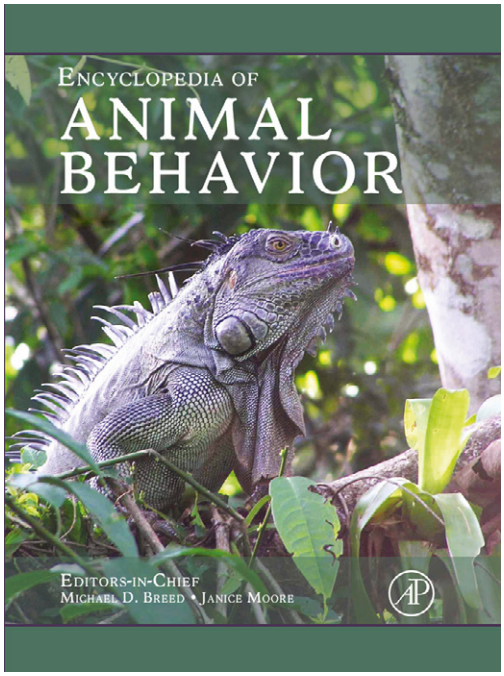

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Neuroethology: Methods

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Introduction

As articulated by Tinbergen in his 'four questions,' a complete understanding of behavior requires an understanding of it at multiple levels of analysis. Neuroethology represents the effort to understand the neurobiology of behavior, what Tinbergen called the causation of behavior. Neuroethologists typically work on a variety of animals, using the natural talents of particular organisms to investigate the basic principles of neurobiology. For example, to understand how the auditory system decodes the location of a sound source, neuroethologists turn to animals that are well adapted to locate sound, such as the barn owl, which relies on acoustic cues to find prey when hunting at night. Using this approach, neuroethology has been very successful in uncovering basic principles of neurobiology. But why should the behavioral ecologist be interested in the findings of the neuroethologist? Understanding the neural mechanisms of behavior not only gives us a more complete understanding of behavior, but can also inform our perspective on behavioral evolution by determining the sensory, cognitive, or motor constraints on the evolution of behavior.

Because neuroethologists are interested in the mechanisms of natural behavior (as opposed to clinically relevant behavior), they address questions that are relevant to the natural history of the organism under study. For example, when neuroethologists ask '*how do animals perceive the world?*' they use behaviorally relevant stimuli to, for example, determine how the toad's visual system discriminates prey from predator. When neuroethologists ask '*how is motor output generated?*' they investigate behaviors that are intimately tied to natural history, such as flight in locusts. Finally, neuroethologists are interested in the plasticity of these mechanisms across different time scales, as plasticity is a major source of individual variation. In temperate breeding songbirds, for example, the neural circuit controlling song may vary dramatically across the year, which helps explain why males are more vociferous in the spring. Even more broadly, experiences of all kinds are encoded by the nervous systems to shape future behavior, as has been elegantly demonstrated in the sea slug, *Aplysia californica*. In some cases, neuroethologists put these questions into an evolutionary perspective in order to better understand the evolution of behavior and its mechanisms. Doing so allows neuroethologists to address the question, *why do individuals or species differ in their behavior?* For example, why are prairie voles monogamous when montane voles are not? These are just some examples of the classic models in neuroethology. In many of these cases, neuroethologists used electrophysiological recordings, electrical stimulation, and lesions to determine the causal relationship between neural activity and behavior. These techniques are still invaluable to neuroethological studies, but they have been augmented in recent years by advances in molecular biology and computational biology.

Advances in Molecular Biology

Technical advances in molecular biology have influenced all aspects of biological research, and neuroethology is no exception. The molecular neuroethologist is typically interested in the genes that are expressed in the nervous system either during development or in adulthood. Understanding genetic differences among individuals or species is an important way of addressing the question, *why do individuals or species differ in their behavior?* In addition, measuring changes in gene expression that are associated with behavior is an important approach to understanding all aspects of the neurobiology of behavior. Both approaches depend, at some point, on knowledge of the relevant gene sequences. For neuroethologists, this can be a challenge. However, recent advances in sequencing technology have made these types of data more accessible than before.

High-Throughput Sequencing

In spite of the explosion of genome sequencing (www.genome.gov), scientists have successfully sequenced the genome of only a fraction of the species under study. Government agencies and institutes typically choose species because they have small genomes and because many scientists have chosen to work on them to answer a particular class of questions. These selection criteria systematically exclude many of the species of interest to neuroethologists, as neuroethologists select their study organisms for very different, often idiosyncratic, reasons (see Introduction). Recent advances in sequencing technology, however, have made large-scale sequencing efforts much more affordable. Thus, it is now feasible for a single laboratory to sequence a genomic or cDNA library of their study organism.

Genomic libraries and cDNA libraries provide different types of information. A cDNA library is a collection of cloned DNA sequences that are complementary to the

mRNA that was extracted from an organism or tissue (the 'c' in cDNA stands for 'complementary'). Thus, the cDNA library represents a so-called transcriptome – that is, a collection of transcribed, or expressed, genes. As such, transcriptomes are tissue and state specific; for example, the transcriptome of a singing bird's brain would be different from the transcriptome of a quiet bird. A database of known cDNA sequences is an invaluable tool in studies that manipulate or measure gene expression. In contrast, a genomic library is a collection of cloned pieces of an organism's genome. As such, a genomic library is organisms-specific and does not vary with the tissue or state of the animal. A genomic library provides information about gene sequences, including regulatory regions that determine when and where a gene is expressed. Genomic libraries are useful tools when a neuroethologist is interested in species differences in gene sequences.

Comparative Gene Analysis

Many neuroethologists are interested in the evolution of behavior, and a powerful way to address this goal is to compare behaviorally relevant genes among closely related species that differ in behavior, or among distantly related species that are convergent in behavior. Neuroethologists can determine gene sequences from a genomic library, or by using PCR. In some cases, selection has acted on the coding region of genes, resulting in changes in the structure and function of proteins. For example, comparative analysis of coding sequences demonstrated that the independent evolution of electric communication signals in two lineages of fish has been accompanied by convergent evolution in the structure of the sodium channels that are important in producing the electric signal. In other cases, selection has acted on the regulatory regions of genes, modifying their expression patterns. For example, differences in the regulatory region of the vasopressin receptor gene results in distinct distribution of the receptor in the monogamous prairie vole compared to the polygamous montane vole, although the receptor itself is identical.

Gene Expression Analysis

One important way we understand brain–behavior relationships is to understand how variation in gene expression relates to variation in behavior within individuals, among individuals, and among species. Although two individuals (or species) may have similar genes, variation in the expression of those genes can have profound impacts on the function of neurons and, therefore, on behavior. Relating gene expression patterns to behavior is facilitated by an understanding of the underlying gene sequences.

A variety of tools are available for quantifying gene expression. Two of the most versatile and widely used are microarray analysis and quantitative reverse transcription PCR (RT-PCR), sometimes called real time RT-PCR. RT-PCR uses primers (short sequences of DNA) to amplify a target sequence from a cDNA pool that represents the genes that were transcribed in the tissue sample. RT-PCR is very sensitive and can detect the presence of very low abundance gene transcripts because the target DNA sequence increases exponentially during the PCR reaction, meaning that it doubles with every cycle. Variation in the number of transcripts in the sample will produce variation in the time at which amplification is detectable; greater numbers of initial transcripts will result in earlier amplification. For example, a tenfold difference in transcript concentration will result in a difference of three cycles in the RT-PCR reaction. Thus, in quantitative RT-PCR, the main interest is the initial cycle number that results in detectable amplification. In order to detect that initial cycle (usually called the cycle threshold), dyes that increase in fluorescence proportionally with the amount of the target DNA are added to the reaction. To use quantitative RT-PCR to measure changes in gene expression, one needs to know the sequence of the gene of interest. In addition, because this is a 'candidate gene' approach, meaning that the researcher has a specific hypothesis about a change in expression of a particular gene, it is typically used to detect changes in expression for a small number of known genes. A complementary approach to measuring changes in gene expression is to use a microarray, which is particularly suited to gene discovery.

In a microarray, or gene chip, tiny spots of DNA are attached to a solid surface, typically a glass slide, in an array. To detect differences in gene expression with a microarray, one hybridizes the array with cDNA synthesized from RNA extracted from experimental tissue samples (e.g., individuals before and after displaying a particular behavior). Before hybridization, one labels the two contrasting samples with different fluorescent dyes and then mixes them in equal volumes. This mixture is then incubated with the array where the two samples compete for hybridization with the DNA spots deposited on the slide. For example, if sample A, labeled with a red dye, has more of a particular transcript than sample B, labeled with a green dye, then the result of the hybridization will be more red dye associated with that transcript on the array. In many cases, these DNA sequences have been previously characterized and they might have been selected from a larger set for a particular experiment. But neuroethologists working on unusual animals may not have this luxury. In such cases, the DNA sequences on the array do not have to be previously characterized. If strong differences in hybridization are found with an array, the experimenter can then return to the identified

DNA sequences to determine their identity and characterize them more thoroughly. In either case, the combination of high-throughput sequencing of a cDNA library and a microarray is a powerful way to discover new genes that are associated with particular behaviors.

Manipulating Gene Expression

Quantitative RT-PCR and microarrays are useful for associating changes in gene expression with changes in behavior. However, because the expression of behavior, itself, can cause changes in gene expression in the behaving animal, it is important to go beyond correlations when testing hypotheses about the causal relationship between genes and behavior. Traditionally, this was accomplished by 'knocking out' a gene in a laboratory mouse. In this approach, a particular gene is silenced in a clonal line of mice. This continues to be an important tool for neuroscientists, but it does not lend itself to investigations of brain–behavior relationships in natural populations of animals. A number of novel approaches now allow neuroethologists to manipulate the presence or absence of genes without the genetic tools of the traditional laboratory mouse model. Two powerful ways to manipulate the expression of genes include the use of viral vectors to introduce novel genes and RNAi to silence the expression of native genes. A key advantage to both techniques is that they can be site-specific. That is, unlike whole-organism knock-outs, these approaches manipulate gene expression of specific brain regions while leaving others intact. This is a key innovation given the complexity of neural tissue because a particular gene product may regulate different behaviors depending on the neural circuit where it is expressed.

If a neuroethologist finds that expression of a particular gene is associated with a particular behavior, he or she may want to silence the gene to determine whether it is a causal factor in the behavior. A relatively simple way of silencing genes in vivo is to capitalize on a cell's RNA regulatory machinery. The RNA interference (RNAi) pathway regulates the likelihood that an RNA molecule will be translated into protein. One way to activate the RNAi pathway is to inject double-stranded RNA corresponding to the target gene into a particular brain region. The double-stranded RNA recruits the RNA regulatory machinery, resulting in the degradation of the target mRNAs, thus leading to gene silencing. Alternatively, a neuroethologist may want to introduce a novel gene, increase expression of a gene, or introduce a native gene into a novel location. One way of doing so is to use viral vectors that insert a gene of interest into the cells of a target brain region. Such viral vectors are engineered to carry the candidate gene and they capitalize on the ability of viruses to deliver their genetic material into foreign cells. This is a particularly useful tool when a neuroethologist wants to test a hypothesis related to behavioral variation between closely related species. In an elegant example of this approach, neuroethologists used a viral vector to introduce a prairie vole gene for the vasopressin receptor into the brains of montane voles to demonstrate that the receptor's unique distribution in prairie voles is an important cause of species-differences in social behavior.

Novel Approaches to Identifying Neural Circuits

The central nervous system is one of the most complex of all organs and understanding its structure is a fundamental endeavor in neurobiology. This is of particular interest to neuroethologists since nervous system structure can vary dramatically among species and such variation is an important cause of species differences in behavior. A major goal of neuroanatomical studies is to determine how individual brain regions are interconnected. In addition, studies that investigate the function of neural circuits during specific behaviors are an important complement to studies of nervous system structure, as brain regions or neural circuits can contribute differentially to different behaviors.

Novel Neural Tracers

Most of what we know about the neural connections among brain regions comes from studies that used lesions, which can identify connections by subsequent degeneration of axonal fibers, or the introduction of tracers. A tracer can be any substance that is taken up by one part of a neuron and transported to another part. Some substances are transported retrogradely (from axon to cell body), anterogradely (from dendrites or cell body to axon), or both. Tracers vary in their effectiveness depending, in part, on how efficiently neurons take them up, and on the method used to visualize them.

There are two major constraints to traditional neural tracers. First, typically only large, robust connections can be identified with discrete injections. Second, only a single set of connections can be identified in a single animal. The application of self-amplifying, transneuronal tracers, such as pseudorabies virus, can potentially solve both these problems. In tracing studies, an attenuated form of the virus is typically used. The pseudorabies virus is taken up by axonal terminals and transported to the cell body where it is replicated. The replication of the virus effectively amplifies the signal and, ideally, results in all neural connections being identified with similar probabilities. Once replicated by the cell, the virus is distributed throughout the dendrites where, importantly, it crosses synapses to subsequently infect connected cells. This process is repeated and, with enough time, should identify

the entire neural network connected, ultimately, to the brain region where the initial injection of virus was made. The pseudorabies virus can be made cell type-specific with genetic modifications that make its expression conditional on the type of neuron within which it is expressed. For example, neuroethologists used this approach to map the afferent network of neurons expressing GnRH, a releasing hormone that regulates reproductive physiology through its action on the pituitary. One constraint on the use of the pseudorabies virus in tracing studies is that it must be infectious in the animal being studied.

Functional Activity Mapping

An important complement to neural tracing studies, which reveal the structural connections among brain regions, are studies that investigate the activity of brain regions during the expression of a behavior or in response to behaviorally relevant stimuli. Most of what we know about nervous system function comes from studies that record electrical activity of neurons in anesthetized or restrained animals. A disadvantage of electrophysiology is that it requires the implantation of electrodes, which constrains the types of behaviors an animal can engage in during recording. In addition, researchers are typically able to study only one or a small number of brain regions at any given time. An alternative to electrophysiology is functional activity mapping, which uses markers that are correlated with neural activity to analyze the pattern of neural activity in multiple brain regions simultaneously. A range of markers are available, including endogenous metabolic markers, such as the mitochondrial enzyme cytochrome oxidase, exogenous metabolic markers, such as radioactively labeled glucose, and endogenous changes in the expression of genes or protein.

Recently, there has been an explosion in studies that utilize changes in expression of genes or proteins that are correlated with neural activity. These studies capitalize on the so-called immediate-early gene response of neurons. The immediate-early gene response was first described when researchers discovered that, in response to external stimulation, cells launch a rapid increase in gene expression that is followed by a wave of protein expression. Further studies showed that expression of immediate-early genes is controlled by cellular signaling cascades that are, in turn, regulated by changes in membrane depolarization. Thus, when neuroethologists detect an increase in immediate-early gene expression (or their protein products), they infer that those cells or brain regions have been recently activated. This approach has proved to be very fruitful, particularly because one can measure changes in neural activity of many brain regions at the same time. In addition, this technique is sometimes more readily adaptable to a variety of organisms than is electrophysiology. However, a major constraint on the interpretation of these studies stems from the time course of changes in immediate-early gene expression. For example, changes in gene expression can typically be detected within minutes and peak levels of gene expression are detected 30 min to 1 h after stimulus onset. Thus, functional activity mapping is very useful for identifying functional attributes of individual brain regions, but is not typically suitable for identifying the underlying neural code.

Advances in Electrophysiology

Neurons communicate with electrical signals, so it should be no surprise that electrophysiology is one of the most important tools of neuroethologists. Electrophysiology is a versatile tool and is favored by many neuroethologists because of its temporal precision. Given the constraints of electrophysiogical recording, most in vivo studies use anesthesia or paralytic chemicals to restrain the animal during recording. In addition to the obvious disadvantage to neuroethologists of working on an animal that cannot move, the use of anesthesia or chemical restraint may pose a more fundamental problem, as neural activity sometimes varies according to the animal's state. For example, parts of the songbird pallium show robust auditory responses to song when the bird is asleep, but no responses when the animal is awake. In addition, in most electrophysiology studies, neuroethologists record from one neuron at a time. Since many functions of the nervous system are likely carried out by ensembles of neurons, rather than individual neurons, this approach may fail to reveal the underlying coding mechanisms. Recent advances in electrophysiology now allow neuroethologists to record from awake, freely behaving animals. The head-mounted equipment is much smaller than before, allowing researchers to work on a wider variety of animals, and the use of commutators or telemetry systems also allows the animal to move about during recording. In addition, the use of multielectrode arrays has facilitated the analysis of networks of neurons. The ability to record from multielectrode arrays has been enabled by advances in electronic technology as well as in the computational methods required to analyze the larger and more complex data sets. Finally, electrophysiology studies have been advanced conceptually by the integration of information theory, a field of mathematics that, when applied to electrophysiology data, can quantify how much information is encoded by a neuron's response.

Advances in Computational Biology

Computational biology represents the integration of computer science, mathematical modeling, and statistics to solve biological problems. As such, its reach is substantial

and highly diverse. Within the field of neuroethology, computational approaches have had an impact in molecular biology, particularly in large-scale sequence analysis and microarray analysis, and in electrophysiology, where computational approaches have facilitated analysis of multielectrode recordings. In addition to advancing these fields, computational biology has also generated new fields of special significance to neuroethologists, namely, computational neuroethology and artificial neural network modeling.

Artificial neural networks are mathematical models that simulate biological neural networks, or nervous systems. They can be used to explore the relationship between nervous systems and behavior and to explore how nervous systems can constrain the evolution of behavior. Generally, neural network modeling is motivated by theory; neural network models are highly simplified and are meant to provide general models that can be used to test ideas. In a neural network, the essential element is a 'node,' conceptually akin to a neuron. Nodes have states and are interconnected with other nodes. The pattern of connections among nodes is referred to as the network architecture. Sometimes, the neural network can change as the result of experience. For example, input nodes may respond to a 'stimulus' and relay this information to output nodes that produce some response or 'behavior.' If the response does not match some standard, the model can specify changes to the nodes and/or their connections that may improve the output.

Computational neuroethology is a related approach to modeling the neural basis of behavior. Like neural network modeling, computational neuroethologists create mathematical models of biological nervous systems to simulate animal behavior. Computational neuroethology emphasizes the interaction of the simulated animal with its environment. Thus, when a simulated animal produces a behavioral response to a stimulus, that behavior changes the nature of the animal's stimulus environment, resulting in the so-called action-perception cycle. In computational neuroethology, the simulated animals may be computer simulations or robotic simulations, and they are generally inspired by specific species and the natural problems they face, such as an insect foraging for food.

See also: Neurobiology, Endocrinology and Behavior; Neuroethology: What is it?; Robot Behavior; Sociogenomics.

Further Reading

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