# REFLECTIONS

⌘ *Author's Choice*

# Mind Molecules

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**Scientific styles vary tremendously. For me, research is largely about the unfettered pursuit of novel ideas and experiments that can test multiple ideas in a day, not a year, an approach that I learned from my mentor Julius "Julie" Axelrod. This focus on creative conceptualizations has been my métier since working in the summers during medical school at the National Institutes of Health, during my two years in the Axelrod laboratory, and throughout my forty-five years at Johns Hopkins University School of Medicine. Equally important has been the "high" that emerges from brainstorming with my students. Nothing can compare with the eureka moments when, together, we sense new insights and, better yet, when high-risk, high-payoff experiments succeed. Although I have studied many different questions over the years, a common theme emerges: simple biochemical approaches to understanding molecular messengers, usually small molecules. Equally important has been identifying, purifying, and cloning the messengers' relevant biosynthetic, degradative, or target proteins, at all times seeking potential therapeutic relevance in the form of drugs. In the interests of brevity, this Reflections article is highly selective, and, with a few exceptions, literature citations are only of findings of our laboratory that illustrate notable themes.**

## Beginnings

In high school, I had no affinity for science but loved reading history and philosophy. As philosophy did not seem like a fit job for a nice Jewish boy, I looked to what the other guys were doing, either premed or engineering. Because I could not stomach math or mechanical drawing, engineering was a turnoff. I hatched the concept that I could do the sort of thinking that one does in philosophy by becoming a psychiatrist, which meant I would have to muddle through the premed science curriculum and medical school at Georgetown University before attaining my goal.

How did this naive reasoning lead to a life studying the biochemistry of the brain? It commenced with what was my greatest passion, the guitar. My teacher, Sophocles Papas, was the best friend and disciple of the great classical guitarist Andrés Segovia, and I was Papas' protégé, even performing once for Segovia. During college, I spent Saturdays giving guitar lessons and minding Mr. Papas' guitar store. One of my students was Don Brown, a young M.D. who was in the first class of research associates at the National Institutes of Health (NIH) and who needed a technician, a job I assumed for the summer of 1958, just prior to entering medical school. Don worked in the laboratory of Marian Kies, who pioneered purification of myelin basic protein, the agent that elicits allergic encephalomyelitis. Don eschewed this effort and instead pursued histidine metabolism as part of Seymour Kety's effort to seek amine-related toxins in schizophrenia. My task was to fractionate the metabolites of [$^{14}$C]histidine by ion-exchange chromatography. In subsequent summers and elective periods, I worked in the laboratory largely on my own, as Don had left for a fellowship in Paris with Jacques Monod. I attempted to identify and purify the enzyme in histidine metabolism, which forms formininoglutamate, thereby linking histidine and glutamate. After

diverse foolish missteps, I managed to partially purify and characterize the enzyme. I wrote on my own a manuscript describing these findings and submitted it to the *Journal of Biological Chemistry*, accepted with no revisions, the only time that has ever happened to a paper of mine (1).

A Milwaukee pediatrician wrote to the laboratory describing his mentally retarded patient whose urine was positive for keto acids, reminiscent of phenylketonuria, but displayed normal phenylalanine levels yet was loaded with histidine. I journeyed to Milwaukee with a vial of [$^{14}$C]histidine, which I administered to the girl, fractionated her urine, and thereby identified a loss of histidase in one of the first cases of histidinemia (2).

The project hooked me on biochemistry, although I still wanted to be a psychiatrist and, indeed, spent much of medical school doing research on perception in schizophrenia, even publishing a few papers in psychiatric journals (3, 4). Hence, the double entendre of the title, as I possessed equal affinities for "minding molecules" and "molecules of the mind."

My hoped-for career in psychiatry confronted a glitch, the "doctors' draft." Every male medical school graduate was obliged to serve two years in the military. NIH, part of the United States Public Health Service, had developed the research associate program wherein young physicians could fulfill their military obligation while doing postdoctoral research in Bethesda, Maryland. As research associate positions were so attractive, securing them was no mean feat, with vast numbers of applicants for the few open slots, the winners all from Harvard and Yale, whereas I was a lowly graduate of Georgetown Medical School. Fortunately, my summer laboratory was across the hall from that of Julie Axelrod, who had collaborated with my mentor Don Brown in identifying the histamine-methylating enzyme and so knew a little of my work. After a year's internship in San Francisco, I joined Julie on July 1, 1963.

### Julie's Laboratory

The early 1960s were the heyday of catecholamine research, with most of Julie's laboratory focused on labeling catecholamine stores with [$^{3}$H]norepinephrine. Julie suggested that I do something different. Building on my earlier experience with histidine, why not examine the disposition in rats of radiolabeled histamine, much as he had done himself with [$^{3}$H]norepinephrine? After [$^{14}$C]histamine administration, I could detect negligible levels of the parent compound but encountered a slew of unidentified metabolites, providing self-taught lessons on how to separate and identify small molecules (5) as well as detecting

inhibition of histamine methylation by psychotropic drugs (6). I also joined Julie's pineal gland project. With another research associate, Dick Wurtman, Julie had identified melatonin as a putative pineal hormone whose formation was regulated by light exposure. Serotonin levels had recently been shown to undergo 10-fold diurnal excursions, but the regulation of this rhythm was obscure. Because the rat's pineal gland is only 1 mg, existing assays for serotonin were not nearly sensitive enough for meaningful analysis. Julie had read a paper reporting that serotonin became highly fluorescent when heated together with ninhydrin, a dye used to stain amino acids. He suggested that I use this as the basis for a new assay, which indeed made it possible for us to measure serotonin in only one or two pineal glands (7). I was able to characterize the circadian rhythm of pineal serotonin, showing it to persist in the absence of light cues but to be strikingly synchronized by external light (8, 9).

### Early Days at Hopkins

Working with Julie was exhilarating. Each of us in the laboratory pursued multiple projects with a surprisingly high yield of successful outcomes. The two years in Julie's laboratory constituted my sole full-time research training, but the impact of his inspirational mentorship on me, as on all of his students, was transformative (Fig. 1). Still, psychiatry remained my career goal; on July 1, 1965, I commenced psychiatry residency training at Johns Hopkins. I secured an arrangement whereby, after an initial year of full-time residency, I became a full-time assistant professor of pharmacology (with a salary allowing my wife, Elaine, and me to start a family) while completing my residency training in an intensive part-time effort. My first project derived from studies I had done "on the side" at the NIH, collaborating with the budding surgeon Joe Fischer to demonstrate a major induction of histidine decarboxylase, the histamine-forming enzyme, after portocaval shunt operations, thereby accounting for the high acid secretion associated with such surgery (10). At Johns Hopkins, I showed that gastric histidine decarboxylase is rapidly induced after treatment with gastrin and explored the enzyme's turnover, a strikingly brief half-life of ~2 h (11), and characterized the disposition of brain histamine (12, 13). This work segued into studies elucidating the extremely rapid turnover of ornithine decarboxylase in regulating polyamine formation (14, 15).

My major early focus was catecholamine uptake. Julie's work employed intravenous injections of [$^{3}$H]norepinephrine. In the case of the brain, with Jacques Glowinski, he administered catecholamines directly into the lateral ven-
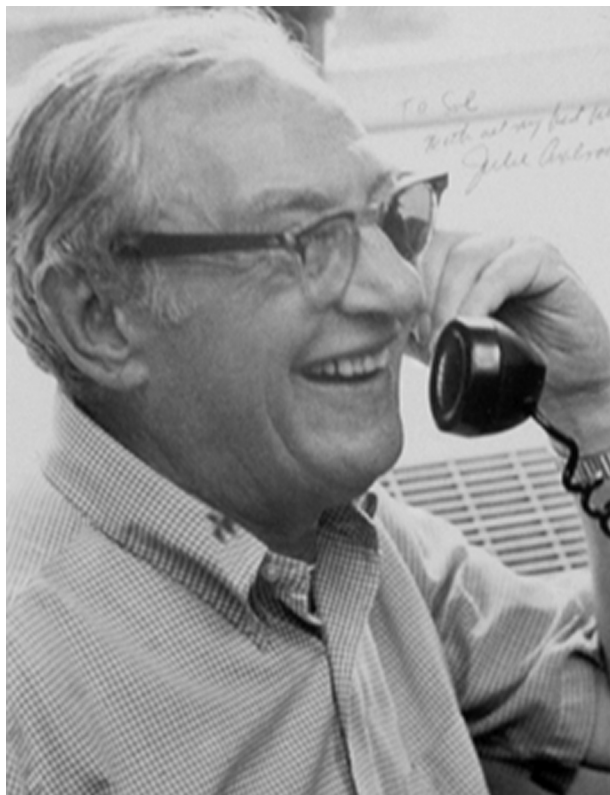
FIGURE 1. **Julie Axelrod.** In this photograph, Julie is accepting a phone call on October 10, 1970, from President Richard Nixon, congratulating him on his receipt that morning of the Nobel Prize in Physiology or Medicine. Considering that Julie received a Ph.D. when he was forty-three years old, his contributions leading to a Nobel Prize at age 59 are all the more impressive. My debt to Julie is inestimable, as my two years in his laboratory (1963–1965) constitute the totality of my full-time research training.

tricles; Jacques then delineated brain catecholamine disposition together with another postdoctoral fellow, Leslie Iversen. As a graduate student, Leslie had analyzed catecholamine uptake kinetics, an exhaustive effort using the isolated perfused rat heart with every data point requiring a separate rat. Leslie was an impeccable scientist and a gifted scientific writer; through our many years of close friendship and collaboration, he affected my efforts to secure scientific rigor as well as to write cogently (Fig. 2). Influenced by Leslie's research, I was impelled to explore catecholamine uptake and sought to study the process in a simpler system, ideally employing pinched-off nerve endings from the brain, "synaptosomes." However, synaptosomes are notably labile in the presence of the salt concentrations required for transport processes. Joe Coyle, then a medical student spending the summer in my laboratory, developed a simple technique, protecting the synaptosomes with sucrose and then adding salt-containing buffers. Besides providing biochemical discrimination of norepinephrine and dopamine uptake in intact animals as well as in synaptosomes (16), we discovered that a number of

antiparkinsonian drugs, thought to act only as anticholinergic agents, were also potent inhibitors of dopamine uptake, which may contribute to their therapeutic actions (17).

We applied the synaptosomal transport strategy to amino acids; the only amino acids with high-affinity, sodium-requiring synaptosomal uptake were glutamate and aspartate and, in the spinal cord, glycine (18, 19). This provided early biochemical evidence for these substances as neurotransmitters, fitting well with neurophysiologic evidence that glycine was a neurotransmitter in the spinal cord but not the cerebral cortex. I maintained a clinical interest in psychotropic drugs and clinical psychiatry, leading to elucidation of novel actions of mescaline/amphetamine derivatives (20).

## Receptors and Neurotransmitter Peptides

In the early 1970s, President Nixon declared "war on heroin" and appointed the psychiatrist Jerome Jaffe as his czar of drug abuse. Jerry was a good friend, and soon other colleagues and I persuaded him to allocate funds for drug abuse research centers, with one of them based at Johns Hopkins. Jerry had been pestering me to do something about opiates, but this was an area of which I knew nothing. The literature on opiates largely consisted of administering morphine to rats and measuring "effects." None of these studies provided definitive molecular explanations of how morphine relieves pain or how heroin causes euphoria. Presumably, opiates acted through specific high-affinity receptors. There had been efforts to find these by radioligand binding with no success, probably because radiolabeled opiates of low specific activity were employed with attendant high concentrations that saturated receptor sites. Our success in identifying opiate receptors stemmed in part from the use of custom preparations of [$^3$H]naloxone at high specific radioactivity. Moreover, we utilized a filter manifold that could handle multiple samples and permit vigorous washing to remove nonspecifically bound ligand without affecting receptor-associated drug (21, 22). Autoradiographic techniques enabled us to localize receptors to discrete nuclei of the brain (23). Receptors were highly concentrated in areas mediating the sorts of pain relieved by opiates as well as in discrete nuclei that mediate other pharmacologic actions of opiates, including euphoria, pupillary constriction, and respiratory depression. One of the most puzzling aspects of pharmacology is the distinction between agonists and antagonists. For opiates, this distinction has clinical importance, as mixed agonist/antagonists offer the greatest possibility of less addicting analgesics. Initial experi-

FIGURE 2. **Iversens.** Elaine and I are pictured with Leslie and Sue Iversen at the wedding of their daughter Amy. Les and I worked together in Julie's laboratory and became close friends and collaborators. I learned good scientific English from Leslie, whose writing was elegant and always cogent.

ments failed to distinguish the binding of agonists and matched antagonists. However, we discovered that sodium ions, and later GTP, nicely discriminated these classes of agents (24). Along with permitting the initiation of high-throughput screening, the "sodium effect" enabled the pharmaceutical industry to identify therapeutically promising agonist/antagonists.

The same general strategy that permitted labeling opiate receptors could be adapted with carefully selected drugs of high affinity to identify receptors for most of the major neurotransmitters in the brain. For instance, dopamine receptors were labeled with [$^3$H]dopamine or with radiolabeled versions of antipsychotic neuroleptic drugs (25), findings obtained independently by Philip Seeman (26). Receptors labeled with [$^3$H]dopamine had different drug specificity than those labeled with $^3$H-neuroleptics, reflecting $D_1$ and $D_2$ receptors, respectively. The relative antipsychotic potencies of neuroleptics were selectively predicted by their ability to block $D_2$ receptors (27, 28). Anticholinergic side effects pose a major problem for antipsychotic and antidepressant drugs. We labeled muscarinic cholinergic receptors with [$^3$H]quinuclidinyl benzilate, which, to this day, is extensively employed by the pharmaceutical industry to screen for anticholinergic actions of candidate psychoactive agents (29). Subtypes of serotonin receptors could be labeled with [$^3$H]serotonin, [$^3$H]LSD, and [$^3$H]spiperone, a neuroleptic that binds to both dopamine $D_2$ and serotonin receptors (30). Combining ligand binding with behavioral experiments, we discriminated functionally distinct serotonin receptor subtypes (31, 32). Drugs acting selectively on serotonin receptor subtypes are now widely employed therapeutically. Other receptors identified by ligand binding included those for glycine (33), GABA (34), nerve growth

factor (35), α-adrenergic actions of norepinephrine (36–38), and adenosine (39). The work on nerve growth factor receptors was done in collaboration with my friend and colleague Pedro Cuatrecasas, who taught me the binding technology that he had employed in characterizing insulin receptors and that greatly facilitated our ligand binding strategies (Fig. 3).

Man was not born with morphine in him. Why do we have opiate receptors? In Aberdeen, Scotland, Hans Kosterlitz and John Hughes took advantage of the influence of opiates upon smooth muscle, developing a bioassay wherein brain extracts exerted naloxone-reversible, opiate-like influences on smooth muscle contractions. We assessed the ability of brain extracts to compete with radiolabeled opiates for receptor binding and showed that the morphine-like effects of brain extracts displayed regional variations throughout the brain correlating to the relative distribution of opiate receptors (40, 41). The Scottish team "won the race" to obtain the structure of the enkephalins as endogenous opioid peptides (42). We purified and sequenced the enkephalins as well, completing our task a couple of months after Hughes and Kosterlitz (43). Purifying small peptides (the enkephalins were five amino acids in length) on ion-exchange columns harked back to my days as a medical student purifying histidine metabolites, reinforcing the importance for the neurosciences of old-fashioned biochemistry. With antibodies to the enkephalins, we mapped their localization immunohistochemically throughout the nervous system. Their discrete localizations correlated nicely with those of opiate receptors and explained specific actions of opiates (44). For instance, opiates can relieve pain at the spinal cord level. We had localized spinal opiate receptors to nerve

FIGURE 3. **Pedro Cuatrecasas and me.** Pedro's laboratory at Johns Hopkins was adjacent to mine. He had developed ligand binding technology in identifying insulin receptors. We collaborated in demonstrating receptors for nerve growth factor, techniques that I adapted for the labeling of opiate receptors.

terminals of sensory pain fibers. Enkephalin occurs in small interneurons in the dorsal portion of the spinal cord, where the sensory nerves enter. Opiates act there by inhibiting the release of pain neurotransmitters, such as the peptide substance P.

An appreciation of the enkephalins as peptide neurotransmitters led to an interest in diverse peptides as neuronal messengers (45). Evidence already existed that substance P is a transmitter. The immunohistochemical techniques developed by Tomas Hökfelt soon were applied to large numbers of peptides, many of which were known in other incarnations as intestinal or brain hormones. For instance, cholecystokinin was first identified as a substance that regulates gallbladder contractility, but it is also an important cerebral cortical transmitter. We characterized bradykinin and its receptors, indicating that it is a major initial stimulus to pain perception (46). Several dozen peptides are now recognized as putative neurotransmitters.

## Odorant-binding Protein

Molecular biology has largely supplanted biochemistry as the principal tool of biomedical science. Our first effort to clone genes came in the mid-1980s, when we began investigations of olfaction. I had become friends with Henry Walter, chief executive officer of International Flavors & Fragrances, the principal generator of odorants for perfumes and other purposes. Walter challenged me, saying, "My dog has more impressive receptors in his nose than all of your neurotransmitter receptors in the brain. Why doesn't anybody study olfaction?" He proposed to fund our laboratory to do such studies: "I'm going to put my money where my nose is." We utilized our ligand binding technology to seek "receptors" for $^3$H-odorants but never found the physiologic odorant receptors. Instead, we discovered a small soluble protein, the odorant-binding protein (OBP), which is secreted from glands in the back of the nose through a tube that sprays OBP, like a perfume atomizer, into the air to bind odorant molecules and transport them back to the receptor sites in the rear portion of the nose (47). With the collaboration of Randall Reed at Johns Hopkins, we successfully purified OBP to homogeneity, obtained a small amount of amino acid sequence, constructed a cDNA probe, and cloned and characterized OBP (48).

## Inositol Phosphates

By the mid-1980s, receptors for most of the major neurotransmitters had been characterized. About that time, Michael Berridge and others elucidated inositol 1,4,5-trisphosphate ($IP_3$) as a major second messenger releasing intracellular calcium. $IP_3$ presumably acted on the surface of intracellular vesicles that contain calcium. We decided to seek such putative $IP_3$ receptors, a task demanding a rich source of the receptors. We conducted a body-wide autoradiographic screen that revealed the highest density of $IP_3$ receptors in the brain, especially the cerebellum. Using cerebellar membranes, we identified and characterized $IP_3$ receptors (49). We decided to address the challenge of solubilizing and purifying the receptors even though, at that time, solubilizing membrane proteins and characterizing them biochemically was a formidable, often hopeless endeavor. Recalling my early efforts at purifying enzymes, I welcomed the challenge. With a lucky choice of appropriate reagents, we solubilized and purified $IP_3$ receptors to homogeneity (50). This enabled us to address what was then an important question about ion-coupled membrane receptors. It was widely held that a neurotransmitter receptor comprises just the recognition site for the transmitter, with the receptor protein moving by lateral

diffusion through the cell membrane to link to an ion channel protein. With Efraim Racker, Richard Huganir had incorporated purified nicotinic acetylcholine receptors into lipid vesicles loaded with radiolabeled sodium and demonstrated that the purified receptor protein contained the relevant sodium channel. As Huganir had recently joined the Johns Hopkins faculty, we collaborated with him, inserting purified $IP_3$ receptors into lipid vesicles loaded with radiolabeled calcium and showing that the purified receptor contained the physiologic calcium channel (51). The same techniques enabled us to elucidate how $IP_3$ receptors mediate the quantal release of calcium (52).

Inositol has six hydroxyl groups, with $IP_3$ utilizing only three of those. $IP_4$, $IP_5$, and $IP_6$ were known to occur in tissues at concentrations even higher than those of $IP_3$. In the mid-1990s, we were entranced by reports that tissues labeled with [$^3$H]inositol formed $IP_7$, an inositol phosphate in which one of the hydroxyls contains a diphosphate, which, by definition, ought to be a pyrophosphate as energetic as ATP. It seemed to me that the only way to learn about the function of inositol pyrophosphates would be to purify and clone their biosynthetic enzymes (Fig. 4). After three years of arduous efforts purifying a very labile $IP_6$ kinase by 30,000-fold (53), we obtained enough protein to secure a partial amino acid sequence and clone a family of three $IP_6$ kinases as well as a related enzyme called inositol polyphosphate multikinase (IPMK) (54). Using conventional biochemistry, RNA interference, gene knockout mice, and other tools, we have developed insight into some of their functions. $IP_7$ can modify proteins by pyrophosphorylating them (55, 56) and can also compete with phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$) as an intracellular signal (57). $IP_6$ kinase-2 (IP6K2) generates $IP_7$ in a pool that interacts with p53 to augment its ability to elicit apoptosis by suppressing the cell arrest actions of p53 (58). The $IP_7$ made by IP6K1 physiologically inhibits the protein kinase Akt (PKB), which can predispose to insulin resistance and obesity (59). Thus, IP6K1 knock-out mice display enhanced sensitivity to insulin and resistance to obesity associated with a high-fat diet. IPMK has multiple actions. It is a physiologic source of $IP_5$ derived by phosphorylating $IP_4$. Additionally, IPMK is a lipid kinase working in coordination with the p110/p85 PI3K to generate $PIP_3$. By dint of its lipid kinase activity, IPMK activates Akt, so IPMK deletion leads to 50–60% declines in Akt signaling (60). By contrast, the inositol phosphate kinase activity of IPMK generates $IP_5$, which is a rate-limiting precursor of $IP_7$ and so leads to diminished Akt activity. We presume that IPMK acts as a molecular switch between these pro- and
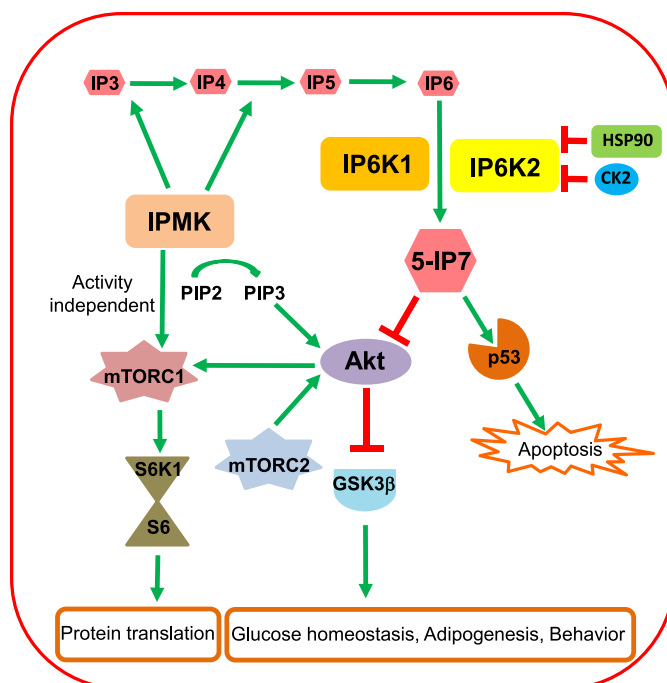


FIGURE 4. **Inositol polyphosphate signaling cascade.** Our work identifying, isolating, and characterizing $IP_3$ receptors (49–52) prompted an interest in higher inositol phosphates, especially $IP_7$, which contains a pyrophosphate bond. Recent findings using mice with targeted deletion of isoforms of $IP_6$ kinase (IP6K) and the related enzyme IPMK have revealed interconnected pathways of inositol phosphates, which pivot cells between trophic and antitrophic functions. Thus, pools of 5-$IP_7$ generated by IP6K2 elicit apoptosis by enhancing actions of p53 (58). HSP90 binds and inactivates IP6K2 (77). Cell stressors disrupt the binding, activating IP6K2 and thereby augmenting cell death. The anti-apoptotic actions of casein kinase-2 (*CK2*) are mediated, at least in part, by phosphorylation of IP6K2 to accelerate its ubiquitin-associated degradation (78). 5-$IP_7$ formed by IP6K1 inhibits Akt, thereby activating GSK3$\beta$ to increase adipogenesis and exert general antitrophic actions (59). IPMK, as a rate-limiting enzyme in the generation of higher inositol phosphates, leads to inhibition of Akt, an antitrophic influence. IPMK is also a physiologic PI3K that forms $PIP_3$ to activate Akt; hence, in this form, it is a trophic enzyme (60). As yet unidentified signals somehow elicit a switch of IPMK between its antitrophic inositol phosphate kinase and trophic PI3K activities. These functions of $IP_6$ kinases and IPMK are exerted primarily in response to growth factors. Nutrients stimulate mTOR and protein translation independently of Akt. Nutrients stimulate the binding of IPMK to mTOR, which non-catalytically stabilizes mTORC1 to augment protein synthesis (61).

antitrophic actions of cells. Additionally, in a non-catalytic fashion, IPMK is a critical component of mTORC1 (mTOR complex 1), a major mediator of protein translation (61). IPMK acts as a scaffold securing the binding of mTOR, as IPMK deletion leads to degradation of the complex and diminished protein translation.

## Gases

In the late 1980s, nitric oxide (NO) was shown to be an endothelium-derived relaxing factor (EDRF) formed from arginine. We wondered whether it might have some function in the brain and were able to show that stimulation of cyclic GMP formation by glutamate in brain extracts

could be inhibited by arginine derivatives that are inhibitors of NO synthesis. As with the inositol pyrophosphates, we could see that meaningful progress required purification and cloning of whatever enzyme converted arginine to NO. The apparent lability of the putative NO synthase had hindered efforts to purify the enzyme. Knowing that calcium played a role in NO formation, we added calmodulin, which stabilized the enzyme, permitting its purification by conventional column techniques (62), followed by its cloning (63). Cloning revealed a family of three NO synthases: neuronal NOS (nNOS), endothelial NOS, and inducible NOS. Targeted deletion of nNOS revealed multiple roles for neuronal NO, including regulating the stomach's pyloric sphincter; hence, NO deficiency appears to participate in the pathophysiology of hypertrophic pyloric stenosis (64). The nNOS mutant male mice also displayed very greatly increased sexual and aggressive behaviors (65). Our immunohistochemical localizations of nNOS (66) to the cavernous nerves that are responsible for penile erection, coupled with the inhibition of penile erection by nNOS inhibitors, established NO as a neurotransmitter of penile erection (67). In relaxing smooth muscle of blood vessels and penile erectile tissue, NO stimulates cyclic GMP formation. Elevating cyclic GMP levels with inhibitors of phosphodiesterase-5, such as sildenafil (Viagra), alleviates erectile dysfunction. Besides activating cyclic GMP, NO signals by *S*-nitrosylating reactive cysteines in target proteins, a process elucidated first by Jonathan Stamler (68) and then by application of the biotin switch assay for endogenous nitrosylation developed in our laboratory (69).

Nitrosylation mediates a novel signal transduction pathway that accounts for cell death. We found that apoptotic stimuli elicit nitrosylation of GAPDH, abolishing its catalytic activity but conferring upon it the ability to bind an E3 ligase, Siah, which translocates GAPDH to the nucleus (70), where it stimulates p300/CBP (cAMP-responsive element-binding protein-binding protein) to acetylate p53, activating its apoptotic influences (71). Nitrosylated GAPDH functions physiologically in the nucleus, *trans*-nitrosylating nuclear proteins, such as the deacetylating enzyme SIRT1, HDAC2 (histone deacetylase-2), and DNA protein kinase (72).

NO is not the sole gasotransmitter. Heme oxygenase (HO) physiologically converts heme in aging red blood cells to biliverdin and then to bilirubin. Cleavage of the heme ring emits carbon monoxide (CO) along with biliverdin. The inducible isoform of HO, highly localized to the spleen, is designated HO1. Another isoform, HO2, is selectively concentrated in the brain and testes and, like HO1, generates CO as a side product. We wondered whether CO might be a neurotransmitter. We demonstrated selective localizations of HO2 to discrete neuronal populations in the brain and peripheral autonomic nervous system (73). We showed that a major part of physiologic intestinal relaxation is attributable to CO formed in myenteric neurons by HO2, with many of the same neurons also forming NO. Utilizing HO2 and nNOS knock-out mice, we obtained evidence that these two gases function coordinately in the intestinal relaxations that underlie peristalsis (74).

More recently, we have been studying hydrogen sulfide ($H_2S$) as a gasotransmitter. $H_2S$ has long been known to be generated in the intestine by bacteria. In test tube systems, the enzymes cystathionine $\gamma$-lyase (CSE) and cystathionine $\beta$-synthase can make $H_2S$ from cysteine. In collaboration with Rui Wang, who developed CSE knock-out mice, we showed that the generation of $H_2S$ by peripheral tissues is abolished in CSE knock-outs (75). In the brain, $H_2S$ appears to be formed predominantly by cystathionine $\beta$-synthase. Like NO, $H_2S$ is a vasorelaxant. In resistance blood vessels of CSE knock-out mice, EDRF activity was reduced by $\sim$75%, and the mice were hypertensive. Thus, $H_2S$ appears to be a major EDRF, perhaps more prominent than NO. $H_2S$ signals by a process of sulfhydration, analogous to nitrosylation, converting the SH of cysteines in proteins to SSH (76). Whereas nitrosylation of cysteines usually inactivates the amino acid, sulfhydration retains the reactive SH of cysteine and makes it more accessible to targets. Hence, sulfhydration typically activates enzymes, for instance, increasing the catalytic activity of GAPDH by 700%. Livers of CSE knock-out mice display major reductions in GAPDH catalytic activity, consistent with evidence that a substantial portion of endogenous GAPDH and other proteins is basally sulfhydrated. Such sulfhydration accounts for the vasorelaxing activities of $H_2S$ mediated by sulfhydration and opening of ATP-sensitive potassium channels. Physiologic vasorelaxation by $H_2S$ reflects hyperpolarization of blood vessels, in contrast to cyclic GMP-mediated vasorelaxation associated with NO.

## Mentoring

Few scientists work in isolation. For me, perhaps more than most, an interplay with graduate students, M.D./Ph.D. students, and postdoctoral fellows has been critical for the creative process. Here, I mention only a few of the more than one hundred to whom I owe my professional successes. Early work on catecholamines in our laboratory was done by Ken Taylor, Alan Green, and, especially, Joe Coyle. Shailesh Banerjee, in collaboration with Pedro Cua-

FIGURE 5. **Family.** Shown are our daughters, Judy (on the guitar) and Debby (on the flute), together with Elaine and me circa 1980. Music remains important: I still play the guitar daily and have served on the board of the Baltimore Symphony Orchestra for nineteen years.

trecasas, discovered ligand binding for nerve growth factor receptors, establishing the technology that we applied to opiate and other receptors. Candace Pert, Gavril Pasternak, and Rabi Simantov were seminal in opiate receptor/enkephalin research. Bob Innis and Don Manning helped elucidate neuropeptide disposition. Dopamine receptor identification was led by Ian Creese and David Burt, whereas Steve Peroutka discriminated subtypes of serotonin receptors, an effort associated also with Jim Bennett. Anne Young identified glycine receptors shortly after the discovery of opiate receptors. The late Hank Yamamura labeled muscarinic cholinergic receptors with [$^3$H]quinuclidinyl benzilate and discovered the high-affinity choline uptake that is the physiologic rate-limiting step in acetylcholine formation. Paul Worley and Jay Baraban identified and characterized $IP_3$ receptors, which were purified to homogeneity by Surachai Supattapone. Chris Ferris functionally reconstituted $IP_3$ receptors into lipid vesicles. Susan Voglmaier heroically purified $IP_6$ kinase, whereas Adolfo Saiardi utilized the purified preparation to clone the family of $IP_6$ kinases and, with Rashna Bhandari, showed that $IP_7$ can pyrophosphorylate target proteins. Anutosh Chakraborty, Michael Koldobskiy, David Maag, Micah Maxwell, Seyun Kim, Krishna Juluri, and Kent Werner have elucidated much of the biology of inositol pyrophosphates. David Bredt identified NO as a neurotransmitter, purified and cloned nNOS, and, in collaboration with Arthur (Bud) Burnett, established NO as a neurotransmitter of penile erection. Charlie Lowenstein cloned inducible NOS, whereas Samie Jaffrey established *S*-nitrosylation as a signaling mechanism, and Michael Kornberg identified *trans*-nitrosylation of nuclear pro-

teins. Ted and Valina Dawson established roles of NO in neurotoxicity. Akira Sawa, Makoto Hara, and Nilkantha Sen elucidated the NO-GAPDH-Siah-p53 signaling cascade. Ajay Verma and Randa Zakhary established CO as a gasotransmitter. Asif Mustafa and Moe Gadalla characterized $H_2S$ functions. Michael Schell and Herman Wolosker identified D-serine as a novel glial transmitter formed by serine racemase, whereas Schell and Paul Kim explicated transmitter functions of D-aspartate generated by aspartate racemase.

Mentoring students is like raising a family. My ability to focus on science was fostered by the loving support of Elaine, my wife of almost fifty years. Our primary training in mentoring happened while raising two wonderful daughters (Fig. 5). Judy, a psychiatrist practicing in Philadelphia, is herself raising Abigail, Emily, and Leo, ages 14, 12, and 9 (in 2011). Debby (Dasha) is a screenwriter living in New York with her wonderful wife, Sonora, a talented actress.

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