A New Link in the Brain's Defenses

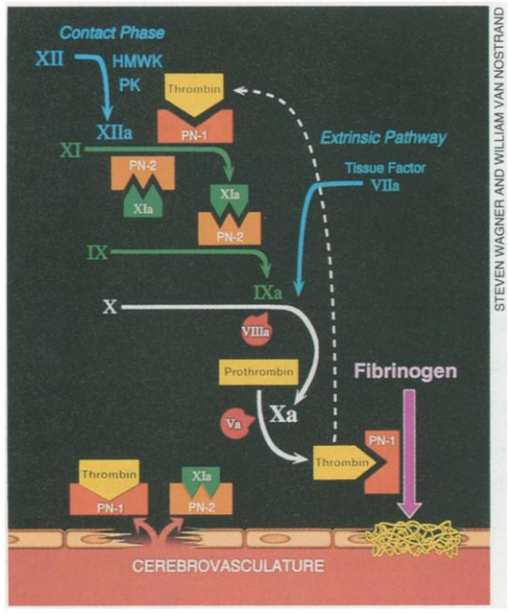
Proteases, such as the clotting factor thrombin, and their inhibitors may help maintain nerve connections in the brain as well as performing their more standard role in blood coagulation

Mother Nature is widely perceived as bountiful and nurturing. But she has another side—one that might be called parsimonious, even stingy. Her philosophy is: Why make a different protein for each and every physiological function, when it's just as easy to adapt one protein for several different jobs? Take the proteins of the body's blood-clotting system. Within the past few years, researchers have been building a case that some of these proteins may do things very far removed from blood clotting, such as helping to regulate normal brain development and defending the brain against damage caused by stroke, trauma, and other injuries. What's more, their results suggest that an imbalance in these proteins' activities may contribute—at least secondarily—to the nerve-cell damage seen in neurodegenerative diseases such as Alzheimer's.

Those findings have brought the proteins much more than academic interest. Information about how they work may assist researchers in designing drugs that diminish stroke damage or prevent the neuronal degeneration of Alzheimer's. No surprise, then, that the blood-clotting proteins have caught the eye of the biotechnology industry. Ivan Lieberburg, who heads the Alzheimer's program at Athena Neurosciences in south San Francisco, describes the proteins and their relatives as "extremely important," and one of the "major therapeutic targets" in Alzheimer's research at Athena and other biotech companies.

Like many other scientific sensations, the idea that clotting proteins might play a role in the brain didn't spring up overnight. Indeed, the story began more than a decade ago with work on the protein thrombin in the lab of biochemist Dennis Cunningham at the University of California, Irvine. Thrombin is a protein-splitting enzyme (or protease) that forms part of the enzymatic cascade that causes mammalian blood to clot. Specifically, thrombin cleaves fibrinogen, releasing the insoluble protein fibrin, which forms the clots. But thrombin also facilitates wound healing by stimulating the division of fibroblasts, the cells that form the connective tissue. Cunningham and his colleagues wanted to find out how thrombin performs that feat.

In the course of that work, they identified two proteins that bind to thrombin and other proteases, inhibiting their enzymatic activity—and in thrombin's case, its ability to stimulate fibroblast division. Because the new proteins bind proteases, the Irvine workers named them with reference to the Latin word for link ("nexus"): protease nexin-1 and -2 (PN-1 and -2). At the time, Cunningham had no idea PN-1 and PN-2 were going to prove to be important brain proteins, nor did he know that, in fact, his group was not the first to identify PN-1.



STEVEN WAGNER AND WILLIAM VAN NOSTRAND

Double duty. The scheme shows how the protease nexins (PN-1 and -2) might regulate the blood-clotting cascade in the brain.

That honor belonged to Denis Monard, at the Friedrich Miescher Institute in Basel, Switzerland. In 1973 Monard identified a protein secreted by glial cells (nonneuronal support cells in the brain) that promotes the growth of neurites, projections nerve cells send out to communicate with one another. But the possibility that PN-1 might be the same as Monard's factor was unsuspected until the mid-1980s, when Monard purified his protein and found that it was also a potent thrombin inhibitor. The suspicion was confirmed in 1988 when Joffre Baker, a former Cunningham postdoc who by then had his own lab at the University of Kansas, Lawrence, in collaboration with researchers at Invitron Corp. in Redwood City, California, cloned the PN-1 gene and determined its entire sequence, which proved to be identical to that of Monard's protein.

Once the identity was established, the Cunningham and Monard groups really dug in, amassing a great deal of evidence suggesting that PN-1 and thrombin play an important role in making and maintaining connections between nerve cells. David Gurwitz, while a postdoc in Cunningham's lab, showed, for example, that thrombin causes cultured nerve cells to retract their neurites, and that PN-1 can reverse that effect by virtue of its ability to inhibit thrombin's protein-cutting activity.

Those effects apparently aren't limited to cells in culture—they seem to take place in the intact brain tissue. The evidence for that includes the fact that PN-1 is made in the brain, and that its concentration is extremely high in the regions that receive and process olfactory signals. And that would fit in with the connection-forming activity, because, says Monard, "in those regions there is tremendous remodeling all the time as neuronal connections are being broken and remade." Thrombin, too, appears to be synthesized in the brain. Although contamination with thrombin from the blood prevents a direct demonstration that brain cells make the clotting factor, Monard's group, in joint work with Cunningham, has shown that the gene that encodes thrombin makes its messenger RNA there.

If the researchers are correct in their hypothesis that PN-1 and thrombin together regulate neurite outgrowth by brain neurons, then an imbalance in the concentration of the two cooperating proteins might well lead to loss of neuronal connections or to nerve cell damage—especially if thrombin, with its neurite-retracting abilities, gets the upper hand.

And the nerve cell damage wreaked by thrombin could take place in some instances even if the supposition that the brain makes its own thrombin turns out to be wrong. Thrombin can induce neurite retraction in very low concentrations—in the picomolar range—and recent results from Monard's group show that the effect happens within a few seconds of nerve cells' exposure to thrombin. "I think this underscores the importance of having a potent inhibitor of thrombin in the brain," Cunningham says. If the barrier that normally prevents proteins from moving from the blood into the brain is damaged by trauma, stroke, or other disease, a small amount of thrombin leaking in could cause serious damage unless an effective inhibitor such as PN-1 is available to counteract thrombin's effects.

Alzheimer's might be one of the diseases where this happens. A few years ago, Steven

Wagner, then a postdoc in Cunningham's lab, showed that PN-1's protease inhibitor activity is much lower in the brains of people who died of the neurodegenerative disease than in control brains, apparently because the PN-1 was tied up in complexes with thrombin. There have been reports that the blood-brain barrier is damaged in Alzheimer's, although the findings are somewhat controversial. Could the persistent inward leakage of thrombin perhaps overwhelm PN-1 defenses and contribute to the neurodegeneration that occurs in the disease? That idea, Cunningham says, is "quite speculative," although nonetheless a possibility.

PN-1 might also be part of the brain's more general defenses against injury. In as yet unpublished work, Patrick Vaughan and Denis Guttridge in Cunningham's group have shown that secretion of PN-1 by brain cells in culture is markedly stimulated by factors, such as interleukin-1 and tumor necrosis factor, that are classically active in injury and inflammation. And Monard, working with Hana Suidan and C. Nitsch of the University of Basel, has evidence from an animal model that injury induces PN-1 production in the brain as well. In these experiments, the researchers tie off the carotid arteries of gerbils for a few minutes and then release them. The brief oxygen deficiency caused by this treatment induces a specific lesion in which the pyramidal nerve cells in the hippocampus region of the gerbil brain die off while other nerve cells are preserved. The result: Monard and his colleagues found that PN-1 is synthesized in glial cells only in the area where the cell death occurs, possibly in an effort by the brain to stimulate new neuronal growth.

For several years while research on PN-1's role in the brain was moving ahead, investigations of PN-2 lagged behind, mainly because it was made in much smaller amounts by fibroblasts and so was harder to purify. William Van Nostrand, another Cunningham postdoc, finally achieved the feat in 1987. He got a partial amino acid sequence but found no related sequences in the databanks at the time that would connect it to the brain—or to anything else for that matter.

Then in 1989, a team of researchers at Athena Neurosciences and also Cunningham's group made a startling discovery—and one that plunged PN-2 right into the thicket of brain research and in particular into Alzheimer's research. The sequence of the amyloid precursor protein (APP), which is the source of β-amyloid, the major protein constituent of the senile plaques that are one of the characteristic pathological features of Alzheimer's brains, had recently been published. And when the two groups compared the APP sequence to the partial PN-2 sequence, they found that they had hit paydirt. They went on to show that PN-2 is in fact part of the APP molecule.

Potential New Alzheimer's Test

As of now, there are no effective therapies for Alzheimer's disease. And that fact makes the only definitive method for diagnosing the disease—brain biopsy—unethical, not to say impractical. But new results presented by William Van Nostrand of the University of California, Irvine, at a meeting on neurodegenerative diseases, which was held in Big Sky, Montana, at the end of March, suggest that it may soon be possible to diagnose Alzheimer's with a relatively simple test on spinal fluid.*

"The data looked good from what I saw. And we certainly need a diagnostic marker that's not invasive," says Alzheimer's researcher Sam Sisodia of Johns Hopkins University School of Medicine, who heard Van Nostrand's talk at Big Sky. Such a test would be invaluable should therapy ever become possible. And even now the test could, at the very least, help distinguish Alzheimer's patients from people with other dementias that might be treatable.

The test developed by Van Nostrand, who works in collaboration with Steven Wagner of Salk Institute Biotechnology/Industrial Associates Inc. in La Jolla, and Dennis Cunningham, also at Irvine, depends on a monoclonal antibody that recognizes the amyloid precursor protein (APP), which contributes to the development of pathological amyloid deposits found in Alzheimer's brains. Using the antibody, the researchers have shown that cerebrospinal fluid from Alzheimer's patients has only one-third as much APP as spinal fluid from normal individuals or from people with other kinds of dementias. With Raymond Roos of the Academic Hospital in Leiden, the Netherlands, they've found similar decreases in APP in patients with hereditary cerebral hemorrhage of the "Dutch type," which is also characterized by abnormal amyloid deposits, but leading to hemorrhagic stroke, rather than dementia. The studies suggest, Van Nostrand says, that the decreases in cerebrospinal APP levels correlate with the degree of amyloid deposition in the brain, especially around blood vessels.

Other researchers who have tried to find changes in APP concentrations in cerebrospinal fluid from Alzheimer's patients have obtained inconsistent results. Some have found small increases, others small decreases. Van Nostrand's antibody may have worked better, he says, because it was made against native protein while the others were made against synthetic APP fragments. Sisodia cautions, however, that the California group needs to look at more controls and more Alzheimer's patients to verify their results—a suggestion with which Van Nostrand heartily agrees. Expanded tests are under way, and if all goes well, better Alzheimer's diagnosis may be on the horizon.

–J.M.

*Some of the results were also described in the April 1992 issue of the *Proceedings of the National Academy of Sciences.*

Of course, a great many labs are trying to figure out just exactly what APP does normally in the brain, as well as what causes the abnormal release of β-amyloid and plaque buildup in Alzheimer's. And while there have been some clues (*Science*, 7 February, p. 689), it's safe to say that both of those matters are still very much up in the air. Many Alzheimer's researchers favor the idea, however, that APP's normal role is to maintain connections between nerve cells in some as yet unclear fashion. But then a logical question arises: If a clotting inhibitor like PN-1 might help regulate neuronal connectivity, could not the reverse be true for the PN-2 portion of the APP molecule? And indeed, that's just what Wagner, who now works at Salk Institute Biotechnology/Industrial Associates Inc. in La Jolla, Cunningham, and Van Nostrand are now proposing. Based on findings in their own labs and elsewhere, they suggest that one of PN-2's normal functions is to cooperate with PN-1 in regulating blood clotting in the brain.

One piece of the evidence for that idea comes, for example, from George Broze's group at Washington University Medical Center in St. Louis, who showed that PN-2 is identical to a factor that they isolated from platelets on the basis of its ability to inhibit clotting factor XIa, a protease that catalyzes a key reaction in the cascade of events leading to thrombin formation. And while both PN-1 and PN-2 are present in platelets, their synthesis is highest in the brain, an organ that may well need their abilities to inhibit blood clotting enzymes, since the blood vessels there are deficient in thrombomodulin, a protein that helps to keep clotting in check in blood vessels elsewhere in the body. What's more, although APP is a membrane protein, it's been well established that the PN-2 part, which is on the outer cell surface, is normally clipped off and secreted so it would be available to act as a clotting inhibitor. "The argument would be strong that the molecules [PN-1 and -2] in the vascular system are involved in some way in the complex series of

events that regulate blood coagulation," Cunningham says. "It's sensible to make that argument for the brain, too."

Whether Alzheimer's researchers generally will find that the suggestion that one of their favorite molecules regulates blood clotting is "sensible" remains to be seen, however. "Making the leap that this is important in blood coagulation is a big if for me," says Sam Sisodia of Johns Hopkins University School of Medicine, who studies APP secretion, among other things. He favors the more standard view that APP somehow maintains connections between nerve cells, but nonetheless concedes that it might have more than one function as so many of the body's other proteins do.

While the California group's proposal may shed some light on APP's normal role, it doesn't help explain how it contributes to Alzheimer's pathology. But as Cunningham points out, maintaining the cerebral blood flow is also important for brain function. A deficiency of a clotting inhibitor might predispose an individual to stroke, for example. All in all, the protease nexins, whether as clotting regulators or maintainers of connections between nerve cells, may have a great deal to do with maintaining the brain's health.

– Jean Marx

EVOLUTION

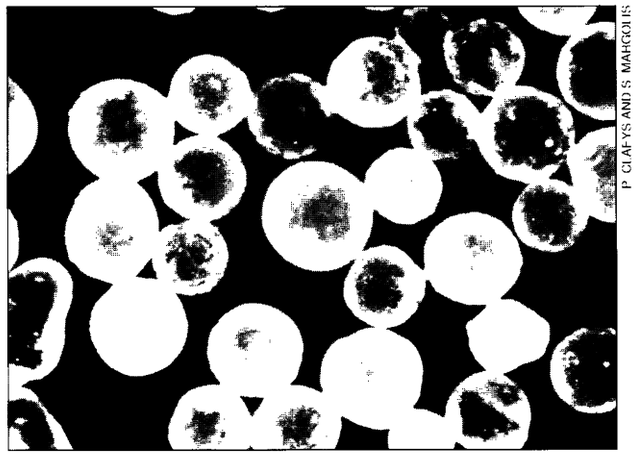
Another Impact Extinction?

At first, they hardly looked like relics of a global cataclysm. The microscopic beads of glass appeared to be little more than a curiosity when Jean-Georges Casier stumbled across them in 1987 among the tiny marine fossils he had extracted from 367-million-year-old rocks in Belgium near the French border. Casier, who works at the Royal Institute of Natural Sciences of Belgium, showed them to a Belgian colleague, who pronounced them to be mere volcanic glass.

That was that—until Casier opened the 29 March 1991 issue of *Science*, where a paper by geochemists Philippe Claeys and Stanley Margolis of the University of California (UC), Davis, and Frank Kyte of UC, Los Angeles, featured spherules that looked strikingly like Casier's. Instead of volcanic glass, though, the paper described them as debris typical of huge asteroid or comet impacts, such as the one 65 million years ago at the geologic boundary between the Cretaceous and Tertiary periods, which marked the end of the age of the dinosaurs. Casier's spherules, though more than 300 million years older, roughly coincided with another of the half-dozen mass extinctions of the past 500 million years—the one marking the boundary between the Frasnian and Famennian stages. That was when most existing species of corals and many other bottom-dwelling marine species went extinct. Could he have stumbled upon another killer impact, Casier wondered?

To test the possibility, Casier teamed up with Margolis and Claeys, and in a poster at this month's American Geophysical Union meeting in Montreal the trio presented evidence that the spherules were indeed formed when molten rock splashed from a giant impact crater. For one thing, the shapes were the same ones—mostly simple spheres but also fused spheres, dumbbells, and teardrops—that show up in the glass beads, or microtektites, from known crater debris. Laboratory analysis of the Belgian spherules also revealed the low water content and extremes of elemental compositions typical of Cretaceous-Tertiary tektites. "The distinctive compositions are impressive," says geochemist Wayne Goodfellow of the Geological Survey of Canada (GSC) in Ottawa, who has searched the F-F boundary elsewhere. "I'm excited about the Belgian microtektites."

No one is rushing to conclude that the impact caused the F-F mass extinction, however. The relative timing is still too uncertain. The F-F boundary has traditionally been located by tracing a change in the assortment of tiny tooth-shaped fossils called conodonts, all that remain of a leech-like creature. But the abundance of conodonts in any particular layer of rock is subject to the vagaries of the fossilization process. Where Casier found the microtektites, conodonts are scarce. At best, says Claeys, the group can say that their thin layer of microtektites falls within a few hundred thousand years of the conodont-defined F-F. That's quite close for events more than one-third of a billion years old, but not clearly coincident.



P. CLAEYS AND S. MARGOLIS

Relics of catastrophe. An asteroid or comet impact spewed these 300-micrometer glass beads 367 million years ago.

So far, workers searching for microtektites at other sites, where the F-F boundary is often more sharply defined, have come up empty-handed. But Goodfellow and sedimentologist Helmut Geldsetzer of the GSC in Calgary are hoping that a different impact indicator may help them tie the impact to the extinctions. In the Montagne Noire region of southern France, Goodfellow and Geldsetzer recently detected enhanced concentrations of the element iridium in a 4-centimeter-thick layer right on a well-defined F-F boundary. Iridium, which is scarce in Earth's crust but abundant in asteroids and comets, turns up in most Cretaceous-Tertiary boundary deposits. Still, the element is not as unequivocal a marker of impacts as microtektites. An iridium-enriched layer found in 1984 near the F-F boundary in western Australia has been dismissed as more likely to be the work of iridium-concentrating microbes than of an impact.

But Goodfellow and Geldsetzer believe their French iridium anomaly is more promising than the Australian find ever was. The concentration of iridium at the French site is far higher than in western Australia, they say, and is comparable to Cretaceous-Tertiary concentrations. And they argue that the ratio of iridium to ruthenium is just what's expected of an asteroid or comet. Whether the Canadians have found a true impact signature could become clearer as they search the iridium layer for microtektites and shocked minerals, another sign of a large impact.

Meanwhile, Claeys and Margolis are on the trail of a different F-F mystery: where the impact struck. They are pursuing the possibility that their microtektites were splashed from a known crater, possibly the nearby Siljan crater in Sweden, which is about as old as the F-F boundary.

Without an impact crater or an indisputable link to the extinction, the signs of impact at the F-F boundary fall in roughly the same category as the shocked minerals recently discovered at the 202-million-year-old Triassic-Jurassic mass extinction (see report in the 24 January *Science*): suggestive, but by no means the clear signature of a killer. But if the case for impact-caused extinction strengthens at either boundary, the long-discussed possibility that repeated killer impacts shaped the history of life may look more compelling.

–Richard A. Kerr

ADDITIONAL READING

• P. Claeys *et al.*, "Microtektites and Mass Extinctions From the Late Devonian of Belgium," *EOS Trans AGU* 73, 328 (1992).