THE NEUROBIOLOGY OF DOPAMINE SIGNALING

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by

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It is estimated that there are about one hundred billion nerve cells in the brain and that on average each of these nerve cells communicates with one thousand other nerve cells. A vigorous debate went on from the 1930s through the 1960s as to whether intercellular communication across the synapses between nerve cells was electrical or chemical in nature. The electrical school of thought held that the nerve impulse or action potential was propagated along the axon to the nerve ending, changed the electrical field across the postsynaptic plasma membrane, and thereby produced a physiological response. The chemical school believed that when the action potential came down the axon to the nerve terminal, it caused the fusion of neurotransmitter-containing vesicles with the presynaptic plasma membrane, releasing a neurotransmitter, which then diffused across the synaptic cleft and, through activation of a (hypothetical) receptor, produced a physiological response. The chemical school won this debate: over 99% of all synapses in the brain use chemical transmission. Based on those earlier studies, I became interested in the biochemical mechanisms by which neurotransmitters, through activation of their receptors, produce their physiological effects within their postsynaptic target nerve cells.

We know today that there are two categories of chemical transmission between nerve cells, which are referred to as fast and slow synaptic transmission. About half of the fast synapses in the brain are excitatory, and most of these fast excitatory synapses utilize glutamate as their neurotransmitter. The other half of the fast synapses are inhibitory and most of these fast inhibitory synapses use GABA as their neurotransmitter. Synaptic transmission at fast synapses occurs in less than one-thousandth of a second, and is attributable to the ability of the fast-acting neurotransmitters to open what are called ligand-operated ion channels present in the plasma membrane of the post-synaptic cells. In fast excitatory transmission, glutamate binds to its receptor, causing a change in the conformation of the receptor, allowing positively charged sodium ions to rush into the cell, causing a depolarizing, i.e. excitatory, signal to be generated in the target cell. In fast inhibitory transmission, GABA binds to its receptor, causing a change in the conformation of the receptor, allowing negatively-charged chloride ions to permeate the cell, causing a hyperpolarizing, i.e. inhibitory, signal to be generated in the target cell.

Slow synaptic transmission, which occurs over periods of tens of milliseconds to seconds, is enormously more complex than fast synaptic transmission. There are about 150 compounds which are now believed to serve as neurotransmitters in the brain. The vast majority of these putative neurotransmitters appear to work through slow synaptic transmission. Thus, it now seems very likely that all of the biogenic amines, and all of the peptide neurotransmitters, produce their effects on their target cells through slow synaptic transmission. And even the fast acting neurotransmitters, including glutamate and GABA, produce many of their effects through slow synaptic transmission pathways.

Our work in trying to elucidate the molecular basis of slow synaptic transmission was inspired by studies carried out by Earl W. Sutherland[1] and Edwin G. Krebs[2]. Sutherland and Krebs were interested in understanding how the hormones glucagon and adrenaline break down glycogen to glucose in liver and muscle cells. Earl Sutherland and his colleagues found that these hormones stimulated the formation of cyclic AMP from ATP by virtue of activating a class of enzymes that they called hormone-sensitive adenylyl cyclases. They then showed that cyclic AMP could mimic the hormones in causing the breakdown of glycogen to glucose. Edwin Krebs and his colleagues subsequently showed that cyclic AMP caused the breakdown of glycogen to glucose by activating an enzyme which they called cyclic AMP-dependent protein kinase. Protein kinases catalyze the reaction:

$$\begin{array}{ccc} \text{Adenosine Triphosphate (ATP)} & & \text{Adenosine Diphosphate (ADP)} \\ \text{+ Substrate} & \rightarrow & \text{+ Phosphosubstrate} \end{array}$$

They further showed that this substrate, when phosphorylated, was an enzyme which caused the breakdown of glycogen to glucose. The action of protein kinases is reversible, an enzyme called a protein phosphatase catalyzing the reaction:

$$\text{Phosphosubstrate} \rightarrow \text{Substrate + Inorganic Phosphate}$$

When my colleagues and I started our work on the molecular basis of synaptic transmission, my hypothesis was that the same signaling machinery used by the endocrine system, e.g. to break down glycogen to glucose, might be used for communication between nerve cells. One major cause for concern with this concept was that in the case of hormones the distance of communication between the sending cell and the receiving cell can be more than two meters, whereas the distance across a synapse is roughly one millionth of a centimeter. Nevertheless, we decided to test this hypothesis. We searched in the brain for signaling enzymes analogous to those which had been found in liver and muscle. We found a family of neurotransmitter-sensitive adenylyl cyclases, analogous to the hormone-sensitive adenylyl cyclases of Earl Sutherland. These adenylyl cyclases converted ATP to cyclic AMP in the presence of neurotransmitters. The first such neurotransmitter-sensitive adenylyl cyclase, found by John W. Kebabian (photo 1), was a dopamine-sensitive adenylyl cyclase: in the presence of dopamine, this membrane-bound enzyme stimulated

Photo 1. John W. Kebabian, 1977.

Photo 2. Eishichi Miyamoto, 2001.

formation of cyclic AMP. Moreover, the data indicated that this enzyme might play a role in synaptic transmission[3].

At about the same time, Eishichi Miyamoto (photo 2) and J. F. Kuo (photo 3) were able to demonstrate cyclic AMP-dependent protein kinase activity in the brain[4]. In addition, the concentration of this enzyme was enormously higher in brain than in liver. Even more intriguing was the fact that the enzyme was concentrated in the synaptic region of nerve cells. These data were consistent with a possible role for cyclic AMP-dependent protein kinase in synaptic transmission.

Photo 3. J. F. Kuo, (undated).

Photo 4. Howard Schulman, 1968.

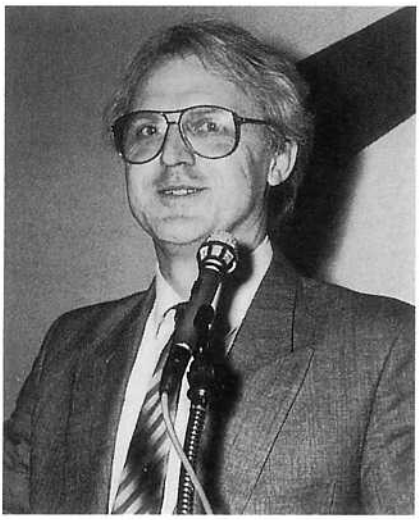

Photo 5. S. Ivar Walaas, 1990.

Photo 6. Angus C. Nairn, 1987.

Soon thereafter, we found a second, distinct class of regulated protein kinases, activated selectively by cyclic GMP, rather than cyclic AMP[5]. This cyclic GMP-dependent protein kinase was present both in brain and in non-neural tissues. Subsequently, Howard Schulman (photo 4) discovered a third group of regulated protein kinases, which were stimulated by calcium, in the presence of an unidentified endogenous heat stable protein that was later shown to be the calcium effector protein calmodulin[6]. The discovery of several neurotransmitter-sensitive enzymes that made cyclic AMP and of several second messenger-dependent protein kinases strengthened our belief that second messengers and protein kinases might be involved in signaling in the brain. This idea was supported by the discovery of a large number of brain-specific substrate proteins for these protein kinases. Thus, S. Ivar Walaas (photo 5) and Angus C. Nairn (photo 6) found more than one hundred substrate proteins for protein kinases which were highly enriched in, or exclusively localized to, the brain, some of which were present in very high concentrations[7,8]. In the ensuing years, we were able to show that injections of various second messengers, protein kinases, protein phosphatases, and activators and inhibitors of these enzymes, were able either to mimic or to antagonize the ability of neurotransmitters to produce physiological responses in nerve cells. Combined, these data have provided overwhelming evidence of a role for those signaling molecules in synaptic transmission[9].

Some of the principal signaling pathways involved in slow synaptic transmission are shown in figure 1. The presynaptic terminal contains synaptic vesicles, which sequester neurotransmitter within them. In response to an action potential, these vesicles fuse with the plasma membrane, releasing the neurotransmitter. In an extensive series of studies, in which Pietro De Camilli (photo 7), Fabio Benfenati (photo 8), Flavia Valtorta (photo 9), and Andrew J. Czernik (photo 10) played leading roles, and which involved a series of col-

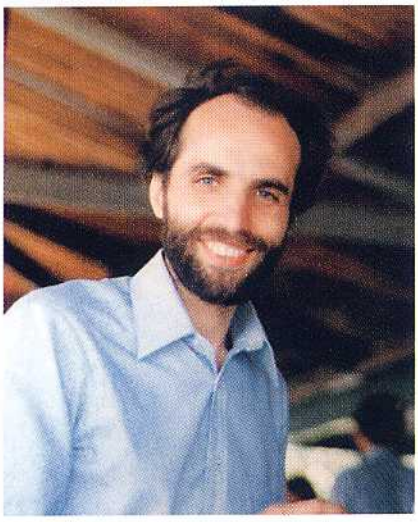

Photo 7. Pietro de Camilli, 1981.

Photo 8. Fabio Benfenati, 1999.

laborative studies with Rodolfo R. Llinas and his colleagues, it was found that the efficacy of release of neurotransmitter from the presynaptic terminal was controlled by regulation of the state of phosphorylation of a family of proteins in the presynaptic nerve terminal[10, 11]. We named these proteins synapsins because they are localized to synaptic vesicles in the presynaptic terminals. The synapsins are the most abundant phosphoproteins found in the brain and, possibly for this reason, were the first to be discovered. Another seminal series of studies on the regulation of neurotransmitter release by cyclic AMP-dependent phosphorylation of nerve terminal proteins was car-

Photo 9. Flavia Valtorta, 2000.

Photo 10. Andrew J. Czernik, 1992.

ried out in a collaboration between Eric R. Kandel's laboratory and my own[12, 13]. The various studies of the physiology of the presynaptic terminal demonstrated conclusively that the efficacy of neurotransmitter release in response to the nerve impulse is regulated by protein phosphorylation/dephosphorylation. Those studies lie outside the scope of this presentation and I shall not discuss them further here. Rather, I am going to concentrate on how slow-acting neurotransmitters, through activation of their receptors, produce appropriate physiological responses in their postsynaptic target cells.

Slow-acting neurotransmitters, upon binding to their receptors, change the level of one or another second messenger, e.g. cyclic AMP, cyclic GMP, calcium, or diacyl glycerol. These second messenger molecules in turn activate distinct classes of protein kinases. The activated protein kinases phosphorylate and thereby change the properties of substrate proteins, which serve as downstream physiological effectors. The substrate proteins of the nervous system can be divided into various classes, four of which are diagrammed in figure 1. One major class of substrates are the receptors for neurotransmitters, both fast- and slow-acting neurotransmitters. These protein kinases also phosphorylate various voltage-gated sodium, potassium, and calcium ion channels. Another class of substrate proteins are ion pumps, which restore ionic equilibrium after a burst of neuronal activity. Still another class are transcription factors present in the cell nucleus, which control new protein synthesis. Protein synthesis is involved in long-term changes in the nerve cell in response to activity, and may form an important component of the molecular basis of learning and memory, a field which was pioneered by Eric Kandel, and is described by him in this volume[14].

Figure 1, in highly simplified form, schematizes the basic principles of slow synaptic transmission. Although this scheme is now a component of the scientific dogma, it was greeted initially with enormous skepticism, and at times down-right hostility, by the scientific community. In retrospect, there were at least two major reasons for this. At the time that we started this work, neuroscience was not a clearly defined field. There were two types of people studying the brain. There were biophysicists, working in physiology departments, who believed that everything significant about the brain could be explained in terms of electrical signaling. And there were biochemists working in biochemistry departments who would happily throw a brain into a homogenizer, with as much abandon as they would a liver, and look for enzymes or lipids. But these biochemists were rarely interested in brain function. And so these two groups rarely spoke to each other, which is just as well because when they did they didn't have nice things to say. So, there was almost no one working on the biochemical basis of how nerve cells function. A second reason for the skepticism that biochemical pathways might be involved in synaptic transmission was more substantive: it was not immediately obvious as to how the relatively slow enzymatic reactions that we were studying, involving protein phosphorylation and dephosphorylation, could be involved in fast synaptic transmission, which occurs in less than one-thousandth of a second. The answer to this apparent paradox was that these slow signaling pathways

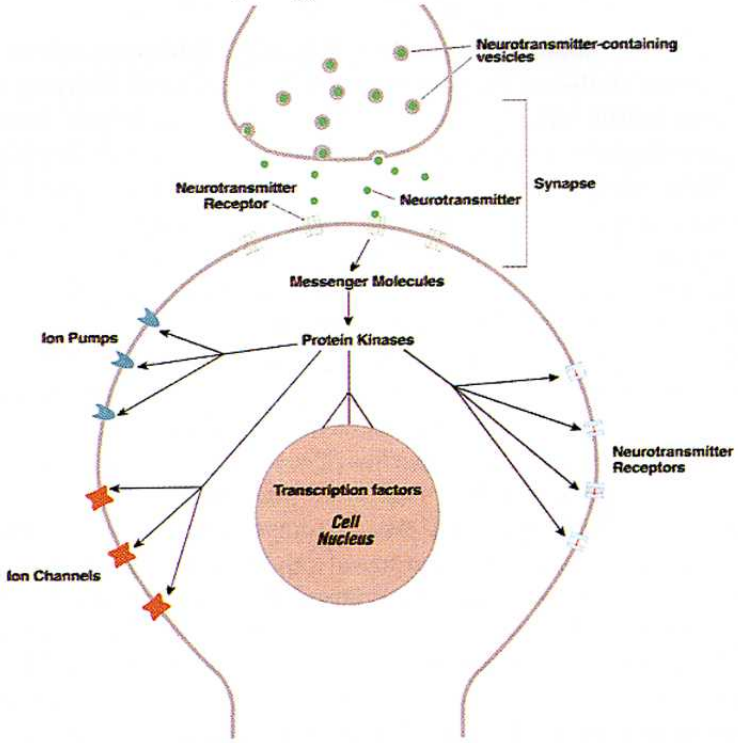

Figure 1. Some of the signaling pathways involved in slow synaptic transmission.

don't *mediate* fast synaptic transmission. Rather they *modulate* fast synaptic transmission, and they do so in two major ways: a) by regulating the state of phosphorylation of synapsins and other key proteins present in the presynaptic terminal, thus modulating the efficacy of neurotransmitter release (the amount of neurotransmitter released from the nerve terminal in response to an action potential), and b) by regulating the state of phosphorylation of neurotransmitter receptors present in the post-synaptic cell, thus modulating the responsivity of these receptors to the released neurotransmitter (responsivity referring to the magnitude of the electrophysiological response to a molecule of neurotransmitter).

The various slow signaling pathways which have been studied to date obey similar principles. The slow-acting neurotransmitter which we have studied most intensively is dopamine. There were several reasons for focusing on this system. First there was the pioneering work of Arvid Carlsson[15], his colleagues, and those who followed in his footsteps, showing that four major and several minor neurological and psychiatric diseases are associated with abnormalities in the dopamine signaling pathway. The four major diseases are Parkinsonism, schizophrenia, Attention Deficit Hyperactivity Disorder (ADHD), and drug abuse. Parkinsonism is associated with the death of dopamine-producing nerve cells, and is treated by giving Levodopa, a precursor of dopamine. Most currently used antipsychotic drugs block a subclass of

Photo 11. Dana W. Aswad, 1979.

Photo 12. Patrick B. Allen, 2001.

dopamine receptors. ADHD is treated by Ritalin, which works in large part by stimulating dopamine release. Virtually all drugs of abuse cause perturbations of dopamine signaling. There were two other reasons for concentrating on dopamine signaling. One was that the neurostriatum, a major target for dopaminergic innervation, is relatively large and homogenous and thus fairly readily permits both electrophysiological and biochemical studies. The other reason is that the simple circuitry of the basal ganglia, compared to the circuitry of the cortex, makes the analysis of signaling systems much more manageable. However, it is worth emphasizing that although much of our research in recent years has concentrated on the dopamine pathways, the basic

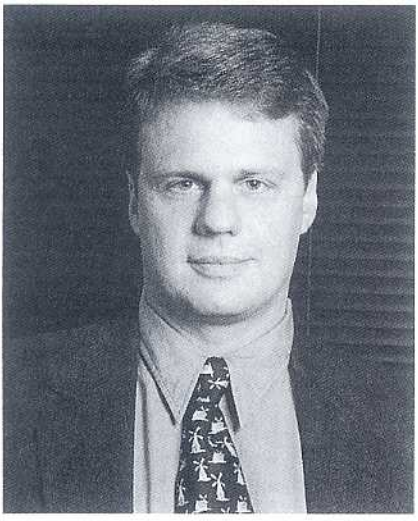

Photo 13. James A. Bibb, 2000.

Photo 14. Allen A. Fienberg, 1996.

Photo 15. Gilberto Fisone, 1995.

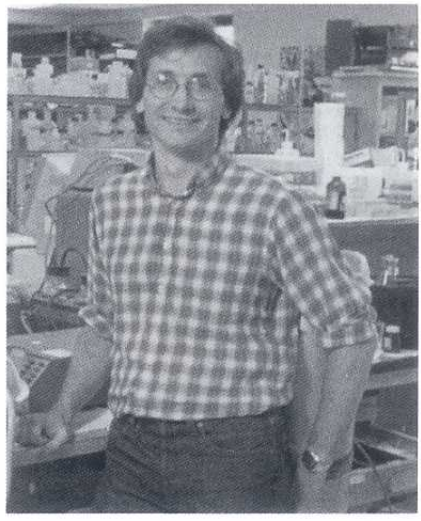

Photo 16. Jean-Antoine Girault, 1986.

principles elucidated in those pathways appear to be applicable to all slow synaptic pathways in the brain.

In our studies of dopamine signaling, we were very fortunate to discover a molecule that we named DARPP-32, an acronym for *d*opamine and cyclic *A*MP *r*egulated *p*hospho*p*rotein MW = *32* kDa. Dopamine, by activation of a subclass of dopamine receptors, causes an increase in the level of cAMP, the activity of PKA and the phosphorylation of threonine 34 of DARPP-32. DARPP-32 plays an obligatory role in mediating the actions of dopamine and has served as a rosetta stone for understanding the interactions of dopamine with other neurotransmitters, therapeutic drugs, and drugs of abuse. DARPP-

Photo 17. Hugh C. Hemmings, Jr. (right) and Angus C. Nairn, 1986.

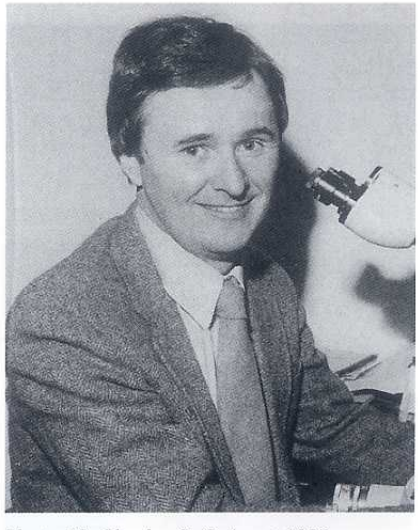

Photo 18. Charles C. Quimet, 1985.

Photo 19. Akinori Nishi, 2000.

Photo 20. Gretchen L. Snyder, 2000.

32 was discovered by S. Ivar Walaas and Dana W. Aswad (photo 11) while searching for region-specific protein kinase substrates in the brain[16]. DARPP-32 has been characterized by Patrick B. Allen (photo 12), James A. Bibb (photo 13), Allen A. Fienberg (photo 14), Gilberto Fisone (photo 15), Jean-Antoine Girault (photo 16), Hugh C. Hemmings, Jr. (photo 17, together with Angus C. Nairn), Angus C. Nairn, Charles C. Ouimet (photo 18), Akinori Nishi (photo 19), Gretchen L. Snyder (photo 20), Per Svenningsson (photo 21), S. Ivar Walaas, and Zhen Yan (photo 22).

DARPP-32 is highly concentrated in the neostriatum (the caudate and the putamen), and the nucleus accumbens. An enormously simplified version of the neostriatal circuitry in which these DARPP-32-containing nerve cells are located is shown in figure 2. This circuitry has provided a useful model for

Photo 21. Per Svenningsson, 2000.

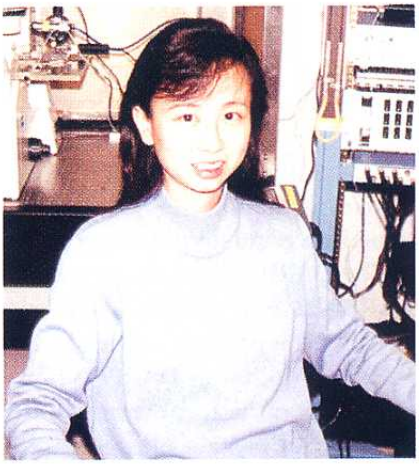

Photo 22. Zhen Yan, 2000.

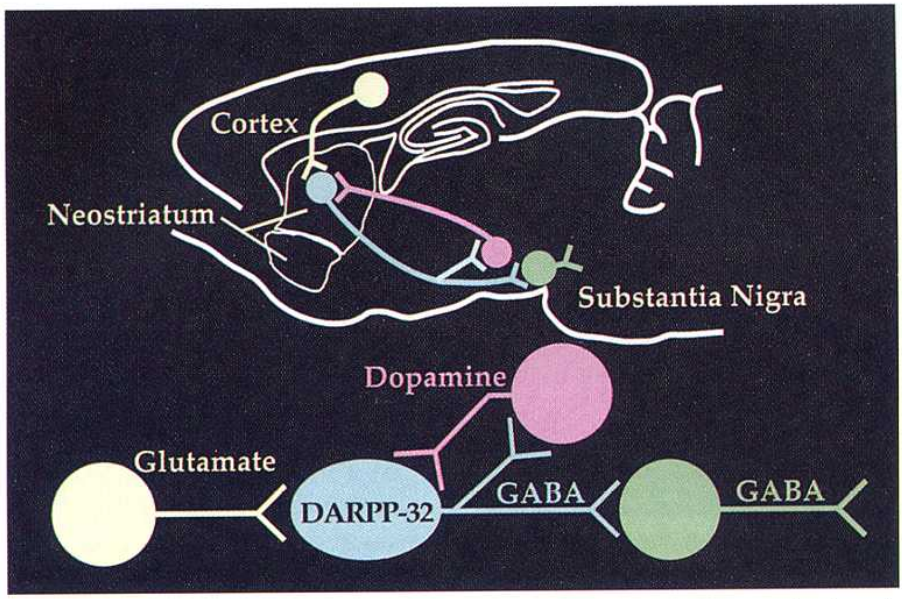

Figure 2. Simplified diagram of some connections between cortex, neostriatum, and substantia nigra.

The upper portion shows the neuroanatomy and the lower portion the corresponding neurochemistry. The cells shown in yellow project from the cortex to the striatum and use glutamate, a fast-acting, excitatory neurotransmitter, to stimulate second-order neurons, shown in blue, which project from the neostriatum to the substantia nigra. These latter neurons contain DARPP-32 and use GABA as their neurotransmitter. In addition to innervating third-order neurons, their axons have branches that innervate dopaminergic neurons, which in turn provide feedback on the DARPP-32-containing cells.

studying the mechanisms by which slow synaptic transmission, as exemplified by dopamine, modulates fast synaptic transmission, as exemplified by glutamate.

There are three major classes of glutamate receptors, designated NMDA, AMPA and Metabotropic, and two major classes of dopamine receptors, designated D1 and D2. The interactions between the dopamine and glutamate signaling pathways are highly complex and in addition are modulated by many other neurotransmitters and their signaling pathways. DARPP-32 plays a central role in the interactions amongst those various complex signaling pathways (figure 3). All four possible classes of mechanisms for regulating the state of phosphorylation of DARPP-32 on threonine 34 have been shown to exist, i.e., increases and decreases in phosphorylation and increases and decreases in dephosphorylation. Why has so much evolutionary machinery gone into regulating the state of phosphorylation of DARPP-32?

The DARPP-32 sequence has been highly conserved within mammals. Rat DARPP-32 is a protein consisting of 205 amino acids. Threonine 34 of DARPP-32 is phosphorylated by PKA or PKG and dephosphorylated by PP2B. Phosphorylation of DARPP-32 on threonine 34 profoundly changes its biological properties, converting it from an inactive molecule into a very potent inhibitor of protein phosphatase 1, with an IC50 (figure 4) and KI of about 10^{-9} M[17]. Since the concentration of DARPP-32 in medium spiny neurons is

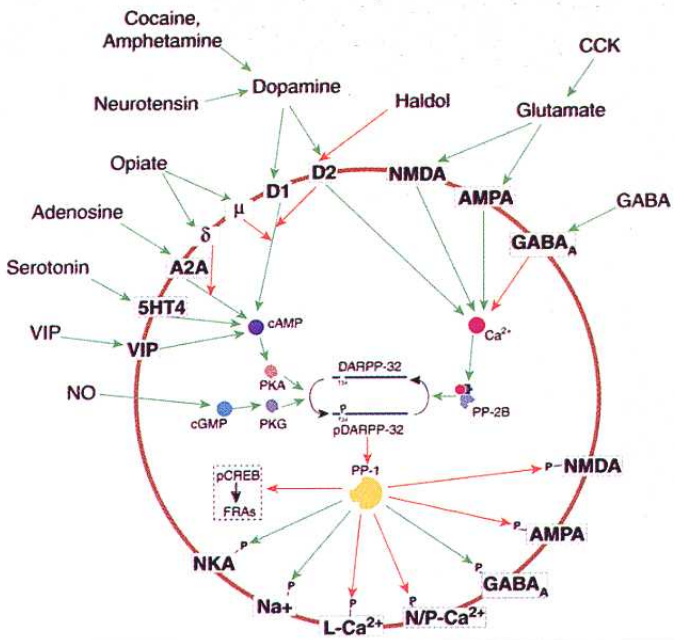

Figure 3. Interactions of signaling pathways in the neostriatum.

Activation by dopamine of the D1 subclass of dopamine receptors stimulates the phosphorylation of DARPP-32 at Thr-34. This is achieved through a pathway involving the activation of adenylyl cyclase, the formation of cyclic AMP, and the activation of cyclic AMP-dependent protein kinase (PKA). Activation by dopamine of the D2 subclass of dopamine receptors causes the dephosphorylation of DARPP-32 through two synergistic mechanisms: D2 receptor activation (a) prevents the D1 receptor-induced increase in cyclic AMP formation, and (b) raises intracellular calcium, which activates a calcium-dependent protein phosphatase, referred to as protein phosphatase 2B (PP2B; calcium/calmodulin-dependent protein phosphatase; calcineurin). Activated PP-2B dephosphorylates DARPP-32 at Thr-34. Glutamate acts as both a fast-acting and slow-acting neurotransmitter. Activation by glutamate of AMPA receptors causes a rapid response through influx of sodium ions, depolarization of the membrane, and firing of an action potential. Slow synaptic transmission, in response to glutamate, results in part from activation of the AMPA and NMDA subclasses of glutamate receptor, which increases intracellular calcium and the activity of PP2B, and causes the dephosphorylation of DARPP-32 on Thr-34. All other neurotransmitters that have been shown to alter the physiology of dopaminoceptive neurons also alter the phosphorylation state of DARPP-32 on Thr-34 in these cells. For example, adenosine acting on the A2A subclass of dopamine receptor, serotonin acting on the 5HT4 subclass of serotonin receptor, and VIP, acting on its receptor, all increase cyclic AMP-dependent phosphorylation of DARPP-32 at Thr-34. Nitric oxide, released from other types of nerve cells, causes an increase in cyclic GMP formation, resulting in activation of cyclic GMP protein kinase (PKG), which like PKA increases DARPP-32 phosphorylation on Thr-34. GABA, acting on the GABA A subclass of GABA receptor, hyperpolarizes the cell membrane, leading to a decrease in intracellular calcium, an inactivation of PP2B, and an increase in the state of phosphorylation of DARPP-32 at Thr-34. Interestingly, dopamine and GABA, both of which are capable of inhibiting activity of these nerve cells, both increase DARPP-32 Thr-34 phosphorylation state, one by increasing phosphorylation, the other by decreasing dephosphorylation. As one would predict, the effects of dopamine and GABA on DARPP-32 phosphorylation are synergistic. Neurotransmitters which act indirectly to affect the physiology of these medium spiny neurons also regulate DARPP-32 phosphorylation: neurotensin, through stimulating the release of dopamine, increases DARPP-32 phosphorylation; conversely cholecystokinin, through stimulating the release of glutamate, decreases DARPP-32 phosphorylation.

Therapeutic drugs and drugs of abuse, all of which affect the physiology of these neurons, also regulate the state of phosphorylation of DARPP-32 on Thr-34. For example antipsychotic drugs, such as Haldol, which block the activation by dopamine of the D2 subclass of dopamine receptor, increase phosphorylation. Agonists for the mu and delta subclasses of opiate receptors block D1 and A2A receptor-mediated increases in cyclic AMP respectively, and the resultant increases in phosphorylation. Cocaine and amphetamine, through increasing extracellular dopamine levels, increase phosphorylation. Marijuana, nicotine, alcohol, and LSD, all of which affect the physiology of the medium spiny neurons, also regulate phosphorylation (unpublished data). Finally, all drugs of abuse have greatly reduced biological effects in animals with targeted deletion of the DARPP-32 gene.

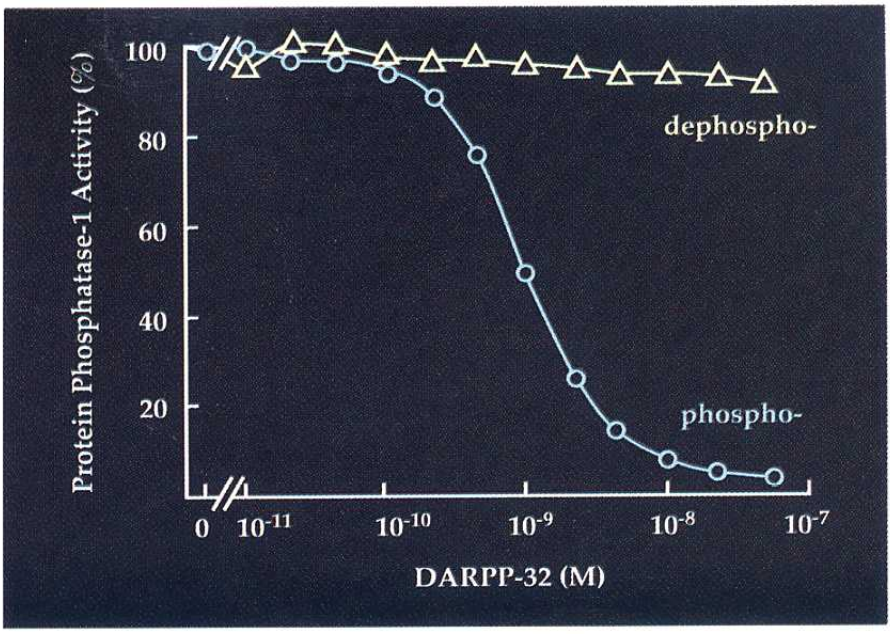

Figure 4. Phospho-DARPP-32, but not dephospho-DARPP-32, inhibits protein phosphatase-1. (Modified from Hemmings, *et al.*, reference 17.)

greater than 10^{-5} M, this means that a small burst of activity in dopaminergic neurons would be expected to result in significant phosphorylation of DARPP-32 and inhibition of protein phosphatase 1. Protein phosphatase 1 has a very broad substrate specificity and controls the state of phosphorylation and activity of a number of physiologically important substrates, including neurotransmitter receptors, voltage-gated ion channels, ion pumps, and transcripton factors. As a result, neurotransmitters which increase or decrease phospho-threonine 34 of DARPP-32, inhibit or activate, respectively, protein phosphatase 1, and thereby increase or decrease the state of phosphorylation and activity of a large array of downstream physiological effectors.

The physiological significance of the DARPP-32/PP1 cascade has been demonstrated in two distinct types of experiments. In one type we injected protein kinases, protein phosphatases, or inhibitors or activators thereof, into medium spiny neurons and obtained physiological responses consistent with the scheme shown in figure 3. The other type of study involved analysis of mice with targeted deletion of the DARPP-32 gene. Allen Fienberg engineered this knockout mouse and, in a multi-institutional collaboration, found that all physiological, biochemical, and pharmacological responses to dopamine, the psychostimulant drugs of abuse, and antischizophrenic drugs, seen in normal mice, were either greatly diminished or abolished in DARPP-32 knockout mice (figure 5)[18].

The scheme shown in figure 3 indicates that the fast excitatory glutamate receptors, AMPA and NMDA, regulate, and are regulated by, the DARPP-32/PP1 cascade. From this scheme one would predict that PP1 would be lo-

Stimulus	Parameter	Examples:
Dopamine	Ion Pumps	Na^+, K^+-ATPase
Dopamine	Ion Channels	Ca^{2+} Channels
Dopamine	Neurotransmitter Release	GABA Release
Dopamine	Neurotransmitter Receptors	GluR1 Receptor
Dopamine; Psychostimulants	Immediate Early Genes	Fos Induction
Psychostimulants	Brain Damage	Reactive Gliosis
Antischizophrenics	Locomotor Activity	Catalepsy
Dopamine; Progesterone	Sexual Receptivity	Female Responsiveness

Figure 5. Responses to dopamine, psychostimulants and antischizophrenic drugs are abolished in DARPP-32 knockout mice.

calized in the vicinity of these glutamate receptors. Immunocytochemical experiments, using antibodies prepared against recombinant PP-1, enabled us to localize this phosphatase in neostriatal cells. PP1 was enriched in the spines of the dendrites of medium spiny neurons[19]. To answer the question as to the basis for the high degree of enrichment of PP1 in spines, Patrick Allen used yeast-two hybrid technology to search for a PP1 targeting protein. He found a molecule, called spinophilin, which has the properties of such a targeting protein. Spinophilin is localized almost exclusively in dendritic spine heads at excitatory synapses (figure 6)[20]. This is precisely where the AMPA and NMDA receptors are concentrated.

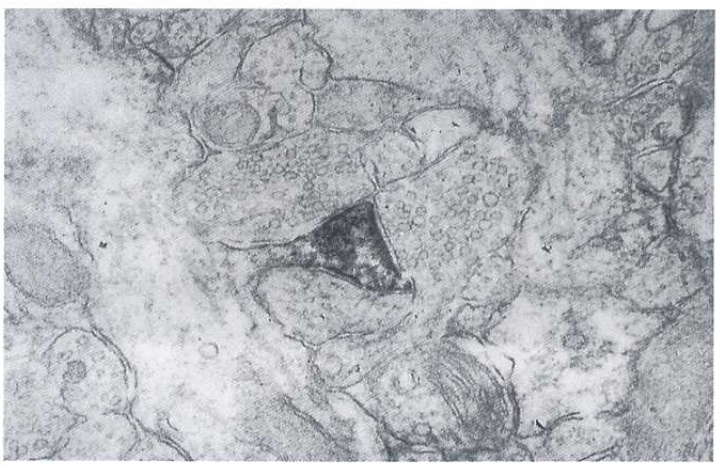

Figure 6. Electron micrograph showing immunoreactivity in a dendritic spine neck and especially in a spine head. Immunoreactivity (black flocculent material) lines the cytoplasmic surface of the spine plasmalemma and coats the postsynaptic density. Immunoreactivity is not present in axon terminals or in the dendritic shaft. (Bar = 100 nm) (Modified from Allen *et al.*, reference 20).

It has recently been demonstrated that spinophilin, through control of PP-1, regulates the conductance properties of AMPA and NMDA receptors[21]. A model to account for the ability of spinophilin and PP1 to regulate the AMPA receptor is shown in figure 7. In its dephosphorylated form, the AMPA receptor is relatively insensitive to activation by glutamate. Its sensitivity to glutamate is greatly enhanced upon phosphorylation by cAMP-dependent protein kinase[23, 24]. In the absence of dopamine, the AMPA receptor is kept in a low conductance state by PP1. Dopamine, through activation of cAMP-dependent protein kinase, causes an increase in the state of phosphorylation of the GluR1 subunit of the AMPA receptor, and this effect is greatly attenuated in the neostriatum of mice lacking the DARPP-32 gene. The activation of PKA in response to dopamine increases the state of phosphorylation of the AMPA receptor by a synergistic mechanism involving direct phosphorylation of the receptor as well as phosphorylation of threonine 34 on DARPP-32, resulting in inhibition of PP-1 catalyzed dephosphorylation of the receptor.

Physiological support for the scheme shown in figure 7 includes evidence that the dephosphorylation, and associated loss of responsivity of AMPA receptors to activation by glutamate, can be prevented by incubation of cells either with a D1 receptor agonist, or with the PP-1 inhibitor okadaic acid, but not with an inactive analog of okadaic acid (figure 7). This scheme is also supported by data demonstrating that injection of a peptide corresponding to residues 6-38 of phospho-DARPP-32, which retains the ability to inhibit PP-1, but not dephospho-DARPP-32 (6-38), prevents loss of AMPA receptor responsitivity. Injection of a spinophilin-based peptide, which prevents

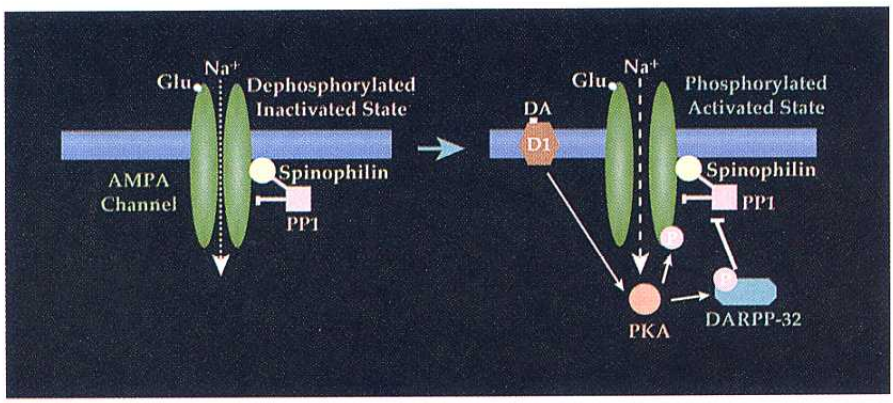

Figure 7. Regulation of AMPA-type glutamate receptors by DARPP-32 and spinophilin.

Model illustrates how regulation of PP-1 could account for the ability of DARPP-32 and spinophilin to control AMPA channels. (Left) Spinophilin, by binding to an unidentified intermediate protein (not shown), localizes PP-1 in the vicinity of the AMPA channel. Under basal conditions, the PP-1/spinophilin complex maintains the AMPA channel in a dephosphorylated, low-activity state. (Right) Following D1 receptor stimulation, AMPA channel phosphorylation is increased due both to direct PKA phosphorylation and to PKA/phospho-DARPP-32-mediated inhibition of PP-1. This synergistic increase in phosphorylation stimulates AMPA channel activity. The kinase anchoring protein (AKAP) believed to localize PKA in the vicinity of the AMPA receptor (reference 22) is not shown. (Modified from Greengard *et al.*, reference 9).

tethering of PP-1 to spinophilin, also prevents AMPA receptor inactivation; a single point mutation abolishes both effects of this peptide. Spinophilin regulates the NMDA receptor in a manner parallel to that by which it regulates the AMPA receptor. Spinophilin does not regulate the fast inhibitory GABA A receptor. Thus spinophilin appears to control fast excitatory, but not fast inhibitory, synaptic transmission. Finally, spinophilin itself is a substrate for PKA[25] and it seems likely that dopamine-induced PKA-mediated phosphorylation of spinophilin plays a role in the complex mechanism by which dopamine controls the efficacy of synaptic transmission at fast excitatory synapses.

MODULATION OF THE BALANCE BETWEEN DOPAMINERGIC AND GLUTAMATERGIC SIGNALING

Our discussion of DARPP-32 up to this point has focused on the ability of various neurotransmitters, by regulating the activity of PKA, PKG, and PP-2B, to regulate DARPP-32 phosphorylation/dephosphorylation at threonine 34 and thereby to control the activity of PP-1, and the state of phosphorylation and activity of a variety of downstream physiological effectors. Clearly, the percentage of DARPP-32 molecules in the phosphorylated state, and the degree of inhibition of PP-1, reflects a balance between the rates of phosphorylation and dephosphorylation of threonine 34 (figure 3). It turns out that the effectiveness of PKA and PP2B in regulating threonine 34 phosphorylation/dephosphorylation is itself regulated by other protein kinases and protein phosphatases. We now know of three sites, in addition to threonine 34, which are phosphorylated in response to activity in other signaling pathways. Each of these three additional pathways modulates the dopamine/D1 receptor/PKA/phospho-threonine 34-DARPP-32/PP1 cascade (figure 8)[26–28]. As is clear from figure 8, DARPP-32 can be either a protein phosphatase inhibitor or a protein kinase inhibitor, depending upon whether threonine 34 or threonine 75 is phosphorylated. Although DARPP-32 is the only bi-functional molecule of this type found to date, it seems likely that other proteins will be found which can serve either as a protein kinase inhibitor or a protein phosphatase inhibitor, depending on the residue phosphorylated.

One major molecular mechanism by which dopamine and glutamate produce opposing physiological effects involves a positive feedback loop that amplifies their mutually antagonistic actions (figure 9). This positive feedback loop involves three components, namely PKA, protein phosphatase 2A (PP2A) and threonine 75 of DARPP-32. In resting animals, threonine 75 is very highly phosphorylated, whereas threonine 34 is only slightly phosphorylated. Tonic activity of the glutamate/CDK5 pathway is probably responsible for keeping threonine 75 phosphorylated, and thereby keeping PKA inhibited. Dopamine, by activating D1 receptors, increases the activity of PKA, leading to phosphorylation of key physiological substrate proteins. Increased PKA activity also increases phosphorylation of threonine 34 on DARPP-32, inhibiting PP1, and thereby decreasing the dephosphorylation of these substrate proteins. PKA also activates PP2A, which dephosphorylates threonine

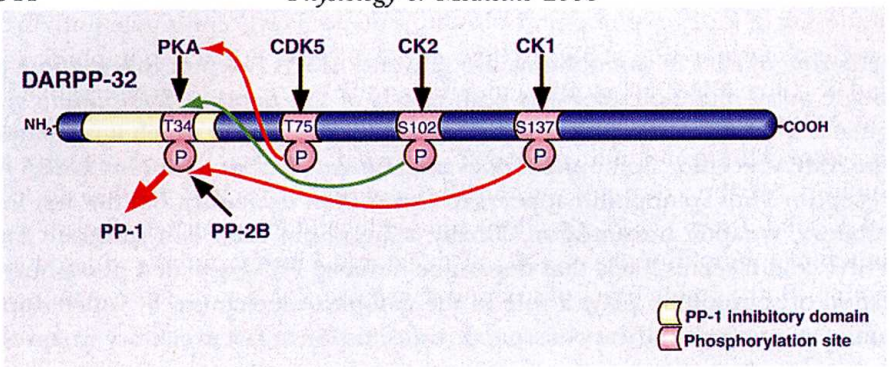

Figure 8. Multiple Phosphorylation sites on DARPP-32.

DARPP-32 is phosphorylated at Thr-34 by PKA (and PKG; not shown) and dephosphorylated by PP-2B (and PP2A; not shown). Phosphorylation at Thr-34 converts DARPP-32 into a potent inhibitor of PP-1. DARPP-32 is phosphorylated on Ser-137 by casein kinase I. Phosphorylation at Ser-137 converts DARPP-32 into a poorer substrate for PP2B-catalyzed dephosphorylation of Thr-34 without affecting phosphorylation of Thr-34 by PKA or PKG and without affecting the ability of PP2B to dephosphorylate other substrates (reference 27). DARPP-32 is phosphorylated on Ser-102 by casein kinase II. Phosphorylation at Ser-102 converts DARPP-32 into a better substrate for phosphorylation by PKA, without affecting its phosphorylation by PKG or its dephosphorylation by PP2B and without affecting the ability of PKA to phosphorylate other substrates (reference 26). Thus, the effects of phospho-Ser-137 DARPP-32 and phospho-Ser-102 DARPP-32 are substrate-directed, not enzyme-directed. DARPP-32 is phosphorylated on Thr-75 by CDK5. Phosphorylation at Thr-75 converts DARPP-32 into an inhibitor of PKA, reducing its ability to phosphorylate any substrate, including DARPP-32 at Thr-34 (reference 28). Thus the effect of phospho-Thr-75 DARPP-32 is enzyme-directed, not substrate-directed. Since dopamine increases and glutamate decreases phosphorylation of DARPP-32 at Thr-34, signaling mediated through phosphorylation/dephosphorylation of Ser-137, Ser-102 and Thr-75 alters the balance between dopaminergic and glutamatergic signaling. Specifically, the casein kinase 1/Ser-137 and casein kinase 2/Ser-102 pathways are prodopaminergic/antiglutamatergic, whereas the CDK5/Thr-75 pathway is antidopaminergic/ proglutamatergic. Phospho-Ser-137 is preferentially dephosphorylated by PP-2C (not shown). NH2-terminal domain of DARPP-32 that binds to PP-1 is shown in yellow. Red arrows indicate inhibition; green arrow indicates stimulation. (Modified from Greengard *et al.*, reference 9).

75, which removes the inhibition of PKA. Thus dopamine causes both an increased activation and a decreased inhibition of PKA. Conversely, glutamate activates CDK5 and, by phosphorylating threonine 75, inhibits PKA, reducing the activity of PP2A, and the resultant dephosphorylation of threonine 75. Through this mechanism, glutamate causes both an increased phosphorylation and a decreased dephosphorylation of threonine 75. Thus the PKA/PP2A/threonine 75 DARPP-32 triad amplifies either the effects of dopamine or the effects of glutamate, whichever is the more dominant neurotransmitter at any given time.

The schemes shown in figures 3, 8 and 9 summarize only a portion of the pathways that we now know to be involved in signal transduction in dopaminoceptive neurons. Both major classes of dopamine receptors and all three major classes of glutamate receptors are involved in the interactions between these neurotransmitters. These signaling pathways are highly complex, and have multiple sites of interaction. For example there are several ways by which the dopamine/D1 receptor/cyclic AMP/PKA/phosphothreonine 34

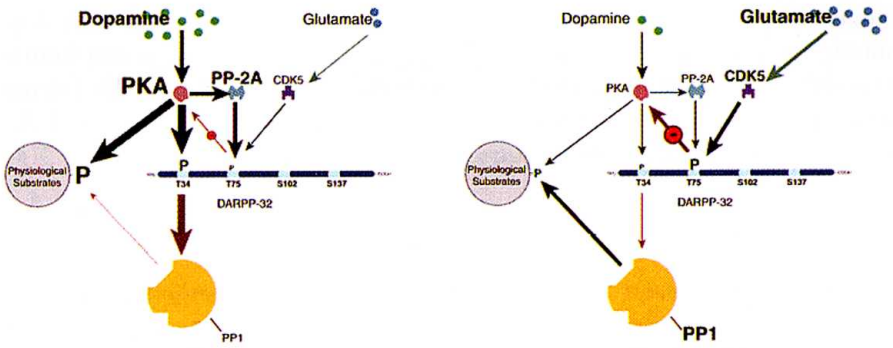

Figure 9. Model illustrating signaling pathways involved in mediating opposing biological actions of the neurotransmitters dopamine and glutamate.

DARPP-32/PP1 pathway can be inactivated. One is by D2 receptor-mediated inhibition of cyclic AMP formation (figure 3), a second is by glutamate-induced phosphothreonine 75 DARPP-32 inhibition of PKA (figure 9), and a third is by AMPA, NMDA or D2-receptor-mediated activation of PP-2B resulting in the dephosphorylation of threonine 34 of DARPP-32 (figure 3).

CONCLUDING REMARKS

The differences between the levels of complexity of fast synaptic transmission, which involves a single ligand-operated ion channel, and the enormously complicated pathways underlying slow synaptic transmission, only part of which we have elucidated, seem amazing. However, when one thinks of fast synaptic transmission as being the hardware of the brain, and slow synaptic transmission as being the software which controls fast transmission, the molecular basis by which nerve cells communicate with each other makes more sense.

The elucidation of the principles underlying slow synaptic transmission, and the discovery of numerous components of the underlying intracellular signaling pathways, have provided a number of novel therapeutic targets for the treatment of neurological and psychiatric illnesses associated with abnormalities of dopamine signaling. Levodopa is effective in most patients with Parkinson's disease. Levodopa is converted to dopamine, which then activates dopamine receptors and alleviates the disease symptoms. Unfortunately, within a short time, many Parkinson's patients become refractory to Levodopa treatment. This refractoriness is probably the result in part of down-regulation of dopamine receptors. It should be possible, through the new-found knowledge of the intracellular signaling pathways by which dopamine produces its physiological effects, to develop various therapeutic substances that activate or inhibit these various intracellular components. Hopefully such agents will be useful for the treatment of Parkinson's disease without the refractoriness associated with Levodopa treatment. Drugs which target intracellular constituents of dopaminoceptive neurons may also be useful for treat-

ment of other neurological and psychiatric disorders associated with dopamine signaling abnormalities. Finally, since the various slow acting neurotransmitters work by similar principles, it should be possible to find drugs working intracellularly for the treatment of diseases affecting parts of the brain where signaling pathways other than dopamine are employed.

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