Molecular Neurobiology

Past, Present, and Future

Solomon H. Snyder

Departments of Neuroscience, Pharmacology, and Molecular Sciences, and Psychiatry and Behavioral Sciences
The Johns Hopkins University School of Medicine
725 North Wolfe Street, Baltimore, MD 21205

Introduction

In providing a perspective of molecular neurobiology, one need not tarry overly long in reviewing past history, since, depending on one's perspective, the past is only a handful of years ago. In medical school and throughout my subsequent medical training, genetics was sorely neglected. There seemed no need to devote much attention to medical genetics, since genetic diseases seemed restricted to a few very uncommon conditions that were usually not treatable. Along with the rapid advances in molecular biology has come an appreciation that many of the most common diseases possess major genetic determinants. For instance, Alzheimer's disease is probably the most common brain disorder in the world. All sorts of etiologies have been propounded, ranging from viruses that enter the brain through the nose to toxic metals, such as aluminum. Genetic studies now indicate a pronounced genetic predisposition toward Alzheimer's disease. When one considers that the incidence of this disorder increases at a rapid rate with advancing age, its age-corrected theoretical incidence may be as high as 25%. Several workers now feel that a diathesis for Alzheimer's disease is transmitted in a genetically dominant pattern. Evidence for genetic propensity toward schizophrenia and affective disease is overwhelming. Thus, if one approaches molecular neurobiology as the study of the nervous system, with a view to understanding the causes and possible therapies of human disease, then there is ample justification to focus particularly on molecular genetics.

Neurotransmitter Receptors

Molecular genetics is rapidly pervading all of neurobiology. Drug and neurotransmitter receptors have not escaped. When we first investigated neurotransmitter receptors by ligand-binding techniques, a number of colleagues advocated that we move rapidly toward solubilization and purification of these membrane proteins. The reasoning was that one could thereby clone the genes for the receptor. At that time it was not clear just what one could learn from

such an enterprise. Recent receptor characterization by numerous laboratories has revealed many surprises. The most extensive work has emerged from investigations of the nicotinic acetylcholine receptor of fish electric organs. The work of several groups, but most especially that of Numa, has shown that the acetylcholine recognition site and the associated sodium channel are part of the same protein molecule. This finding was somewhat unexpected, since one would have thought that Nature would prefer to sculpt acetylcholine-recognition sites and ion channels separately for different purposes and then allow the distinct proteins to work together in some sort of allosteric fashion.

By eliciting molecular mutations of the receptor in precisely defined sequences, researchers are now clarifying exactly which portions of the molecule mediate specific synaptic actions. There are many questions to be asked, such as where local anesthetics act to alter synaptic function. Similarly, investigations of receptor phosphorylation have provided insights into cholinergic receptor desensitization. Strikingly, in the electric organ receptor one can detect endogenous tyrosine kinases that phosphorylate the acetylcholine receptor, linking this receptor protein with the many other targets of oncogene-coded tyrosine kinases.

The nicotinic acetylcholine receptor is so abundant, thousands of times more concentrated in the electric organ than are most neurotransmitter receptors in the brain, that one worries whether precedents established with the acetylcholine receptor can be translated to other systems. Very recently it has been possible to clone genes for other neurotransmitter receptors. Particular success has come with studies of beta-adrenergic receptors, based on the work of Lefkowitz at Duke University and a variety of researchers at the Merck and Genentech Companies. Properties of the cloned beta recepters provide a number of novel insights. The structure of beta receptors closely resembles that of rhodopsin, indicating a link between the recognition of light by the eye and of neurotransmitters by synaptic receptors. Of course, both beta receptors and rhodopsin interact with GTP-binding proteins. Indeed, one would anticipate close similarities between the intracellular portion of all G-protein-associated receptors. Surprisingly, the two distinct beta receptors that have been cloned and rhodopsin display the greatest homology in the membrane-spanning region and not in the intracellular domain where interactions presumbly take place with G proteins. Even more unexpected are the extracellular domains of the two beta receptors. To recognize catecholamines, presumably, they should be very similar. Yet, they resemble each other less than do the membrane-spanning regions.

The very recent cloning of genes for muscarinic cholinergic receptors reveals an extremely close resemblance to the structure of beta receptors. Indeed, muscarinic and beta receptors are about as similar as the two subtypes of beta receptors. Acetylcholine and the catecholamines are thought of as very different neurotransmitters so that the close similarity of their receptors is perhaps counterintuitive. On the other hand, both are associated with G proteins. Moreover, norepinephrine and acetylcholine are the major transmitters of the autonomic nervous system. The receptor similarities suggest that the entire autonomic nervous system evolved in a unitary fashion and that muscarinic and cholinergic functions separated later in evolution. This notion would accord with the numerous demonstrations that cholinergic and adrenergic properies are interchangeable in developing autonomic neurons. Such a concept also predicts that alpha-adrenergic receptors will turn out to resemble beta and muscarinic receptors.

Elegant studies from the laboratory of Barnard have resulted in the isolation of the GABA–benzodiazepine receptor complex and quite recently in the cloning of the genes for this receptor complex (Burt, personal communication). One might anticipate valuable insights. Conventional ligand-binding techniques have

revealed a number of distinct recognition sites on this receptor. Benzodiazepines, barbiturates, and GABA all bind to distinct but allosterically interacting sites. This explains the pharmacologic evidence that barbiturates and benzodiazepines act to facilitate the synaptic effects of GABA. Yet another site on this receptor presumably interacts with ethyl alcohol. Recent research reveals that one of the benzodiazepines developed by the Roche Drug Company, Ro15-4513, blocks the effects of alcohol. These actions involve a subtype of a benzodiazepine receptor at which Ro15-4513 must act as some sort of "partial" inverse agonist. Thus, pure benzodiazepine antagonists, such as Ro15-1788, reverse the alcohol blocking effects of Ro15-4513. Other drugs, usually of a carboline structure, are said to be inverse agonists at benzodiazepine receptors because they cause profound anxiety responses in humans and animals that are antoagonized by Ro15-1788. The term 'inverse agonist' refers to the fact that the anxiety-relieving effects of drugs such as diazepam represent "conventional agonist" effects at the receptor and are blocked by Ro15-1788. The molecular genetics of a GABA–benzodiazepine receptor should considerably enhance our understanding of the relationship of the various drug recognition sites and the nature of agonism, antagonism, and inverse agonism.

One of the principal puzzles of opiate receptors that might be clarified by molecular genetic approaches relates to receptor subtypes. The differences in opiate structures that gave rise to varying types of opiate effects are quite subtle. Indeed, on the basis of drug effects in intact animals and humans, many investigators were skeptical that there really exist different receptor subtypes. Instead, one might postulate only a single opiate receptor, with differences in clinical effects of certain opiates involving sites other than opiate receptors, Indeed, this appears to be the case for the psychotomimetic effects of opiates that involve sigma receptors, presumably unrelated in a fundamental sense to opiate receptors. In ligand-binding studies, differences in the drug selectivity of the various receptor sites are also sufficiently minor that for several years a number of researchers, including myself, felt that one could account for these differences on the basis of a single receptor with several binding sites. A given drug or enkephalin-related peptide might interact with all or only some of the attachment points, thereby producing a distinctive binding profile. The elegant studies of Kosterlitz, combining binding and bioassay, established definitively the existence of distinct mu-, delta-, and kappa-receptor subtypes. In addition to these three, well-established receptors, several other receptor subtypes have been characterized and may also represent separate molecular entities. As of this writing (early 1987), several laboratories have come close to completing the cloning of genes for some subtypes of opiate receptors. It may be hoped that a full characterization of opiate receptor genes will reveal the molecular basis for receptor subtypes as well as sites of intereractions with G proteins.

Molecular Neuroanatomy

Ligand-binding techniques used first with tissue homogenates to characterize neurotransmitter receptors have been employed to image receptors by light microscopic autoradiography. The resultant localization of many receptors for neurotransmitters and drugs has assisted greatly in our understanding of how drugs exert particular pharmacologic effects. For instance, the localization of opiate receptors to areas of the brain involved in emotional regulation, pain perception, pupillary diameter regulation, and respiratory centers, can explain most of the major pharmacologic actions of opiate drugs.

Techniques that were employed successfully for neurotransmitter-receptor autoradiography have been extended to other types of ligands

relevant to neurobiology. In our own laboratory extensive use has been made of these techniques for investigating second-messenger systems. Forskolin binds with nanomolar affinity to adenylate cyclase. Autoradiography of [^{3}H]-forskolin bound to brain slices permits a delineation of the principal areas utilizing the adenylate cyclase system. Although adenylate cyclase occurs all over the brain, the highest densities by far are in the descending striatonigral pathway. The extremely high concentration of adenylate cyclase in these neurons reflects a linkage to dopamine D_1 receptors on caudate neurons associated with the dopamine-sensitive adenylate cyclase.

Some of the most impressive new insights into second-messenger systems have employed autoradiographic techniques to localize elements of the phosphoinositide cycle (PI). The tumor-promoting phorbol esters bind with nanomolar affinity to protein kinase C and can be employed as ligands to image this enzyme. Protein kinase C also occurs in many parts of the brain, but with extremely high densities in specific localizations, such as the Purkinje cells of the cerebellum, the pyramidal cells of the hippocampus, and the descending striatonigral pathway. The PI cycle comprises two "arms." Hydrolysis of phosphatidylinositol-bisphosphate (PIP_2) by phospholipase C gives rise to diacylglycerol, which stimulates protein kinase C to constitute one of the arms. The other product of PIP_2 hydrolysis is inositoltrisphosphate (IP_3), the other arm of the PI cycle. By binding to specific receptor sites on the endoplasmic reticulum, IP_3 is thought to mobilize intracellular calcium. This calcium may facilitate the phosphorylation by protein kinase C of various substrates. An abundance of evidence suggests that some cells utilize only one or the other arm of the PI cycle. Worley, Baraban, and I were able to identify IP_3 receptors both biochemically and by autoradiography. The map of IP_3 receptors is extremely similar to that of protein kinase C, with certain exceptions. Thus, the substantia gelatinosa of the spinal cord and the olfactory bulb possess intense bands of protein kinase C, but no IP_3, whereas the Purkinje cells of the cerebellum and the pyramidal cells of the hippocampus are enriched in both elements of the PI cycle. These differences in localization fit with evidence accumulating that some cells utilize only one or the other arm of the PI cycle.

G proteins are emerging as crucial second messengers in numerous systems. They link many receptors to adenylate cyclase. G proteins also are required for the phospholipase C activity of the PI cycle. Some G proteins appear to be linked directly to ion channel activation. In the eye, transducin, a G protein, is crucial for cyclic GMP hydrolysis in photoreception. The exact role of several other G proteins is not clear. The identity of the G protein involved in the PI cycle is somewhat uncertain, but molecular neuroanatomical techniques provide strong hints. An immunohistochemical map of the distribution of the major G protein in the brain, G_0, by Worley, Baraban, Van Dop, Neer, and me reveals a close association of G_0 localizations in the brain with the localization of protein kinase C.

In situ hybridization promises to be a particularly powerful technique of molecular neuroanatomy. In this procedure various types of nucleotide probes, comprising 20–30 bases, can be synthesized based on the partial amino acid sequence of a protein. Alternatively, if gene cloning has already taken place, a much longer ribonucleotide probe can be employed. Since the rate of peptide and protein synthesis is determined by levels of messenger RNA, one can obtain an index of biosynthetic rates for peptides and proteins by monitoring levels of messenger RNA. Already many instances are known in which drug treatments or pathophysiologic alterations do not affect concentrations of a peptide, such as enkephalin, but provoke marked changes in messenger RNA levels. One can measure messenger RNA by biochem-

ical techniques such as the "dot blot." *In situ* hybridization adds another dimension, namely microscopic localization. The importance of *in situ* hybridization for dynamic studies lies in the likelihood that alterations in the turnover of a particular neuropeptide or protein might take place in only one of a number of peptide- or protein-containing pathways.

At the present state of the art, *in situ* hybridization is not efficient in detecting messenger RNA and may reveal only a few percent of the total messenger RNA content for a particular peptide or protein. Accordingly, the greatest success has come with peptides whose messenger RNA levels are abundant, as is the case for vasopressin. Several laboratories have imaged vasopressin messenger RNA in the supraoptic and paraventricular nuclei and have shown dramatic changes in messenger RNA level as vasopressin turnover is altered by procedures such as dehydration.

In situ hybridization can also be used to image elements of second messenger systems, such as G proteins. Based on the conservation of the GTP recognition site among many GTP binding proteins, it has been possible to clone genes for a number of G proteins, including several for which the protein itself has not yet been isolated. The exact functions of the half dozen or more presently recognized G proteins is not clear. Moreover, it seems likely that within a few years one- or two-dozen G proteins will be identified. *In situ* hybridization may provide a rapid route to identifying function. Mapping the messenger RNA for a given G protein will establish its coincidence with known second messenger systems, such as the PI cycle or adenylate cyclase, and its link to various neurotransmitters.

In situ hybridization can be employed for other problems in neurobiology. Tubulin is a major axonal protein whose turnover changes dramatically as neurons are destroyed and regenerate. In our laboratory, Pearson has conducted *in situ* hybridization of tubulin messenger RNA to clarify the neuronal regeneration process. Lesions of the hypoglossal nerve are followed by its gradual regeneration. New protein synthesis in the hypoglossal nucleus of the lower brainstem is crucial for this process, and tubulin would be expected to be particularly prominent as a major axonal constituent. Thus, it has not been surprising to find that hypoglossal nerve lesions on one side of a rat's neck are followed by massive increases in messenger RNA in the ipsilateral hypoglossal nucleus. In this way, *in situ* hybridization provides a technique for evaluating the dynamics of turnover of a major protein as a function of neuronal regeneration and links messenger RNA turnover to changes in the growth of the axon itself.

This brief review has perforce skimped in many areas. Molecular neuroendocrinology has been a particularly productive field. Cloning of the genes to pro-opiomelanocortin, the precursor of ACTH and beta endorphin, was one of the first applications of molecular biology to neuropeptides. Regulation of peptide hormone synthesis and the actions of numerous hormones at their receptors have been clarified greatly by molecular genetics. Particularly exciting has been the recent application of molecular genetics to neurodegenerative diseases. Identification of a molecular genetic marker for Huntington's disease has been followed by similar advances in muscular dystrophy and cystic fibrosis. Many laboratories are now attempting to utilize these techniques to seek the genetic abnormalities in Alzheimer's disease, schizophrenia, and depression.

Though I have focused on molecular biological strategies, gene cloning is by no means the be-all and end-all of molecular neurobiology. Indeed, molecular biology should be regarded only as a body of techniques to provide us with insights into the fundamental functions of neurons, glia, and other elements of the nervous system.

Acknowledgments

Supported by USPHS grants DA-00266, NS-16375, MH-18501, and Research Scientist Award DA-00074.