The Audacity Principle in Science[1]

SOLOMON H. SNYDER
Department of Neuroscience
Johns Hopkins University
School of Medicine

DEDICATED TO JULIUS AXELROD
a humble, gentle scientist who nonetheless epitomized the Audacity Principle in Science. He died 29 December 2004.

MY MENTOR, Julius Axelrod, often commented, "Ninety-nine percent of the discoveries are made by one percent of the scientists." This may sound like an exaggeration. However, a brief examination of the major advances in any branch of science reveals the truth of this dictum. Axelrod himself is a prime example. In the field of molecular pharmacology, many of the key advances are attributable to his own efforts. He elucidated the metabolism of the major psychoactive drugs, in the process laying the groundwork for the emergence of acetominophen (Tylenol) as a major analgesic and then uncovering the family of drug-metabolizing enzymes, now known as the P450 enzymes. He accomplished most of this while working as a laboratory technician without a Ph.D. Following receipt of his doctorate at age forty-two, Axelrod proceeded to revolutionize neurotransmitter research. Consider the catecholamine neurotransmitters such as norepinephrine and dopamine. After norepinephrine was established as the neurotransmitter of sympathetic nerves in the late 1940s, advances were relatively modest. The enzymatic processes leading to its biosynthesis from the dietary amino acid tyrosine were gradually elucidated by multiple investigators over a period of several decades. Classical pharmacologic studies comparing the effects of different drugs on sympathetic neurotransmission had led to an appreciation that there were at least two subtypes of receptors for norepinephrine, designated alpha

[1] A version of this paper was read at the Autumn General Meeting on 9 November 2002.
Acknowledgments: Supported by USPHS grants MH18501, DA00266, MH068830 and Research Scientist Award DA00074. I thank Susan Arellano for helpful suggestions.

and beta, which subsequently led to important new drugs. Then, in roughly half a decade, Axelrod had a series of insights that drastically altered our thinking about norepinephrine and, indeed, all neurotransmitters. In 1957 he discovered catechol-O-methyltransferase, a key enzyme in metabolizing the catecholamines. This led him to question the prevailing assumption that the only other known enzyme that metabolizes catecholamines, monoamine oxidase, accounts for inactivation of norepinephrine after it is released by sympathetic nerves. Inactivating neurotransmitters is of crucial importance, for it serves to remove them from the vicinity of receptors on adjacent neurons so that successive nerve impulses will be effective. In the early 1960s the only neurotransmitter known besides norepinephrine was acetylcholine, discovered in the late 1920s. It was well established that the actions of acetylcholine are terminated by enzymatic degradation via an enzyme called acetylcholinesterase. Drugs that inhibit this enzyme potentiate the actions of acetylcholine at synapses, sites where nerves communicate with each other. Such acetylcholinesterase inhibitors provide important therapy for diseases such as myasthenia gravis that are characterized by muscle weakness because of deficient neurotransmission at acetylcholine synapses.

Because of the well-established role of acetylcholinesterase in inactivating acetylcholine, it was accepted wisdom that enzymes would inactivate norepinephrine at its synapses. But no one had directly evaluated whether monoamine oxidase was in fact responsible for norepinephrine inactivation. To compare the roles of catechol-O-methyltransferase and acetylcholinesterase, Axelrod utilized drugs that inhibit the two enzymes and was surprised to find that neither enzyme could explain synaptic inactivation. About this time, radioactive forms of norepinephrine became available. Instead of pursuing convoluted biochemical experiments with the radiolabeled molecule, Axelrod simply injected it into rats. He was amazed to find that organs enriched in sympathetic nerves, such as the heart, enormously concentrated the radioactive norepinephrine. When sympathetic nerves were severed, these organs no longer took up the neurotransmitter, indicating that it was the sympathetic nerve endings that had been concentrating norepinephrine. Based on his experimental findings, Axelrod formulated a simple model to explain neurotransmitter inactivation. He proposed that uptake of the norepinephrine into the nerve endings that had released it mediates synaptic inactivation. His theory was rapidly proven right by experiments showing that cutting the nerve endings to eliminate the uptake process markedly potentiated neurotransmission. He wondered whether drugs that could mimic the effects of sympathetic nerve firing might act by inhibiting this uptake process, thereby potentiating actions of norepinephrine. Utilizing radiolabeled norepinephrine, he soon showed that

agents such as cocaine, amphetamines, and, most important, the major antidepressants, exert their effects by inhibiting the uptake process. Soon, other scientists showed that uptake mechanisms account for inactivation of virtually all the major neurotransmitters, with enzymatic degradation, as with acetylcholine, being the exception rather than the rule.

For these contributions, Axelrod shared the Nobel Prize in Physiology or Medicine in 1970 with Ulf von Euler, who established norepinephrine as a transmitter, and Bernard Katz, who showed that neurotransmitters are stored in and released from synaptic vesicles.

What do we learn from the above anecdote? First, in this field one scientist with a tiny laboratory comprising no more than three or four postdoctoral fellows could make many if not most of the key breakthroughs in a large field of research. Second, we learn to wonder what differentiates individuals such as Axelrod from others in the field. This leads us to the focus of this essay: what makes for greatness in scientific research?

Clearly Axelrod manifested an abundance of creative insights. He conceptualized principles never previously enunciated. The notion that neurotransmitters were inactivated by being taken back into the nerve that had released them initially met with ridicule. Axelrod saw through dogma as gerrymandered as the Ptolemaic planetary system and, like Galileo, provided radical simplification. But creativity isn't enough. Whenever a major new discovery is published, dozens of scientists exclaim, "I thought of that a long time ago but just didn't do the right experiment." Original ideas are only a part of the story. A special sort of energy is required to overcome the fear or inertia that hinders scientists from essaying risky, unprecedented experiments. Of course, devising the optimal experiment is crucial, and all experimental breakthroughs involve simplification. One could conceptualize intricate, year-long approaches to experiments to explore neurotransmitter uptake. Axelrod simply injected radiolabeled norepinephrine into rats and came up with the "answer" in a day or two. Experimental ingenuity, a shrewdness in experimental design, and "good hands" all play a role in coming up with the "right" experiment. But I have known many talented experimentalists who never make major advances.

Much has been written about the nature of scientific discovery. The rigor of scientific hypothesis formulation and testing, as well as critical thinking to rule out artefactual explanations of data, is often highlighted. My personal experience over three to four decades tells me that the real breakthroughs don't happen this way. The greatest scientists tend to resemble artists in certain ways, but with notable differences. Artists see the world differently than the rest of us. They find wonder in the seemingly mundane. They detect commonalities among objects

that most viewers see as notably disparate. Similarly, the finest researchers view with puzzlement established principles that are taken for granted by the scientific community. In particular, they become irritated by overly convoluted explanatory principles. They seek simplicity, whereupon novel concepts emerge. The ability to divine unifying notions out of a morass of data seems critical.

Equally important is the intellectual and often emotional courage to enunciate such simplifications. Courage is requisite for many reasons. Challenging established authority is always risky. The challenge is even more complex in science because, when first presented, a new unifying concept can rarely account for all the available data. One must be willing to proffer and defend a novel hypothesis in the face of some contradictions, based simply on the argument that the virtues of a new model, compared with older formulations, exceed its drawbacks. Often history bears out the validity of the new paradigm, but sometimes the innovative notion proves false; hence the risk. Positing something new even in the presence of discrepancies is justified, for often such discrepancies fade away as new data emerge. The late Francis Crick was a lover of elegant, simple, and revolutionary hypotheses. He believed strongly in the beauty and simplicity of nature and thus favored simple explanatory models. In an informal group in which he and I participated years ago, he put it roughly this way, "If the theory has a beautiful feel and makes good sense despite some ugly data which don't agree, perhaps the data are wrong!"

All these factors seem to be relevant. Originality and simplicity are certainly crucial elements. Even more important are the intellectual fearlessness and emotional drive to put it all together, step forward, do the right experiment, promulgate it to the world, defend the new insights, and go forward to further innovation. A simple designation for this behavioral pattern might be the Audacity Principle. Audacious behavior is usually regarded as a form of hubris or chuzpah, an offputting and overly aggressive behavior that we don't usually link to creativity. Here I use the term to focus on the intellectual qualities of audacity that enable individuals of talent to implement their own native ability. In other words, scientific originality may not be so rare a commodity as is the capacity to appreciate the importance of one's own ideas and to put them into practice.

Lineage

One way to seek the qualities that make for scientific discovery is to examine what is conveyed in the mentor-apprentice relationship. The eminent sociologist Harriet Zuckerman has provided compelling evidence

for the importance of scientific lineage. She compared the careers of Nobel laureates with others, matched for closely similar educational background, intelligence, association with distinguished universities, and other factors, who were very good but not "Nobel quality" (Zuckerman 1996). The single factor that most clearly differentiated Nobel laureates from outstanding but lesser scientists was training with another Nobel laureate. Zuckerman distinguished the mentor-student personal relationship from the political contacts offered by a prominent scientist, as most future Nobelists trained with their mentors long before the latter had attained scientific eminence, far less a Nobel Prize.

Individual testimonies convey the role of mentorship. The biochemist Hans Krebs commented, "If I ask myself how it came about that one day I found myself in Stockholm, I have not the slightest doubt that I owe this good fortune to the circumstance that I had an outstanding teacher" (Zuckerman 1996, 124). What do mentors convey? The best teaching is done by example. There is an important emotional element whereby the mentor enables the student to feel self-confident enough to pour forth his/her original ideas. This mode of teaching is similar to what the best psychotherapists do with their patients, or parents with children. For the psychologist Carl Rogers the essence of psychotherapy was conveying to the patient an attitude that he designated "unconditional positive regard." All good parents know that their children are never "bad children" but are instead good children who occasionally do bad things. Reinforcing the "good" and not punishing but merely disregarding the "bad" enhances self-esteem. I remember presenting my mentor, Julie Axelrod, with a bundle of experimental results that seemed like a total failure. Julie looked through the results and commented, "Sol, why are you so glum? Some of the findings aren't so great, but, look! I see kernels of exciting ideas we can explore further." When one considers that easily nine out of every ten experiments fail, such pep talks are invaluable.

A number of Zuckerman's findings can be subsumed under the Audacity Principle, e.g., being sufficiently courageous to ask "important" questions. One might think that anybody's grandmother can tell you what is important: "Go find the cure to cancer!" In reality, choices are far more subtle. Many molecular pathways lead to cancer, so it is hard to know which is "more important." Moreover, scientists like everyone else are subject to a powerful herd instinct, jumping on the current, fashionable area, one for which experimental tools are usually readily available so that obtaining publishable data is relatively risk-free. Thus, asking the important question requires a combination of creative insight and audacity. The great biochemist Otto Warburg, who studied with Emil Fischer, the second scientist to be awarded the Nobel Prize in

chemistry, commented, "I learned (from Fischer) that a scientist must have the courage to attack the great unsolved problems of his time . . . without much critical hesitation" (Zuckerman 1996, 128). This courage to "go for it" without obsessive procrastination and excessive self-criticism is a hallmark of the greatest scientists. As Zuckerman said in summarizing her observations, "Among the elite scientists, the prime criteria of scientific taste are a sense for the 'important problem' and an appreciation of stylish solutions."

The writer Robert Kanigel, who has chronicled the careers of leading scientists, distills their lessons: "Just go with your hunch, your scientific intuition and isolate that single, elegant, pointed experiment that will tell you in a flash whether you are on the right track." He also noted, "*Just do it*, don't spend all year in the library getting ready to do it. Don't wait until you've gotten all the boring little preparatory experiments out of the way. Don't worry about scientific controls except the most rudimentary" (Kanigel 1993, 236).

There is a knack to identifying important questions, which good mentors convey. Robert Kanigel described it this way: "Don't bother with the routine scientific problems. . . . Leave those to others. Don't bother, either, with big, fundamental problems which are simply not approachable with available techniques and knowledge; why beat your head against the wall? Half the battle is asking the right question at the right time" (Kanigel 1993, 235). Axelrod said similar things: "One of the most important qualities in doing research, I found, was to ask the right questions at the right time. I learned that it takes the same effort to work on an important problem as on a pedestrian or trivial one. When opportunities came, I made the right choices" (Axelrod 1988).

Let me illustrate how original ideas, simplification, experimental ingenuity, and willingness to take risks manifest the Audacity Principle in my own work. I do not claim to possess these qualities in greater abundance than other scientists, but cite my own examples simply because I know them best.

Receptors

Much of my professional life has dealt with studies of neurotransmitters. In 1970 scientists knew a great deal about their biochemistry, how they are formed, stored, released, and inactivated by reuptake. Virtually nothing was known about the most critical actions of neurotransmitters, namely, their ability to act in a lock-and-key fashion upon receptors on adjacent target cells. Then, within a span of a few months, several research groups reported success in identifying a receptor for acetylcholine, the first-discovered and best-characterized transmitter.

The investigators took advantage of a remarkable structure, the electric organ of the electric eel. The electric organ is extraordinarily enriched in these receptors, so much so that they generate sometimes lethal shocks to the eel's prey. The researchers also took advantage of an extraordinarily potent snake toxin, alpha-bungarotoxin, which binds with high affinity to the receptors. The unique properties of this system suggested that it would be impossible ever to identify biochemically the receptors for neurotransmitters in the brain. Thus, the acetylcholine receptors constitute 20 percent by weight of the electric organ of some eels, whereas one could calculate that most neurotransmitter receptors in the mammalian brain would be only about one millionth by weight. Moreover, there were no magical toxins available for most neurotransmitter receptors.

I was aware of the importance of identifying neurotransmitter receptors, but chose instead to address receptors for opiate drugs, largely because research in our laboratory was funded by the drug abuse division of the National Institutes of Health. At that time nothing was known about the nature of receptors for drugs such as opiates, other than their ability to bind the drugs. One could obtain radiolabeled drugs and monitor their binding to brain membranes, but armchair calculations would tell you that this should be impossible. Based on the known potencies of opiate drugs in intact mammals, the distinguished pharmacologist Vincent Dole had estimated the quantity of presumed opiate receptors in the brain and had come up with a number that was about the same as the expected density of most neurotransmitter receptors, namely, about one millionth by weight. Opiate drugs possess positively charged nitrogen atoms that might bind nonspecifically to negatively charged tissue constituents. The drugs also possess benzene rings that would bind nonspecifically to lipid-containing constituents. Such nonspecific binding would surely vastly exceed the number of specific receptor binding sites.

To overcome such challenges, my students and I reasoned that the biologically relevant opiate receptors should have a far higher affinity for opiate drugs than the non-specific binding sites. One could take advantage of this by utilizing opiates of high specific radioactivity, i.e., with a great deal of radioactive label per molecule, so that one could employ very low concentrations of the radiolabeled drug that would bind more selectively to specific receptors than to nonspecific sites. Moreover, drug molecules that were bound with high affinity to receptors would wash away more slowly than nonspecifically bound drug molecules. Thus, one would wash the brain membranes extensively to remove nonspecific binding while preserving true receptor interactions. Of course, such washing would have to be done very rapidly, lest the

radiolabeled opiate also wash off the receptors. This strategy enabled us to identify opiate receptors (Pert and Snyder 1973).

To implement this receptor-binding strategy on a large scale, I borrowed from my colleague Pedro Cuatrecasas a vacuum filtration manifold he devised for his pioneering studies of insulin receptors. With this apparatus we were able to process hundreds of samples in an hour, enabling us to do far more than simply establish that the brain possesses pharmacologically relevant opiate receptors. We could examine large numbers of drugs rapidly. This permitted us to explore the actions of numerous drugs. For instance, we were able to explain key pharmacologic actions of codeine and heroin. Codeine, which acts gradually to relieve pain and cough, didn't bind to opiate receptors at all. Codeine differs from morphine solely by the addition of a methyl group that covers up a hydroxyl structure that is critical for receptor interactions. After ingestion, the methyl group of codeine is removed in the liver, generating morphine that then enters the brain. Hence, codeine is nothing but a pro-drug for morphine. The fact that it must be metabolized before it can act explains its gradual onset of action. Any drug that enters the brain gradually is much less likely to produce a "high." This explains the greater safety of codeine as compared to morphine in terms of abuse. Heroin also failed to bind to the opiate receptor. Interestingly, the pharmacology of heroin is the opposite of that of codeine. After intravenous injection heroin enters the brain very rapidly, causing the "rush" of euphoria that underlies its massive addictive potential. Heroin doesn't bind to opiate receptors, because it possesses an acetyl group that overlies the critical hydroxyl of morphine. In contrast to the stability of the methyl group in codeine, which requires removal by enzymes of the liver, the acetyl group of heroin is spontaneously liberated when heroin enters the brain. Thus, heroin, like codeine, is a pro-drug of morphine, but it delivers the morphine far more rapidly.

Since we could measure large numbers of samples rapidly, we were able to dissect monkey brains into tiny regions, and thus discovered that the relative densities of opiate receptors in various areas of the brain could fully account for major pharmacologic actions of the drug (Kuhar et al. 1973). For instance, opiate receptors were extremely concentrated in the lateral region of the thalamus, an area where sensory information is processed. Moreover, the lateral thalamus processes information about chronic, aching pain, the sort that is relieved by opiates, in contrast to the medial thalamus, which deals with brief, sharp pin-prick types of pain that don't respond to the drugs. Opiates are well known to constrict the pupils of the eye, enabling police to identify an addict at a glance. Opiate receptors were highly enriched in certain nuclei of the brain stem that regulate pupillary diameter.

These are but a few of the myriad insights into opiate actions that derived from the ability to measure them in simple test-tube systems. What does this reveal about behaviors that make for scientific success? First of all, most scientists would never have embarked on the search in the first place. Their carefully considered armchair reasoning would have told them that the task was fruitless. Moreover, much preplanning was required. To seek opiate receptors, we had to obtain, at great expense, custom-prepared radiolabeled forms of the opiate antagonist naloxone. Mastering the vacuum filter manifold took time. Nonetheless, with the "uncritical" foolhardiness of youthful optimists, my students and I obtained the appropriate radiolabeled drug and moved forward. Suspending one's critical faculties at key times and moving forward with seeming impetuosity have contributed to many major advances in science. In almost all instances known to me, the most successful scientists have addressed the riskiest projects and have thus encountered more failure than success. But, as in venture capital investing, a few major successes more than compensate for large numbers of failures.

Another component of success in science is what detractors refer to as opportunistic exploitation of discoveries. By this, they mean applying the fruits of one discovery to other, related "easy" findings. Thus, when Axelrod discovered catechol-O-methyltransferase, the enzyme that adds a methyl group to norepinephrine, he reasoned that there must be many other methylating enzymes. He thus went about seeking and finding the enzyme that generates the pineal gland hormone melatonin, the enzyme that forms the adrenal gland hormone adrenaline/epinephrine, and many others. His scientific critics described this approach as "going for the low-hanging fruit." On the contrary, I regard Axelrod's approach as a way to uncover major new insights with minimal effort and I have never shirked from such scientific opportunism. Since the properties of the opiate receptor were very much like what one would expect of a neurotransmitter receptor, we applied receptor technology, with various modifications, to an assault on all the major neurotransmitter receptors in the brain and, by the mid-1970s, had successfully labeled most of them. For instance, in the mid-1970s there was great interest in the neurotransmitter dopamine. Degeneration of dopamine neurons in the brain accounts for the principal symptoms of Parkinson's Disease. L-Dopa, the amino acid precursor of dopamine, was first employed therapeutically in the late 1960s as a drug that replaces the missing dopamine and alleviates most symptoms. There were also hints, from the work of the Swedish pharmacologist Arvid Carlsson, that the antischizophrenic actions of the class of drugs called the neuroleptics, exemplified by agents such as chlorpromazine and

haloperidol, might act by blocking dopamine receptors. Inability to measure dopamine receptors directly precluded an investigation of this hypothesis. Utilizing radiolabeled dopamine and haloperidol, we identified dopamine receptors by the same binding technology that worked with opiate receptors (Creese et al. 1975), findings obtained independently by Philip Seeman in Toronto (Seeman et al. 1975). We showed that the relative potencies of neuroleptic drugs in blocking dopamine receptors paralleled closely their potencies in relieving schizophrenic symptoms, establishing the mechanism of the antipsychotic actions of the drug. The "opportunism" involved in transferring opiate receptor technology to a variety of other receptors seems to be another manifestation of the Audacity Principle.

Man was not born with morphine in him. The fact that opiate receptors resembled neurotransmitter receptors suggested that there must exist opiate-like neurotransmitters. We were able to demonstrate that such substances exist (Pasternak et al. 1975), as did the Swedish pharmacologist Lars Terenius (Terenius and Wahlstrom 1975) and Scottish investigators John Hughes and Hans Kosterlitz (Hughes et al. 1975a). The Scottish group and our group developed approaches to isolating the substances and obtaining their chemical structure. In a brilliant opus the Hughes-Kosterlitz team reported in December 1975 the chemical structure of the two enkephalins, the first of the endorphins, neurotransmitters that mimic the actions of morphine and are major regulators of pain and emotional perception (Hughes et al. 1975b), findings we obtained as well soon thereafter (Simantov and Snyder 1976).

Conceptualizing that endogenous morphine-like substances should exist was important and reflected an appreciation of "the big question," but many scientists had come to similar conclusions. One needed the gumption to "do something." Moreover, divining how to transform the big question into small, soluble parcels was particularly critical.

Our lab and the Kosterlitz group designed very different ways of approaching the problem. As experts on opiate receptor binding, we looked in brain extracts for materials that would compete with radioactive opiates for binding to the receptors. A master of classical pharmacology, Kosterlitz took advantage of the known constipating actions of morphine, monitoring in a simple organ bath the ability of brain extracts to mimic the capacity of morphine to inhibit electrically induced contractions of the gut. With a simple system to measure the morphine-like activity of brain extracts, both groups could purify the enkephalins.

When we embarked on the search for the enkephalins, we already knew that Hughes and Kosterlitz had identified such substances and had made considerable progress in purifying them. Many scientists would

have shied away from the competition. They would not even have conceptualized a way of addressing the problem, for they would assume that others had already covered the ground. Our willingness to accept the challenge again reflects the Audacity Principle.

Gases as Neurotransmitters

In the late 1980s I read a paper in *Nature* by Salvador Moncada (Palmer et al. 1987) describing his experiments establishing conclusively that nitric oxide (NO) was the physiologic molecule that accounts for endothelial-derived relaxing factor activity. I was captivated by the elegance of the binding and wondered if nitric oxide might do something in the brain. First let me describe the background.

Robert Furchgott, a pharmacologist at Downstate Medical Center in Brooklyn, had been studying blood vessel relaxation. The classic neurotransmitter acetylcholine relaxes blood vessels. To study this process directly, Furchgott "cleaned" his blood vessels, removing the inner endothelial layer to provide direct access of acetylcholine to the underlying muscle. When he did this, acetylcholine no longer elicited relaxation. Reasoning that something from the endothelial layer was critical, he restored it, and the relaxation returned. He concluded that the acetylcholine must have triggered the release from the endothelium of a chemical that causes blood vessel relaxation, which he dubbed "endothelial derived relaxing factor (EDRF)" (Furchgott and Zawadzki 1980). For a number of years numerous investigators strove to identify the substance, but it seemed to be extraordinarily labile and to have a variety of peculiar features. Identification of EDRF as NO might never have taken place except for the convergence of different lines of research. The pharmacologist Ferid Murad sought to know how nitroglycerin potently relaxes blood vessels to elicit its therapeutic actions in cardiac angina. He demonstrated that nitroglycerin must first be converted to NO as an active metabolic product (Arnold et al. 1977). Louis Ignarro, a pharmacologist at UCLA, had been pursuing a similar line of investigation with similar conclusions. Simultaneously, his laboratory had been trying to identify EDRF. He had detected various similarities between EDRF and nitric oxide, such as antagonism of their actions by the dye methylene blue and their heme-dependent augmentation of the activity of the cyclic GMP-forming enzyme guanylyl cyclase. Emboldened by these clues, Ignarro devised experiments proving that EDRF and NO are identical in terms of their ability to influence contractions of a wide range of smooth muscle preparations as well as to influence the absorption spectrum of hemoglobin (Ignarro et al. 1987).

The Nobel Prize in Physiology and Medicine was awarded to Furch-

gott, Murad, and Ignarro in 1998. Their efforts reflect the Audacity Principle. When Furchgott carried out his work identifying EDRF, few biomedical scientists cared about the nuances of blood vessel relaxation. Furchgott's paper describing EDRF lacked any evidence for its chemical structure, which rendered the enterprise somewhat dubious. The search for the identity of EDRF, which consumed many years, was fraught with major hurdles in seeking a molecule so labile as NO. But for the link to nitroglycerin, the search might well have failed. Most scientists would have dropped the project in favor of one with a greater guarantee of short-term success.

When I read about NO, its remarkable properties spawned the fantasy that this molecule might do something in the brain. The British biochemist John Garthwaite had published a paper showing that brain cultures could synthesize a molecule with the properties of EDRF (Garthwaite et al. 1988). My M.D. Ph.D. student David Bredt and I decided that it was worth exploring the functions of NO in the brain. We took lessons from what was already known about blood vessels. NO relaxes blood vessels by stimulating formation of the second messenger molecule cyclic GMP formation. In the brain the major excitatory neurotransmitter, glutamic acid, stimulates the enzyme guanylyl cyclase, which makes cyclic GMP. We wondered whether NO was involved. We knew that NO is formed from the amino acid arginine and that arginine derivatives act as inhibitors of the enzyme. One of these arginine derivatives prevented cyclic GMP formation in proportion to its ability to block the formation of NO. Clearly NO somehow mediated the actions of glutamic acid, generally regarded as the most prominent neurotransmitter in the brain. This convinced us that we were dealing with an area of importance for brain function.

What were we to do? We knew it was important to isolate and clone the enzyme that converts arginine to NO, but numerous other laboratories had already failed in this endeavor. First, it was notoriously difficult to monitor the conversion of arginine to NO, since the labile NO is not readily detected by most techniques. Existing assays to monitor the activity of the NO synthase enzyme were insensitive and cumbersome, which would have precluded the large number of assays required for enzyme purification. We decided to measure the conversion of radiolabeled arginine to the amino acid citrulline, which is formed as a byproduct, and developed a simple ion exchange column procedure enabling us to conduct fifty assays in an hour.

Next was the challenge of purifying the enzyme. Why should we succeed where so many others had failed? In our first experiment we found what all other investigators had encountered. When one tried to purify a brain extract over an ion exchange column separating large

numbers of fractions, all enzyme activity vanished. The enzyme seemed hopelessly unstable. David wondered whether the enzyme was in fact stable, but the purification procedure had separated out a crucial cofactor. When he reconstituted the test tube fractions, enzyme activity reappeared, establishing that something had been lost in the purification process. How would we purify and isolate the mysterious cofactor? One could presumably pursue the laborious process of determining the substance's properties and trying to isolate it, which could take months. David chose instead to guess. He knew that calcium played some role in augmenting NO formation. Calcium interacts with a large number of proteins, but the best characterized is a small protein called calmodulin, required for the activity of many enzymes. David added calmodulin, and enzyme activity returned (Bredt and Snyder 1990b).

The discovery of calmodulin as a crucial factor for NO synthase clarified certain mysteries. Acetylcholine must trigger the formation of nitric oxide very rapidly to mediate the normally rapid blood vessel reactivity. If NO were a neurotransmitter, a similarly swift activation of its formation would be required with each nerve impulse. We knew already that neuronal firing is associated with an immediate influx of calcium, and acetylcholine augments calcium levels inside blood vessel cells. For NO to be a neurotransmitter, its biosynthetic enzyme would have to be activated every time NO-containing nerves fired, because, unlike classical neurotransmitters, NO, a gas, couldn't exist in large storage pools in synaptic vesicles. The link to calmodulin resolved these dilemmas. NO can be formed "on-line" with nerve or neurotransmitter activity, as the associated rapid calcium entry activates its biosynthetic enzyme.

With NO synthase stabilized by calmodulin, David was able to purify the enzyme protein to homogeneity, obtain its amino acid sequence, and clone its gene (Bredt et al. 1991). This led to the discrimination of three separate forms of the enzyme, a neuronal form, a blood vessel or endothelial form, and an inducible one that occurs in all cells of the body. Myriad advances in NO biology would not be possible without the cloned enzymes.

Establishing definitively that NO is a neurotransmitter did not employ the brain, so complex that it is the poorest preparation for clarifying neurotransmitter properties. The classic neurotransmitters acetylcholine and norepinephrine were all characterized in the peripheral nervous system many decades before their role in neurotransmission of the brain was established. In the case of NO, our ability to purify its biosynthetic enzyme enabled us to generate antibodies to NO synthase. With these antibodies we could visualize the enzyme by immunohistochemistry (Bredt et al. 1990a). First, we examined blood vessels and found the

enzyme highly concentrated in the endothelial layer, providing definitive evidence that this is the place where NO is generated in response to acetylcholine. In peripheral organs we found the enzyme localized to neuronal structures. In the intestine, NO synthase occurs in the myenteric plexus of neurons, which regulates the contraction and relaxation that underlie digestive peristalsis. Since the 1940s scientists tried unsuccessfully to implicate the classic neurotransmitters acetylcholine and norepinephrine in peristaltic relaxation. They concluded that some other neurotransmitter mediates what they referred to as Non-Adrenergic, Non-Cholinergic (NANC) relaxation. Several laboratories simply added NO synthase inhibitors and observed a substantial reduction in NANC relaxation, indicating a transmitter role for nitric oxide. Our cloning of the gene for neuronal NO synthase led to the generation of mice with targeted deletion "knockout" of the gene. We showed that NANC relaxation was reduced about 50 percent in these gene knockout mice, consistent with the findings using enzyme inhibitors.

Even more dramatic than intestinal neuronal localizations of the enzyme was David's observation that the cavernous nerves that project to the penis are extremely enriched in NO synthase. The cavernous nerve is required for penile erection. This led us to collaborate with Dr. Arthur Burnett, a colleague in the urology department. Burnett had developed an elegant system in which electrical stimulation of the cavernous nerves of intact rodents produces robust penile erection. NO synthase inhibitors abolished erection (Burnett et al. 1992). Interestingly, penile erection involves a process analogous to blood vessel dilation. Erection takes place when the smooth muscle of the venous sinuses of the penis relaxes, enabling them to become engorged with blood. As with conventional blood vessels, engorgement of penile venous sinuses involves cyclic GMP. Cyclic GMP is rapidly degraded by the enzyme phosphodiesterase (PDE), and PDE inhibitors elevate cyclic GMP levels. When publications appeared establishing NO as a neurotransmitter of erection, scientists at the Pfizer drug company resurrected one of their PDE inhibitor drugs, which had been ineffective in treating angina. Their clinical trials validated the drug as a treatment for erectile dysfunction, and it was subsequently marketed as Viagra.

The dramatic properties of NO as a gaseous neurotransmitter led to a seemingly simple but not necessarily obvious question: "Might there be other gaseous neurotransmitters?" Neurotransmitters come in chemical classes. First were the biogenic amines such as acetylcholine, norepinephrine, dopamine, and serotonin. Next were amino acid neurotransmitters such as gamma-aminobutyric acid (GABA) and glutamic acid, followed by peptides such as the enkephalins, substance P, cholecystokinin, and many others. What other gases should we explore as

possible neurotransmitters? Ethylene is a gas with important functions in plants, but we could find no evidence for a role in animal tissues. Ajay Verma, an M.D. Ph.D. student, suggested carbon monoxide (CO). He had noted in biochemistry textbooks that when the enzyme heme oxygenase degrades heme emerging from aging red blood cells, it breaks open the heme ring to form the green pigment biliverdin, which is rapidly reduced to the yellow pigment bilirubin. In the process, a one-carbon fragment is released as CO. Though this biochemical pathway had been well established for decades, neither I nor most of my colleagues were aware of it, presumably because nobody had ever given any thought to biological functions for CO.

We explored what was known of heme oxygenase (HO). The enzyme was first described in the spleen, where aging red blood cells congregate. Formation of the principal form of the enzyme is induced by heme and many other substances. We found a publication describing a second form of HO that emerged during purification of the classic form. This enzyme, designated HO2, was not inducible, and was hence of little interest to the hematologists who were the principal students of heme degradation. Ajay found HO2 highly concentrated in discrete areas of the brain in neurons with localizations closely resembling guanylyl cyclase, suggesting that CO, like NO, might normally stimulate cyclic GMP formation (Verma et al. 1993).

With antibodies to HO2, we visualized the enzyme by immunohistochemistry. In the intestine HO2 occurs in the same myenteric plexus neurons as neuronal NO synthase. Hence, NO and CO might well function as co-neurotransmitters. Definitive evidence that CO is a neurotransmitter came from our experiments with NANC relaxation of the intestine in HO2 knockout and/or neuronal NO synthase knockout mice. As mentioned already, the neuronal NO synthase knockout mice display a 50 percent decrease in NANC relaxation. The HO2 knockout mice also showed a 50 percent decline in the process (Zakhary et al. 1997). Subsequently we cross-bred the two gene knockout mice. Loss of both NO and CO generating systems led to virtual abolition of NANC relaxation. Thus, NO and CO are major neurotransmitters of the intestinal relaxation that mediates the peristaltic process so crucial for digesting food. Subsequent work has shown that NO and CO are likely neurotransmitters in multiple sites in the brain and throughout the body.

Many investigators would never have dreamed of suggesting that gases such as NO and CO might be neurotransmitters. Such a proposition would overturn myriad principles of neurotransmission as fundamental to the field as Newton's Laws are to physics. Here are some of the classical criteria for establishing a chemical as a neurotransmitter.

Neurotransmitters must be stored in synaptic vesicles and released by a process called exocytosis, in which the walls of the synaptic vesicles fuse with the plasma membrane of the cell spewing out the transmitter molecule. Indeed, Bernard Katz's Nobel Prize was awarded for his experiments showing that neurotransmitters are released in little packets or quanta that emerge from synaptic vesicles. Neurotransmitters are classically assumed to act upon specific receptor sites, proteins on the external membrane of the adjacent neurons. For neurons to respond to rapid firing patterns, there must exist large storage pools of the neurotransmitter molecule to prevent a neuron from losing the ability to respond to neuronal stimulation.

Clearly NO and CO overturn these "rules." Neither can be stored in synaptic vesicles; they must be resynthesized with each new nerve impulse. As indicated above, calcium influx associated with neuronal depolarization activates neuronal NO synthase. Fairly recently, we found that a similar process takes place for HO2. NO and CO don't act upon classic receptor proteins on adjacent neurons. Instead, they diffuse into the adjacent cell and bind to guanylyl cyclase, activating it to form more cyclic GMP. More recently, it has been established that NO can also act by chemically modifying target proteins via a process called S-nitrosylation of cysteines in the proteins.

When we embarked on our studies of NO and then of CO as neurotransmitters, we were aware that they did not fit established paradigms. We knew that anything we did in this area would therefore be met with great skepticism, if not derision. However, each experiment led to another with an inexorability that was irresistible. Such "disregard" for what others might think presumably reflects the Audacity Principle.

Conclusion

Perhaps the most audacious aspect of this essay is my chuzpah in titling it "The Audacity Principle." Many distinguished students of the scientific discovery process have conducted scholarly studies, while my presentation is anecdotal and derived largely from personal experience. I do not claim to have identified the most crucial talent underlying important scientific advances. Rather, I felt it of interest to provoke thinking about the various factors that separate the scientific "men from boys." Clearly the mentor-apprentice relationship is paramount. Scientists do not learn originality from a book any more than do artists and composers. Innate gifts are equally important, for only a small percentage of the students of great scientists manifest comparable greatness. The essence of those gifts is hard to divine, but originality, simplicity,

and audaciousness overlying a bedrock of intellect seem to be consistent ingredients. Blending these qualities in just the right proportions appears to do the trick. I have emphasized audacity, because, in its absence, the other qualities fail. Needless to say, audacity on the part of unintelligent, uncreative individuals is disastrous. However, in the right mix, audacity can make all the difference.

References

Arnold, W. P., C. K. Mittal, S. Katsuki, and F. Murad. 1977. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proc. Natl. Acad. Sci. U.S.A. 74: 3203–07.

Axelrod, J. 1988. An unexpected life in research. Ann. Rev. Pharmacol. Toxicol. 28: 1–23.

Bredt, D. S., P. M. Hwang, C. E. Glatt, C. Lowenstein, R. R. Reed, and S. H. Snyder. 1991. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 351: 714–18.

Bredt, D. S., P. M. Hwang, and S. H. Snyder. 1990a. Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 347: 768–70.

Bredt, D. S., and S. H. Snyder. 1990b. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. U.S.A. 87: 682–85.

Burnett, A. L., C. J. Lowenstein, D. S. Bredt, T. S. Chang, and S. H. Snyder. 1992. Nitric oxide: a physiologic mediator of penile erection. Science 257: 401–03.

Creese, I., D. R. Burt, and S. H. Snyder. 1975. Dopamine receptor binding: differentiation of agonist and antagonist states with 3H-dopamine and 3H-haloperidol. Life Sci. 17: 933–1001.

Furchgott, R. F., and J. V. Zawadzki. 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288: 373–76.

Garthwaite, J., S. L. Charles, and R. Chess-Williams. 1988. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 336: 385–88.

Hughes, J., T. Smith, B. Morgan, and L. Fothergill. 1975a. Purification and properties of enkephalin—the possible endogenous ligand for the morphine receptor. Life Sci. 16: 1753–58.

Hughes, J., T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, and H. R. Morris. 1975b. Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature 258: 577–80.

Ignarro, L. J., G. M. Buga, K. S. Wood, R. E. Byrns, and G. Chaudhuri. 1987. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. U.S.A. 84: 9265–69.

Kanigel, R. 1993. *Apprentice to Genius*. Baltimore, Md.: Johns Hopkins University Press.

Kuhar, M. J., C. B. Pert, and S. H. Snyder. 1973. Regional distribution of opiate receptor binding in monkey and human brain. Nature 245: 447–50.

Palmer, R. M., A. G. Ferrige, and S. Moncada. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327: 524–26.

Pasternak, G. W., R. Goodman, and S. H. Snyder. 1975. An endogenous morphine-like factor in mammalian brain. Life Sci. 16: 1765–69.

Pert, C. B., and S. H. Snyder. 1973. Opiate receptor: demonstration in nervous tissue. Science 179: 1011–14.

Seeman, P., M. Chau-Wong, J. Tedesco, and K. Wong. 1975. Brain receptors for antipsychotic drugs and dopamine: direct binding assays. Proc. Natl. Acad. Sci. U.S.A. 72: 4376–80.

Simantov, R., and S. H. Snyder. 1976. Morphine-like peptides in mammalian brain: isolation, structure elucidation, and interactions with the opiate receptor. Proc. Natl. Acad. Sci. U.S.A. 73: 2515–19.

Terenius, L., and A. Wahlstrom. 1975. Search for an endogenous ligand for the opiate receptor. Acta Physiol. Scand. 94: 74–81.

Verma, A., D. J. Hirsch, C. E. Glatt, G. V. Ronnett, and S. H. Snyder. 1993. Carbon monoxide: a putative neural messenger. Science 259: 381–84.

Zakhary, R., K. D. Poss, S. R. Jaffrey, C. D. Ferris, S. Tonegawa, and S. H. Snyder. 1997. Targeted gene deletion of heme oxygenase 2 reveals neural role for carbon monoxide. Proc. Natl. Acad. Sci. U.S.A. 94: 14848–53.

Zuckerman, H. 1996. *Scientific Elite*. New Brunswick, N.J.: Transaction Publishers.