Proteins for All Seasons

Studies of the highly diverse phosphoproteins of the brain are contributing new insights into how neurotransmitters work

"The work of the last 20 years has made it clear that almost every biological process is regulated by protein phosphorylation," says Paul Greengard of Rockefeller University. A list of the regulatory agents that produce their cellular effects either directly or indirectly by causing the addition of phosphate groups to proteins includes numerous hormones, growth factors, and the neurotransmitters that carry chemical signals between nerve cells.

For some of these regulatory agents, the way in which the phosphorylation brings about the biological response is clear. The classic example is the stimulation of glycogen breakdown in response to the hormone epinephrine. Binding of the hormone to its receptors sets off a chain of events that culminates in the phosphorylation and subsequent activation of the enzyme phosphorylase, which breaks down the glucose-storage substance glycogen, yielding a glucose product that can be readily converted to energy by the cell. The phosphorylase can then be inactivated by removal of the phosphate group by an enzyme called a protein phosphatase.

Although work on phosphorylase and other enzymes established that adding and removing phosphate is an effective way of controlling enzyme activities, the precise roles of the proteins that undergo phosphorylation in response to many regulatory agents have often proved difficult to pin down. This has been true for growth-factor responses and also for those to neurotransmitters, although the latter at least are now beginning to yield up their secrets. Greengard, working first at Yale University School of Medicine and since 1983 at Rockefeller, has been among the leaders in the research on neurotransmitters and protein phosphorylation. In a recent interview with *Science*, he talked about his group's current progress in isolating and characterizing phosphoproteins from mammalian brain and what the work reveals about how neurotransmitters produce their effects.

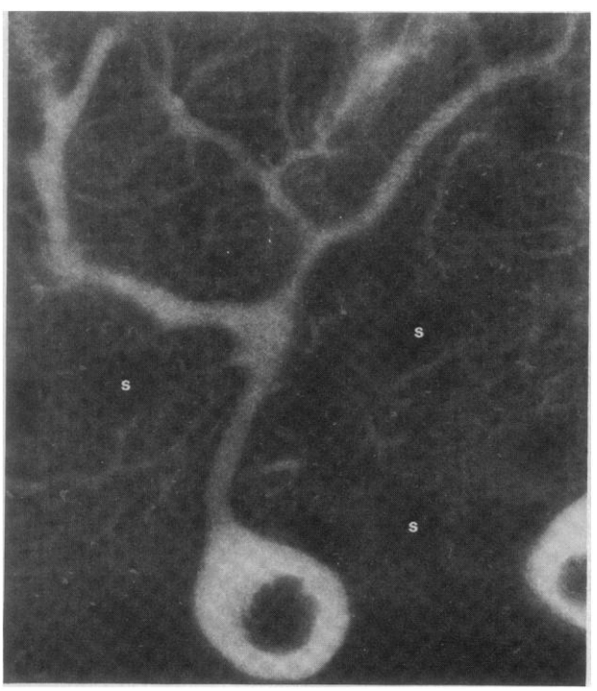
One of the main conclusions of the research is that the proteins phosphorylated as a result of neurotransmitter action in brain are a very diverse group, much more diverse than the other known participants in neurotransmitter responses. The specific reaction of a particular nerve cell to a neurotransmitter may thus depend more on the types of proteins phosphorylated than on the other components of the regulatory machinery. Moreover, the proteins can serve as markers for labeling nerve cells and tracing nerve connections and are also potential targets for pyschoactive drugs.

The actions of all neurotransmitters begin with their binding to specific receptors on their target cells. Over the years, Greengard and his colleagues have found that a consequence of the binding of many neurotransmitters, among them dopamine, serotonin, and norepinephrine, is the activation of enzymes called kinases that attach phosphate groups to proteins.

Until recently, the evidence that the kinases and the proteins that they phosphorylate actually mediate the effects of the neurotransmitters was largely indirect, based on correlations between a particular response and the phosphorylation state of one or another of the proteins. Only within the past few years have Greengard and his Rockefeller colleague Angus Nairn been able to garner more direct evidence. In collaboration with a number of researchers who specialize in the electrophysiology of neurons, they have found that they can duplicate the responses of nerve cells to neurotransmitters by injecting the cells with individual kinases. In contrast, injection of a specific inhibitor of one of the kinases prevents the normal response to the appropriate neurotransmitter.

The neurotransmitters do not activate the kinases directly, however, but work through "second messengers" as they are called. The second messengers include cyclic AMP (cyclic adenosine monophosphate), cyclic GMP (cyclic guanosine monophosphate), calcium ions, and the lipid diacylglycerol.

The numbers of second messengers and of neurotransmitter-regulated kinases are limited. Only a few of each have been identified so far and, with the exception of one kinase, all are widely distributed in brain neurons. Greengard and his colleagues are now finding a very different situation for the proteins that are phosphorylated by the kinases. "In contrast to the handful of kinases, there are an enormous number of proteins that serve as substrates for the kinases," Greengard explains. "We have partially characterized 70 already. I think that there are at least hundreds and possibly thousands in the nervous system." The work has revealed that the protein substrates can differ greatly, in the kinases by which they are phosphorylated, in their molecular properties, in their functions, and lastly in their cellular and intracellular locations.



Purkinje cell

View of Purkinje cell stained with an antibody to a cell-specific kinase. The "s" indicate unstained cells of other types. [Source: P. De Camilli, P. E. Miller, P. Levitts, U. Walter, and P. Greengard, Neuroscience *11, 761 (1984)]*

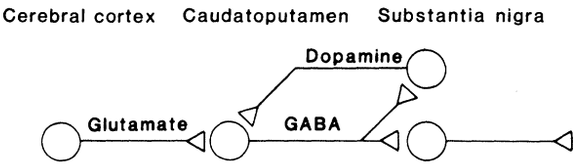
Some are found in virtually all neurons, whereas others occur in just a few types of neuron or even in a single type. All this adds to the evidence that neurons show tremendous biochemical heterogeneity, Greengard notes, a picture which contrasts with the old view that one neuron is much like another.

The existence of so many biochemically distinct types of nerve cells has potential implications for the design of neuroactive drugs. It may be possible to develop drugs that specifically enhance or inhibit the function of a kinase substrate in just one kind of neuron, or at least in just a few neuronal types. Such drugs might be more specific in their action than agents directed against the more widely distributed kinases or against the neurotransmitters, which may act on several different target cells. Potential targets for drug therapy include a family of phosphoproteins that was identified by the Greengard group in the neurons involved in Parkinson's and Huntington's diseases.

Drugs that act directly on the phosphoproteins have yet to be produced. Meanwhile, some of the proteins, especially those found in only one type of neuron, are already proving valuable for tracing the anatomy of nerve connections. "They color-code the neurons for you so you can sort out what is going on," as Greengard puts it. He cites as examples a cyclic GMP–dependent kinase and its substrate protein, both of which are specific markers for the Purkinje cells that carry outgoing signals from the cerebellum and are thus important in coordinating movements. Antibodies to the purified kinase worked very well for tracing the neurons. "With one antibody my colleague Pietro De Camilli was able to map out the projections of all the Purkinje cells in the central nervous system," Greengard says.

Other workers have previously traced Purkinje cell projections, but the methods are tedious. Identification of cell-specific markers can greatly facilitate the mapping of neuronal connections and has also been extremely useful in following the fates of neurons during development.

The big challenge is determining the cellular functions of such diverse proteins. "There's a tremendous amount of work to be done," Greengard says. "We have done in-depth studies on only a few of the proteins." In particular, they have concentrated on two proteins, synapsin I and DARPP-32 (for dopamine- and cyclic AMP–regulated phosphoprotein with a molecular weight of 32,000).



Neuronal connections

The nerve cells release the neurotransmitters glutamate, dopamine, or GABA (γ-aminobutyric acid) as indicated. DARPP-32 occurs in the GABA-releasing neurons that respond to dopamine.

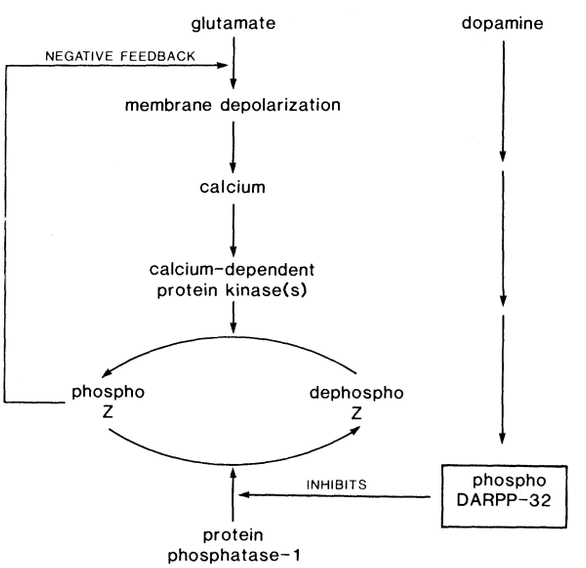
The cellular and subcellular locations of the phosphoproteins can provide clues to their functions. Synapsin I, which was characterized by De Camilli, Tetsufumi Ueda, Wieland Huttner, and Eric Nestler of the Rockefeller group, is a phosphoprotein with a molecular weight of about 83,000. It apparently occurs in all neurons where it is concentrated in the axon terminals that form the connections with the neuronal target cells. In particular, synapsin I covers the surfaces of small particles in the terminals, which are called synaptic vesicles. The protein's distribution indicates that it may play a role in neurotransmitter release, Greengard hypothesizes.

When a neuron is stimulated, the terminals release their neurotransmitter, which diffuses across a small gap (synapse) between the terminals and the target cells where it binds to its receptors and evokes its response. Neurotransmitters are stored in the synaptic vesicles and most neurobiologists currently think that the chemicals are released when the vesicles fuse with the cell membrane at the axon terminal in response to an appropriate stimulus and discharge their contents into the synapse.

Earlier work had provided indirect evidence for synapsin I involvement in neurotransmitter release. There was a correlation between nerve cell activity, neurotransmitter release, and phosphorylation of the protein. More recently, Rodolfo Llinas and his colleagues at New York University Medical Center in collaboration with the Greengard group injected individual nerve terminals either with synapsin I or with the calcium-activated kinase that phosphorylates it. They found that unphosphorylated synapsin I inhibits neurotransmitter discharge, whereas the kinase potentiates it. Moreover, Werner Schiebler and Rinehard Jahn of the Rockefeller group find that synapsin I dissociates from the vesicles when it is phosphorylated by the kinase.

The investigators propose a model based on these results in which synapsin I in its unphosphorylated state forms a cage around the vesicles. Then, when a neuron receives an appropriate stimulus, the calcium ion concentration increases inside the cell and the kinase is activated. The kinase in turn phosphorylates synapsin I, which dissociates from the vesicles and consequently increases their availability. As a result the vesicles are more able to fuse to the cellular membrane and discharge their neurotransmitter.

The distribution of DARPP-32 is more restricted that than of synapsin I. According to Greengard and his colleagues S. Ivar Walaas, Hugh Hemmings, Jr., and Charles Ouimet, DARPP-32 occurs primarily in certain neurons that respond to dopamine. This neurotransmitter binds to two types of receptor, but



Neurotransmitter interactions

The scheme shows how DARPP-32 might mediate interactions between dopamine and glutamate. [Source: Hugh Hemmings, Jr., and Paul Greengard, Rockefeller University]

DARPP-32 is found only in neurons with the D_1 type of receptor. It does not appear to occur in nerve cells with D_2 receptors. The cells that contain DARPP-32 include the medium-sized spiny neurons in the caudatoputamen region of the brain, which deteriorate in patients with Huntington's disease. These same neurons are targets of the dopamine-releasing cells that degenerate in Parkinson's disease.

As a consequence of dopamine binding to D_1 receptors, the cyclic AMP concentration increases, resulting in activation of the cyclic AMP–dependent kinase, which is the enzyme that phosphorylates DARPP-32. Greengard suggests that the protein mediates certain responses to dopamine acting through the D_1 receptor. Whereas synapsin I appears to participate in releasing neurotransmitter signals from all neurons, DARPP-32 may be involved in receiving them in a limited group of neurons.

Similarities between DARPP-32 and a protein that inhibits the activity of protein phosphatase-1, one of the enzymes that removes phosphate groups from proteins, gave a clue to how DARPP-32 might work. The cyclic AMP–dependent kinase phosphorylates several proteins in addition to DARPP-32 in response to dopamine. DARPP-32 in its phosphorylated state, but not when unphosphorylated, proved to be a very efficient inhibitor of protein phosphatase-1 when this was tested directly. Phosphorylated DARPP-32 may thus potentiate dopamine's effects by preventing phosphate removal from other dopamine-regulated phosphoproteins.

DARPP-32 may also provide a means of integrating dopamine's effects with those of other neutrotransmitters. For example, the medium-sized spiny neurons of the caudatoputamen are innervated both by dopamine-releasing and glutamate-releasing neurons. Glutamate, acting through calcium ions as a second messenger, probably stimulates a calcium-dependent kinase. Phosphorylated DARPP-32 may inhibit the removal of phosphate from these kinase substrates, too. If that is the case, then DARPP-32 may account for the ability of dopamine to potentiate the effects of glutamate.

These possible interactions are still speculative, Greengard notes, and require further confirmation. Nevertheless, he maintains, "Even if some details of these interactions are wrong, I still think that phosphatase inhibition will prove to be an important component of the molecular mechanisms underlying interactions between neurotransmitters."

—JEAN L. MARX

Catastrophism Not Yet Dead

The recently announced demise of the notion that major extinction events punctuate the history of life at some 26-million-year intervals is, as Mark Twain put it, greatly exaggerated.

In his paper in *Nature* (*1*), Antoni Hoffman of the Lamont-Doherty Geological Laboratory, New York, outlined some of the frustrating uncertainties inherent in dealing with the fossil record in any large-scale quantitative analysis. He went on to conclude that the 26-million-year cycle of extinction reported in February 1984 by David Raup and John Sepkoski of the University of Chicago is the inevitable outcome of the nature of the data and the analytical manipulation employed upon them. An editorial in the same issue (*2*) emphasizes Hoffman's message and declares that "Last year's fashion for explaining a supposed 26-million-year periodicity in mass extinctions of species has been made to seem a little spurious."

Hoffman's criticisms rest on three main points: that the database used by Raup and Sepkoski is culled, which distorts comparison of the record through the 250 million years ago to the present; that uncertainties in the geological time scale, and of the stages within it, introduce large potential errors; and that the artificial nature of the measuring unit used—the paleontological stage— makes periodicity inevitably fall out of any statistical analysis.

Raup and Sepkoski's original analysis was based on a subset of 567 families of marine organisms that was extracted from a total of some 3500 available in a recent compilation. The data set was culled so as to remove all families of uncertain taxonomic or stratigraphic provenance. In addition, all extant families were removed so as to avoid the damping effect of "the pull of the recent." Hoffman notes that one effect of this culling is to allow the disappearance of five families in recent times to be classified as a possible mass extinction compared with many times that number earlier in the record. One counter to this criticism is that there has in fact been a substantial reduction in overall extinction rates in the marine record through time. A more direct response comes from the demonstration that even when the data set is maintained intact the 26-million-year signal still emerges, though less sharply.

Uncertainties in the timing of the geological time scale and its components are of course a constant frustration to those who use it. Raup and Sepkoski argue, however, that it is more reasonable to note that the 26-million-year signal comes through in spite of these uncertainties, not because of them, and to be impressed by that fact.

Hoffman's third point—on the question of paleontological stages—is clearly attractive. Each stage is defined by the special features of the fossil assemblage within it, and, by definition, each must differ from the next. Stages range from just a couple of million years in duration to more than 15 million, though many are in the region of 6 to 7 million. Given the restriction that adjacent stages must differ, Hoffman argues, there is a 1 in 4 probability that any single stage will stand out as a major extinction, given a random distribution; and with stages averaging 6.2 million years long, a 26-million-year signal (4×6.2) is statisically inevitable. In fact, although some kind of nonrandom pattern would emerge from a random distribution of extinctions between stages, a clear 26-million-year cycle is unlikely. But, again, the most telling counter to this challenge is that Raup and Sepkoski's analysis included a comparison of the real data against a random distribution of the data between stages: a random distribution was the null hypothesis, which was statistically rejected.

The *Nature* editorial, in supporting Hoffman's challenge to periodicity notes that " . . . human nature being what it is, it seems unlikely that the enthusiasts for catastrophism will now abandon their quest." The new catastrophism may well have to be abandoned, but not yet.—ROGER LEWIN

References

1. A. Hoffman, *Nature* (*London*) **315**, 659 (1985).
2. J. Maddox, *ibid.*, p. 627.