The neurons may even be able to survive this damage—for a while. As Cotman and others point out, cells have mechanisms for keeping apoptosis in check. Otherwise they might die when they shouldn't. And neurons, which don't normally divide, may be better at this than other cells.

Ultimately, though, as a nerve cell loses more and more of its terminals, it will die, because it needs so-called trophic factors, produced by the other neurons with which it connects, to survive. "In my view, there is a long period of [neuronal] dysfunction that may contribute to cognitive decline before there's frank neuronal loss," Yankner says. Even skeptic Smith deems the idea of such slow neuronal death "reasonable," although he still maintains that this shouldn't be considered classic apoptosis.

Researchers clearly have a lot more work to do to sort out what all those caspases are doing and how they might interact with one another. Another big question concerns how Aβ might trigger caspase activation, although there are some clues. In the June issue of the *Journal of Neurochemistry*, Shelanski's team at Columbia reports that in cultured neurons the peptide appears to act through one of the cell's many kinase enzymes, the one known as JNK (for c-Jun N-terminal kinase), which has been tagged in other work as an intermediary in cell pathways leading to apoptosis.

And in still unpublished work, Mary Savage and her colleagues at Cephalon Inc., a biotech firm in Brandywine, Pennsylvania, have found increased amounts of activated JNK in an intriguing location in the brains of a mouse Alzheimer's model. Savage says they are "located right around the amyloid deposits," where the nerve terminals are degenerating. "This may be an example of distal activation of an apoptotic pathway leading to synapse loss," Cole speculates.

If Aβ does in fact trigger apoptosis through JNK, then the kinase as well as the caspases would be a potential target for drugs aimed at stopping, or at least slowing, Alzheimer's development. No one expects that developing such drugs will be easy, however, given the fact that both the caspases and the kinases play key roles in cell regulation throughout the body. For example, by helping eliminate cells with damaged DNA, the caspases protect against cancer.

So there are worries that caspase or kinase inhibitors aimed at stopping apoptosis in Alzheimer's could have unacceptable side effects, especially because they may have to be given for a long time, even beginning well before symptoms become severe. And Yankner suspects that anti-apoptosis drugs by themselves might not be sufficient. Still, he says, they "may give an edge to repairing [damaged] neurons that haven't died." In any case, if researchers finally do pin the death of neurons in Alzheimer's on apoptosis, these cellular "medical examiners" will at least have helped nab the culprit in this dread disease.

–JEAN MARX

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