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The Study of Behavioral Endocrinology

The phrase "raging hormones" is a common expression that has been used to explain or excuse many types of behavior. For example, inappropriate behaviors, especially sexual behaviors, displayed by adolescent boys are often attributed to raging hormones. Women who display aggressive or assertive behaviors, especially in association with their pre-menstrual period, are often said to be affected by raging hormones. What are these hormones, and can they really take over the nervous system to direct behavior?

You may have discussed this and other questions about hormones and behavior with friends and family members. For example, does anabolic steroid abuse cause violent behavior? Does an individual's sex drive wane with aging? How does exposure to acute or chronic stress affect sexual behavior? Is the sex drive of men higher or lower than the sex drive of women? Can melatonin cure jet lag? Do seasonal cycles of depression occur in people? Why are men much more likely than women to commit violent crimes? Does postpartum depression really exist? Is homosexuality caused by hormone concentrations that are too low or too high? Is the sexual behavior of women influenced by menopause? Can leptin curb our food intake? Contrary to popular beliefs, hormones do not *cause* behavioral changes per se. Rather, hormones change the *probability* that a specific behavior will be emitted in the appropriate behavioral or social context.

Researchers in the field of **behavioral endocrinology**, the study of the interaction between hormones and behavior, attempt to address these kinds of questions in

a formal, scientific manner. The study of hormones and behavior has been truly interdisciplinary: methods and techniques from one scientific discipline have been borrowed and refined by researchers in other fields. Psychologists, endocrinologists, neuroscientists, entomologists, zoologists, anatomists, physiologists, psychiatrists, and other behavioral biologists have all made contributions to the understanding of hormone–behavior interactions. This exciting commingling of scientific interests and approaches, with its ongoing synthesis of knowledge, has led to the emergence of behavioral endocrinology as a distinct and important field of study (Beach, 1975b).

Historical Roots of Behavioral Endocrinology

Psychology, as Ebbinghaus (1908) stated, has a short history but a long past, and the same can be said of behavioral endocrinology (Beach, 1974a). Although the modern era of the discipline is generally recognized to have emerged during the middle of the 20th century, with the publication of the classic book *Hormones and Behavior* (Beach, 1948), some of the relationships among the endocrine glands, their hormone products, and behavior have been implicitly recognized for centuries.

The male sex organs, or **testes**, produce and secrete a hormone called testosterone, which influences sexual behavior, aggression, territoriality, hibernation, and migration, as well as many other behaviors that differentiate males from females. The testes of mammals are usually located outside of the body cavity and can easily be damaged or removed. Thus, **castration**, the surgical removal of the testes, has historically been the most common manipulation of the endocrine system. For millennia, individuals of many species of domestic animals have been castrated to make them better to eat or easier to control, and the behavioral and physical effects of castration have been known since antiquity. Indeed, these effects were known to Aristotle, who described the effects of castration in roosters (and humans) with great detail and accuracy. For example, in *History of Animals*, written about 350 B.C., Aristotle reported that

> Birds have their testicles inside . . . Birds are castrated at the rump at the part where the two sexes unite in copulation. If you burn this area twice or thrice with hot irons, then, if the bird be full-grown, his crest grows sallow, he ceases to crow, and foregoes sexual passion; but if you cauterize the bird when young, none of these male attributes or propensities will come to him as he grows up. The case is the same for men: if you mutilate them in boyhood, the later-growing hair never comes, and the voice never changes but remains high-pitched; if they be mutilated in early manhood, the late-growths of hair quit them except the growth on the groin, and that diminishes but does not entirely depart.

Human males have been castrated for a number of reasons throughout history. For centuries, royalty employed men castrated before puberty, called **eunuchs**, to guard women from other men. For example, the Old Testament reports that these emasculated males were used to guard the women's quarters of Hebrew kings and

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1.1 Eunuch of the last imperial court, photographed in China by Henri Cartier-Bresson in 1949. Note the lack of facial hair and unusually long arms.

princes (Esther 1:10). Castration in humans often has little or no effect on physical appearance or future sexual behavior when performed after the unfortunate individual attains sexual maturation; however, if human males are castrated before puberty, they will develop a characteristic physical appearance, marked by short stature and long arms (Figure 1.1), and sexual behaviors are unlikely to develop. The typical secondary male sex characters are also affected by prepubertal removal of the testes. For example, as noted by Aristotle, eunuchs never develop beards, and the pubertal change in voice does not occur. Normally during puberty, the vocal cords of males thicken in response to testosterone secreted by the testes. It is the thickened vocal cords that produce the deeper-pitched voice characteristic of males, just as the thick strings of a guitar produce deeper-pitched notes than the thin strings.

Castration was once a common practice in Europe and Asia (Box 1.1). Young boys with exceptional singing voices were castrated to prevent the pubertal changes in pitch. These singers became known as *castrati.* Although castrati were prized by church choirs for centuries, their popularity reached a peak in Europe during the 17th and 18th centuries with the development of opera, which made castrati the first superstars of the entertainment world (Heriot, 1974). The first castrato opera star, Baldassare Ferri, died in 1680 at the age of 70 with a fortune that was worth the equivalent of $3 million today. In hopes of attaining this level of wealth and fame, young boys with musical aptitude were identified early, and poor families offered their sons outright to church leaders, singing teachers, and

BOX 1.1 The Hijras of India

Over a million eunuchs, called Hijras, are estimated to be currently living in India. Traditionally, the cultural function of Indian Hijras has been to sing and dance at weddings and ceremonies associated with the birth of male children. The Hijras have become associated with fertility and form a sect within Hinduism; they worship Bahachara, a manifestation of the Hindu mother goddess. However, a significant minority of Hijras are Muslem. Although the Hijras claim the religious status of "*sannyasis*" (or celibates) because their genitalia (i.e., testes and penis) have been surgically removed, many work as prostitutes.

The Hijras are a heterogeneous population; most individuals have sexually ambiguous histories. Commonly, their genitalia were ambiguous at birth, or their sexual development was atypical during puberty (Nanda, 1990). Some Hijras engaged in homosexual sex during adolescence, and virtually all were reared as boys. The surgery is usually performed during an outdoor ritual that is accompanied by singing and dancing Hijras. Both the penis and scrotum are removed in the rapid, but nonsterile, operation. Rumors persist that young, homeless boys are taken in by the Hijras, who provide friendship and material goods, then transform them into eunuchs (e.g., Diamond, 1984). However, recent accounts of the Hijras failed to uncover any evidence of coerced or involuntary acceptance of the demasculinizing surgery (Nanda, 1990).

The most common occupation of the Hijras today is removing "bad luck." Because the Hijras consider themselves to have already suffered the very worst luck that can befall a man (despite the "elective" nature of the surgery), they will accept a little more bad luck for a fee. Thus, it is customary for new homeowners to hire Hijras to dance through all the rooms to absorb any potential bad luck. Similarly, small groups of Hijras appear uninvited at weddings to dance away any potential bad fortune for the bride and groom. Despite a certain amount of traditional charm, the arrival of the garishly dressed Hijras at a wedding is seldom a welcome sight. If they are not given a substantial fee, the Hijras will disrupt the wedding ceremony by cursing and exposing their disfigured genitalia. Not surprisingly, most families pay the fee to avoid the anatomy lesson, but these small extortions have made the Hijras rather unpopular in the cities of India.

Photo courtesy of Takeshi Ishikawa.

music academies. Thousands of boys lost their testes but never gained the celebrity or riches of the star castrati.

What did a castrato sound like? Essentially, the castrati had the range of a soprano, but the greater development of the male lungs gave their singing remarkable power. An early critic remarked, "Their timbre is as clear and pierc-

ing as that of choirboys and much more powerful; they appear to sing an octave above the natural voice of women. Their voices . . . are brilliant, light, full of sparkle, very loud, and astound with a very wide range" (Heriot, 1974).

After 200 years, the tastes of the opera-loving public changed. The rise in popularity of the female soprano voice reduced the demand for castrati, and they soon became an oddity. In 1849, the last great castrato, Giovanni Velluti, retired from opera to his villa in Venice. The last known castrato, Alessandro Moreschi, who served as the director of the Sistine Chapel Choir, as well as one of the choir's soloists, died in 1922. Before his death, he made 17 recordings that, although of poor quality by today's standards, still provide a remarkable example of the castrato voice.

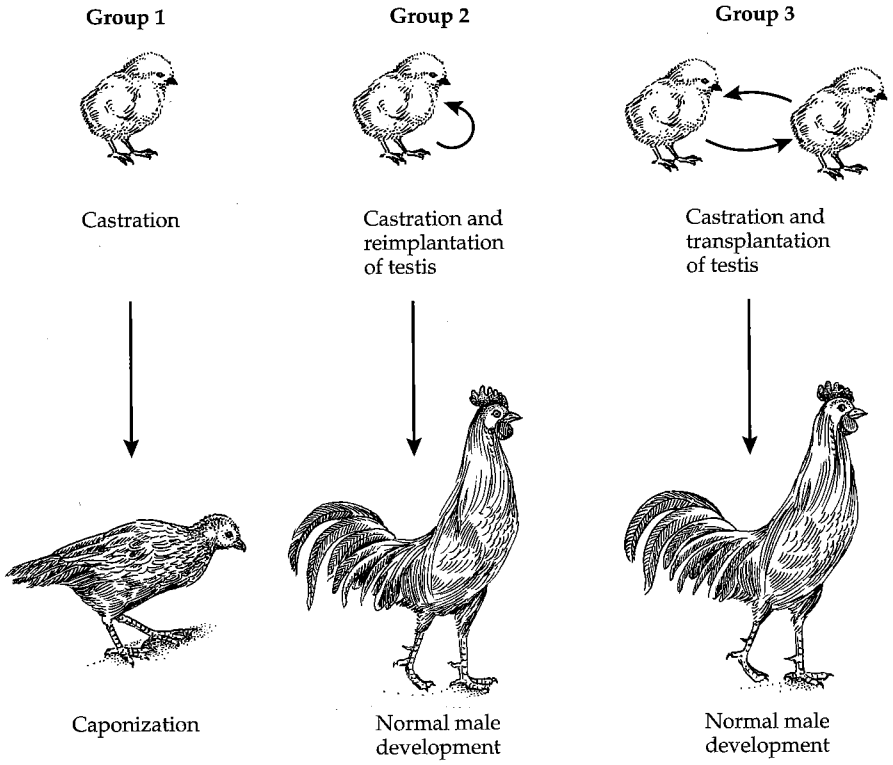
Berthold's Experiment

A useful starting point for understanding research in hormones and behavior is a classic 19th-century experiment that is now considered to be the first formal study of endocrinology. This remarkable experiment conclusively demonstrated that a substance produced by the testes could travel through the bloodstream and eventually affect behavior. Professor Arnold Adolph Berthold, a Swiss–German physician and professor of physiology at the University of Göttingen (Figure 1.2), demonstrated experimentally that a product of the testes was necessary for a cockerel (an immature male chicken) to develop into a normal adult rooster.

As you probably know, roosters display several characteristic behaviors that are not typically seen among hens or immature chicks of either sex. Roosters mate with hens, they fight with other roosters, and of course, roosters crow. Moreover, roosters are larger than hens and immature birds and have distinctive plumage. On the other hand, capons, male chickens that have been castrated prior to adulthood in order to make their meat more tender, do not show many of the behavioral and physical characteristics of roosters. They do not attempt to mate with hens and are not very aggressive toward other males. Indeed, they avoid aggressive encounters, and if conditions force them to fight, they do so in a "half-hearted" manner. Finally, capons do not crow like roosters.

The behavioral and physical differences among roosters, hens, capons, and immature chickens were undoubtedly familiar to Berthold when he planned his study, which began on the second day of August, 1848, and lasted for sev-

1.2 Arnold Adolph Berthold of the University of Göttingen, who in 1849 conducted what is now recognized as the first formal experiment in endocrinology.



1.3 Berthold's experiment. The two birds in group 1 were castrated, and when observed several months later, were smaller than normal roosters and failed to engage in rooster-typical behaviors. The two birds in group 2 were also castrated, but one of each bird's own testes was reimplanted in its abdominal cavity. These birds looked and behaved like normal roosters when adults. The two birds in group 3 were also castrated, and one testis from each bird was transplanted into the abdomen of the other. Several months later, these birds also looked and behaved liked normal roosters. Berthold found that the reimplanted and transplanted testes in groups 2 and 3 developed vascular connections and generated sperm.

eral months (Figure 1.3; Berthold, 1849). He placed six cockerels in three experimental groups, each consisting of two birds. He removed both testes from each of the two cockerels in the first group, and as expected, these birds eventually developed as capons. They never fought with other males after castration, and they failed to crow; instead, Professor Berthold reported, they developed the "monotone voice of the capon." They avoided females and never exhibited mating behavior. Finally, these birds *looked* different from intact (uncastrated) adult males. Their bodies and heads were small, and their combs and wattles were atrophied and pale in color.

The second pair of cockerels was also castrated, but Berthold reimplanted one testis from each bird in its abdominal cavity after ensuring that all of the original vascular and neural connections had been cut. Interestingly, both birds in this

group developed normal rooster behavior. According to Berthold, they "crowed lustily, often engaged in battle with each other and with other cockerels, and showed the usual reactions to hens." Their physical appearance was indistinguishable from that of other young roosters; they grew normally and possessed highly developed combs and wattles that were bright red in color.

The remaining two birds were also castrated, but after the testes were removed, Berthold placed a single testis from each bird in the other's abdominal cavity. Like the cockerels in the second experimental group, these birds also developed the "voice, sexual urge, belligerence, and growth of combs and wattles" characteristic of intact males.

After observing all six birds for several months, Berthold dissected one of the cockerels from the second group and found that the implanted testis had attached itself to the intestines, developed a vascular supply, and nearly doubled in size. Eventually, he examined all the implanted testes under a microscope and noted the presence of sperm.

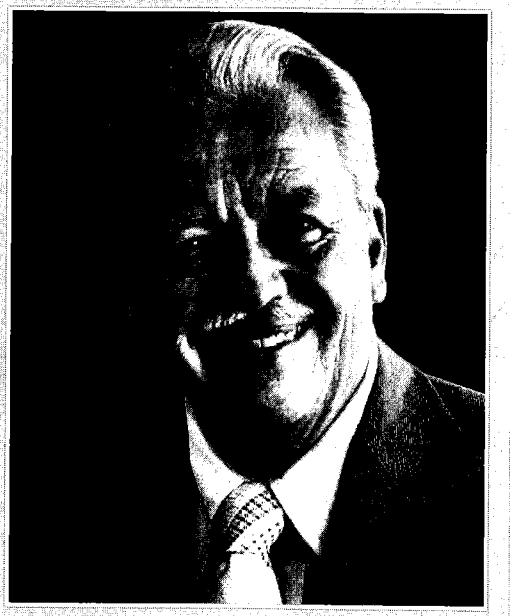
Based on the results of this experiment, Berthold drew three major conclusions: (1) the testes are transplantable organs; (2) transplanted testes can function and produce sperm (Berthold drew the analogy to a tree branch that produces its own fruit after having been grafted to another tree); and (3) because the testes functioned normally after all nerves were severed, there are no specific nerves directing testicular function. To account for these findings, Berthold proposed that a secretory, blood-borne product of the transplanted testes (*productive Verhältniss der Hoden*) was responsible for the normal development of the birds in the second and third groups. It is worth noting that three of the four parameters Berthold used to formulate this hormonal hypothesis—mating, vocalization, aggression, and distinctive appearance—were behavioral.

In recent years, Berthold's experiment has been credited as the genesis of the field of endocrinology (and thus of behavioral endocrinology; Box 1.2), but his intriguing demonstration of non-neural control of behavior was apparently not embraced with great enthusiasm by his scientific contemporaries, as his paper does not seem to have been cited for nearly 60 years after its publication. Why, then, did Berthold conduct his study? His experiment was elegant in its simplicity, but unfortunately his published report was brief and had no introduction, so we cannot know for certain what questions motivated him to conduct the work (Forbes, 1949). He seems unaware of the work of John Hunter, who had reported by 1771 the successful transplantation of a testis from a rooster into a hen (with no obvious changes resulting; Forbes, 1947). We do know that Berthold had previously authored a well-known physiology textbook and had actively conducted biological research. A reading of his textbook makes it apparent that Berthold was a proponent of the pangenesis theory of inheritance. This theory, endorsed by many biologists prior to the discovery of chromosomes and genes, held that all body parts actively discharge bits and pieces of themselves into the blood system, where they are transported to the ovaries or testes and assembled into miniature offspring resembling the parents. As a consequence of this theoretical stance, Berthold had two concepts at hand when evaluating the results of his testicular

BOX 1.2 Frank A. Beach and the Origins of the Modern Era of Behavioral Endocrinology

For some time before behavioral endocrinology emerged as a recognized field, its foundations were being laid by researchers in other fields. The anatomists, physiologists, and zoologists who were doing the majority of the work on "internal secretions" prior to 1930 often used behavioral parameters in their studies. Soon thereafter, psychologists began making important contributions to the study of hormones and behavior. In the early decades of the 20th century, American psychology was undergoing a major change, both in ideology and in methodology. Led by John B. Watson, students of the "science of the mind" were casting aside introspection as a method in favor of observation and experimentation. Watson argued that only overt behavior was observable, and psychologists began describing and quantifying all types of overt behavior.

Karl S. Lashley did his graduate work under Watson at Johns Hopkins University and eventually joined the faculty at the University of Chicago. Lashley investigated the effects of removing parts of rats' brains to discover where in the brain vari-



Frank A. Beach (1911–1988)

transplantation study: (1) various parts of the body release specific agents into the blood; and (2) these agents travel through the bloodstream to particular target organs. Why Berthold did not go any further with his interesting finding is not known; he died 12 years later in 1861 without following up on his now-famous study.

What Are Hormones?

Berthold took the first step in the study of behavioral endocrinology by demonstrating that the well-known effects of the testes were due to their production of a substance that circulated in the blood. Modern studies in behavioral endocrinology have documented the effects of substances from many different glands affecting an ever greater range of behaviors.

We now refer to Berthold's "secretory blood-borne product" as a hormone, a termed coined by Bayliss and Starling in 1902. **Hormones** are organic chemical

ous psychological processes were carried out; he was particularly interested in finding where memories were stored. Although he never published any reports on the interaction between hormones and behavior, Lashley was clearly interested in the subject (e.g., Lashley, 1938), and several of Lashley's students became important contributors to behavioral endocrinology, including Calvin P. Stone, Josephine Ball, and Frank A. Beach.

Beach, William C. Young (see Box 3.2), and Daniel Lehrman (see Box 7.2) were especially influential during the early studies of behavioral endocrinology. Beach's dissertation at Chicago, "The Neural Basis for Innate Behavior," examined the effects of cortical tissue destruction on the maternal behavior of first-time mother rats. In 1937, Beach began working as a curator in the Department of Experimental Biology at the American Museum of Natural History in New York and began contributing to the museum's tradition of comparative behavioral experimentation. One study completed at the museum, which was a logical extension of Beach's dissertation work, is of special note: he began investigating the effects of cortical lesions on the mating behavior of male rats. He found that some brain-damaged rats continued to mate, whereas others failed to do so. Beach was concerned that his lesions were interfering indirectly with the endocrine system, so he injected the nonmating brain-injured rats with testosterone, the primary hormone secreted from the testes. The treatment evoked mating behavior in some of the lesioned rats, and this modification of behavior by hormones prompted Beach to learn more about endocrinology.

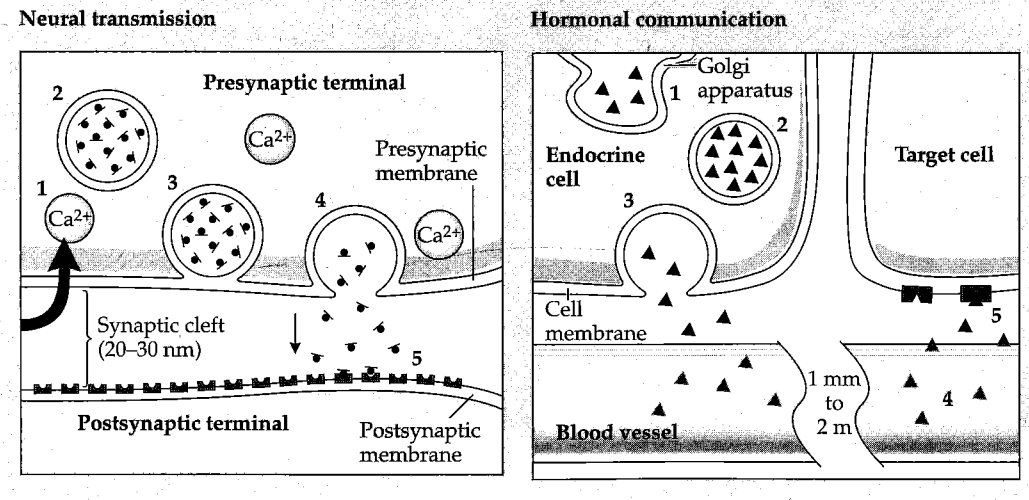
Beach audited a course in endocrinology at New York University, but was distressed by the lack of information about the behavioral effects of hormones; the professor responded to Beach's complaint by allowing him to teach one session. While preparing for the lecture, Beach discovered that no comprehensive summary of hormone–behavior interactions existed, and he prepared such a review as a term paper for the endocrinology course. Several years later, Beach expanded his paper into an influential book, *Hormones and Behavior* (Beach, 1948). The publication of this book marked the beginning of the formal study of behavioral endocrinology. Beach is credited with the genesis of this scientific discipline, and he continued to provide intellectual leadership in shaping the field for the next 40 years.

messengers produced and released by specialized glands called **endocrine glands**. Hormones are released from these glands into the bloodstream (or the tissue fluid system in invertebrates), where they may then act on target organs (or tissues) at some distance from their origin. Hormones coordinate the physiology and behavior of an animal by regulating, integrating, and controlling its bodily functions. For example, the same hormones that cause gametic (egg or sperm) maturation also promote mating behavior in many species. This dual hormonal function ensures that mating behavior occurs when animals have mature gametes available for fertilization. Another example of endocrine regulation of physiological and behavioral function is provided by the metabolic system. Several metabolic hormones work together to elevate blood glucose levels prior to awakening in anticipation of increased activity and energy demand. This "programmed" elevation of fuel availability coordinates the animal's physiology with its behavior.

Hormones are similar in function to **neurotransmitters**, the chemicals used by the nervous system in coordinating animals' activities. However, hormones

BOX 1.3 Neural Transmission versus Hormonal Communication

Although neural and hormonal communication both rely on chemical signals, there are several prominent differences between them. Communication in the nervous system is analogous to traveling on a train. You can use the train in your travel plans as long as tracks exist between your proposed origin and destination. Likewise, neural messages can travel only to destinations along existing nerve tracts. Hormonal communication, on the other hand, is like traveling in a car. You can drive to many more destinations than train travel allows because there are many more roads than railroad tracks. Likewise, hormonal messages can travel anywhere in the body via the circulatory system; any



can operate over a greater distance and over a much greater temporal range than neurotransmitters (Box 1.3). Hormones are also similar to **cytokines**, chemical signals produced by cells of the immune system, and may interact with cytokines to affect behavior, especially when individuals are ill or unduly stressed.

Not all cells are influenced by each and every hormone. Rather, any given hormone can directly influence only cells that have specific **receptors** for that hormone. Cells that have these specific receptors are called **target cells** for the hormone. The interaction of a hormone with its receptor often begins a series of cellular events that eventually affect gene expression and protein synthesis. The newly synthesized proteins may activate or deactivate other genes, causing yet another cascade of cellular events. Recently, some effects of hormones on behavior have been reported that are not caused by activation of the genetic machin-

cell receiving blood is potentially able to receive a hormonal message.

Neural and hormonal communication differ in other ways as well. To illustrate them, consider the differences between digital and analog technologies. Neural messages are digital, all-or-none events that have rapid onset and offset: neural signals can take place in milliseconds. Accordingly, the nervous system mediates changes in the body that are relatively rapid. For example, the nervous system regulates immediate food intake and directs body movement. In contrast, hormonal messages are analog, graded events that may take seconds, minutes, or even hours to occur. Hormones can mediate long-term processes, such as growth, development, reproduction, and metabolism.

Hormonal and neural messages are both chemical in nature, and they are released and received by cells in a similar manner; however, there are important differences as well. As shown in the left panel of the accompanying figure, when a neural impulse arrives at a presynaptic terminal, there is an influx of calcium ions (Ca^{2+}) into the cell (1) that causes vesicles containing neural chemical messengers called neurotransmitters to move toward the presynaptic cell membrane (2). The vesicles fuse with the membrane (3) and release the neurotransmitters into the synaptic cleft (4). The neurotransmitters travel a distance of only 20–30 nanometers (30×10^{-9} m) to the membrane of the postsynaptic neuron, where they bind to receptors (5). Hormones, as shown in the right panel of the figure are manufactured in the Golgi apparatus of an endocrine cell (1). They also move toward the cell membrane in vesicles (2), which fuse with the membrane, releasing the hormone (3). However, hormones then enter the circulatory system, through which they may travel from 1 millimeter to 2 meters (4) before arriving at a cell of a target tissue, where they bind with specific receptors (5).

Another distinction between neural and hormonal communication is the degree of voluntary control that can be exerted over their functioning. In general, there is more voluntary control of neural than of hormonal signals. It is virtually impossible to will a change in your thyroid hormone levels, for example, whereas moving your limbs on command is easy.

Although these differences are significant, the division between the nervous system and the endocrine system is becoming more blurred as we learn more about how the nervous system regulates hormonal communication. A better understanding of the interface between the endocrine system and the nervous system is likely to yield important advances in the future study of the interaction between hormones and behavior.

ery; these so-called nongenomic effects of hormones on behavior will be reviewed in later chapters.

Importantly, sufficient numbers of appropriate hormone receptors must be available for a specific hormone to produce any effects. For example, if a capon had no receptors for testosterone, then implanting another testis (or giving testosterone hormone therapy) would not cause it to mate, fight, or crow. Furthermore, a common bias in behavioral endocrinology is the assumption that individual differences in the expression of a behavior reflect differences in hormone concentrations in the blood. In other words, it is assumed that roosters that crow frequently have higher blood testosterone concentrations than roosters that rarely crow. To a certain extent, this assumption is true, but such individual differences usually reflect complex influences of hormone concentrations, patterns of hormone release, numbers and loca-

tions of hormone receptors, and the efficiency of those receptors in triggering the signal transduction pathways that ultimately affect gene transcription.

Hormones commonly alter the rate of normal cellular function. Another way that hormones can affect cells is to change their morphology or size. For example, some athletes abuse anabolic steroids, which are synthetic hormones, because muscle cells grow larger after exposure to these substances. Hormones also may affect neuronal growth and development, as well as cell death throughout the nervous system. Whereas the examples we have discussed so far have all demonstrated how the presence or absence of a hormone may affect behavior, it is important to appreciate that the interactive relationship between hormones and behavior is bidirectional: hormones obviously affect behavior, but, as we will see in later chapters, behavior can also influence hormone concentrations.

The Study of Behavior

Behavioral endocrinologists are interested in how the general physiological effects of hormones may eventually alter behavior, and how behavior may influence the effects of hormones. This book will describe, both phenomenologically and functionally, how hormones affect behavior.

What is behavior? Generally, we think of behavior as "output," and because muscles are the most common output organs, or **effectors**, we tend to think of behavior as coordinated movement. Sometimes lack of movement is an important behavior, especially when stalking prey or avoiding predators, or during mating for females of many species. The excretion of scents and chemicals, changes in skin coloration, the flashing lights of fireflies, and the production of electrical signals by various species are also types of behavior.

Problems of Behavioral Research

The goals of behavioral scientists are to determine what behaviors are relevant to the question being asked, to describe those behaviors, and to interpret their function. These goals are not as simple to achieve as they may sound, and there are several pitfalls that behavioral investigators must avoid. Let us examine several of these problems in the context of a simple example: how might we begin to explain the singing of a robin (*Turdus migratorius*) that visits our backyard bird feeder each morning?

As soon as any observer begins to look at behavior, some degree of abstraction and bias is inevitable. When hens or roosters vocalize, we say that they "cluck" or "crow." However, the vocalizations of many birds are so melodious that we actually call them "singing." We may thus explain the bird's singing as a result of its being happy because we often sing when we are happy. This is obviously an anthropomorphic bias on our part. Behavioral scientists must take care not to attribute motives (hunger, fear, happiness) to animals based only on introspection, and must make an effort to observe behavior as objectively as possible.

Second, we must determine what other behaviors may be relevant to elucidating the behavior being examined. Even if an animal's behavior is videotaped contin-

uously or observed directly for 24 hours a day, decisions must be made by the observer regarding which behaviors are meaningful and which are trivial in terms of answering the question at hand. In the case of the robin, for example, we must determine whether its presence at the bird feeder bears any relation to its singing.

Finally, to understand the causes (hormonal or otherwise) of any behavior, we must thoroughly describe that behavior (Tinbergen, 1951). When does the robin sing? What elicits its singing? Do all robins, or only some, engage in singing? As we saw in our discussion of Berthold's experiment, we must have a reasonably complete description of normal behavior before we can accurately assess the effects of any experimental manipulations on behavior.

How Is Behavior Described?

We may classify descriptions of behavior within two broad categories: (1) descriptions of action and (2) descriptions of consequence (Dewsbury, 1979). In the former, the pattern of an animal's behavioral output is described with little or no reference to the effects of the behavior on the environment. Thus, descriptions of a lion's bared teeth, a firefly's flash of light, or a songbird's singing are descriptions of action. When classifying behavior by consequence, an observer notes the effect of the behavior on the environment; the observer may describe an animal's behavior as "gathering up nest material," "depressing a lever," or "inducing a female to visit." In practice, the two types of descriptive classifications can be combined to provide a rich description of animal behavior.

The Simple System Approach

Even though we may ultimately be curious about the influence of hormones on human behavior, the unique and complex interactions of genes and environment in humans make behavioral endocrinology studies of our own species very difficult to interpret. For example, suppose we observed that people in the upper 25th percentile of body mass tend to be more socially aggressive than individuals in the lower 25th percentile of body mass. The cause of the difference in behavior might reflect differences in body size, which might result from differences in concentrations of hormones that regulate growth and development. Alternatively, the differences in body mass might reflect differences in nutrition that affect brain development and thus the development of confidence in social situations. There is also the "chicken and egg" problem to resolve; that is, do hormones affect behavior directly by affecting the brain, indirectly by affecting body size, which in turn affects brain and behavior, or does acting aggressively affect hormone concentrations, which in turn affect body development? Furthermore, humans are reared in a wide variety of environments, which complicates the assignment of causation to individual variation in behavior.

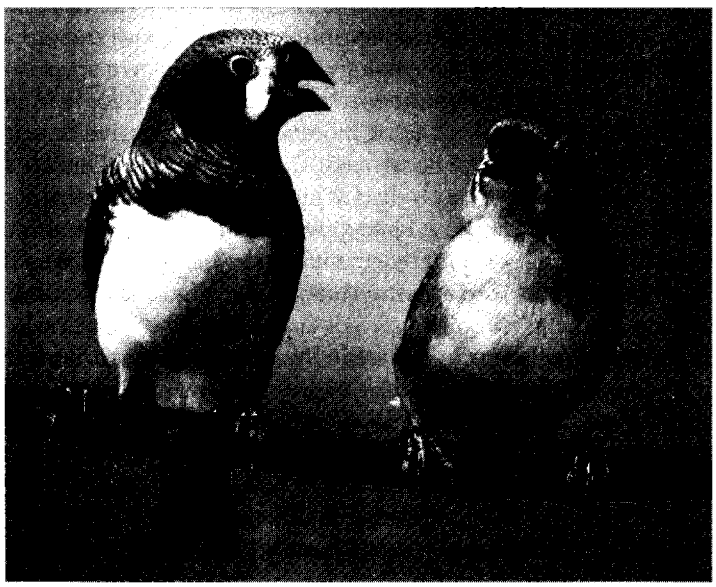
In order to untangle the contributions of various factors to hormone–behavior interactions, behavioral endocrinologists generally perform experiments on genetically identical animals in controlled environments. The development of behaviors and changes in hormone concentrations can be monitored throughout life under these conditions. Similar controlled experiments are virtually impossible

to conduct on humans. Even though nonhuman animals represent "simple systems" relative to humans, it is important to appreciate that the behavioral repertoires of animals are extraordinarily varied and complex.

Most research in behavioral endocrinology involves only a few types of simple behavior. This narrow focus on only a few behavioral measures is partially a response to the enormous variation inherent in observations of complex behaviors. There are advantages and disadvantages to this approach. The advantages of using simple behaviors include ease of replication and quantification. In this way, the simple system approach parallels the reductionist approach prevalent in physiological and biochemical analyses. On the other hand, the most apparent disadvantage to studying simple behaviors is the possibility that subtle but important interactions between hormones and behavior will be neglected and overlooked. Social and other environmental factors are often absent, or significantly reduced, in the laboratory, but may also be important in attaining a complete understanding of hormone–behavior interactions. There are certainly cases in which investigators have endeavored to observe the hormonal correlates of complex behavior. But commonly, the behavioral end point studied is as simple as the presence or absence of bird song, or the occurrence of mounting behavior among male rodents.

Levels of Analysis

Once behaviors have been adequately described, we may proceed to ask about their causes (Alcock, 2001; Dewsbury, 1979; Tinbergen, 1951). For example, the zebra finch (*Taeniopygia guttata*; Figure 1.4), a native Australian songbird, is one of the



1.4 Zebra finches. These small birds have been used extensively in the study of the hormonal and neural bases of bird song. As in most songbird species, only the male zebra finch (right) sings in nature. Courtesy of Atsuko Takahashi.

species most frequently used to study bird song. The various notes zebra finches produce, the circumstances in which they sing, and even the specific muscles they use during singing have been extensively studied and described. Based on these descriptions, many researchers have begun to explore the causes of singing in male zebra finches by developing hypotheses and testing them through observation and experimentation. (As in most songbird species, females and immature zebra finches do not sing in nature.)

The generic question an animal behaviorist asks at this point in the research may be simply expressed as "What causes animal A to emit behavior X?" (Sherman, 1988), so many researchers have asked, in effect, "What causes zebra finches to sing?" You may be surprised to learn that there may be four types of correct answers to this basic question, based on four different **levels of analysis**: immediate causation, development, evolution, and adaptive function (Tinbergen, 1951).

IMMEDIATE CAUSATION The level of **immediate causation** encompasses the underlying physiological, or proximate, mechanisms responsible for a given behavior. Typically these mechanisms are mediated by the nervous and endocrine systems, which influence behavior on a moment-to-moment basis during the life of an individual. Various internal and environmental stimuli, as well as sensory and perceptual processes, are involved in the short-term regulation of behavior. Accordingly, experiments designed to address questions of immediate causation often use physiological methods such as alterations of hormone concentrations or direct manipulations of the brain. In the case of zebra finches, these kinds of experiments have revealed that elevated blood concentrations of testosterone and increased rates of neural activity in certain areas of the brain are immediate causes of singing, so a correct answer to the question posed above might be that zebra finches sing because the level of testosterone in their blood is high. This class of explanation is the one most frequently used by behavioral endocrinologists, and will be the primary focus of this book.

DEVELOPMENT The behavioral responses and repertoires of animals change throughout their lives as a result of the interaction between genes and environmental factors. Questions of **development** concern the full range of the organism's lifetime from conception to death. For example, the behavior of newborns is quite rudimentary in many species, but becomes more complex as they grow and interact with the environment. Hormonal events affecting the fetal and newborn animal can have pervasive influences later in life. Although the majority of research at the developmental level of analysis has focused on how events early in development influence animals later in life, the decay of behavioral patterns during aging is also of interest to behavioral biologists pursuing developmental questions. Possible answers to our question from the developmental perspective might be that zebra finches sing because they have undergone puberty or because they learned their songs from their fathers.

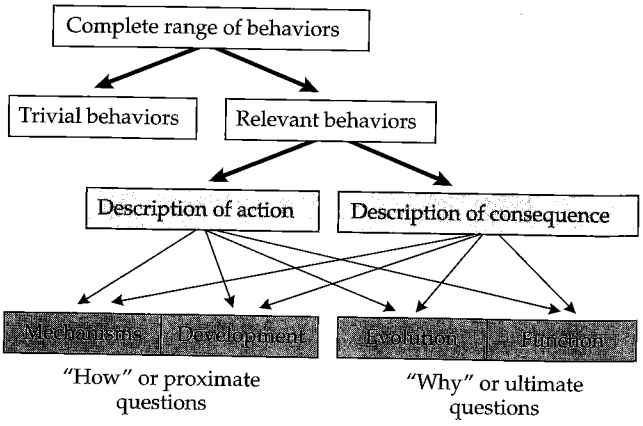
EVOLUTION **Evolutionary approaches** involve many generations of animals and address the ways that specific behaviors change during the course of natural

selection. Behavioral biologists study the evolutionary bases of behavior to learn why behavior varies between closely related species as well as to understand the specific behavioral changes that occur during the evolution of new species. Behaviors rarely leave interpretable traces in the fossil record, so the study of the evolution of behavior relies on comparing existing species that vary in relatedness. An investigator working at the evolutionary level might say that zebra finches sing because they are finches, and that all finches sing because they have evolved from a common ancestral species that sang.

ADAPTIVE FUNCTION Questions of **adaptive function** are synonymous with questions of adaptive significance: they are concerned with the role that behavior plays in the adaptation of animals to their environments and with the selective forces that currently maintain behavior. At this level of analysis, it might be argued that male zebra finches sing because singing increases the likelihood that they will reproduce by attracting females to their territories or dissuading competing males from entering their territories.

Thus, there are four different types of causal explanations for a particular behavior, and there may be many correct answers to the question, "What causes male zebra finches to sing?" No one type of explanation is better or more complete than another, and in practice, the levels of questions and explanations overlap and interact in many situations. Nevertheless, it is important that researchers specify clearly the level of analysis within which they are working when they are generating hypotheses for testing. Care must be taken to avoid comparing noncompeting hypotheses at the different levels of analysis (Sherman, 1988).

For the sake of simplicity, these four levels of analysis can be grouped into sets of two, with questions of immediate causation and development grouped as "how questions" ("*How* does an animal engage in a behavior?") and questions of evolution and adaptive function as "why questions" ("*Why* does an animal engage in a particular behavior?") (Alcock, 2001). "How questions" have also been referred to as questions of *proximate causation*, and "why questions" as questions of *ultimate causation* (Wilson, 1975). To construct an exhaustive explanation of



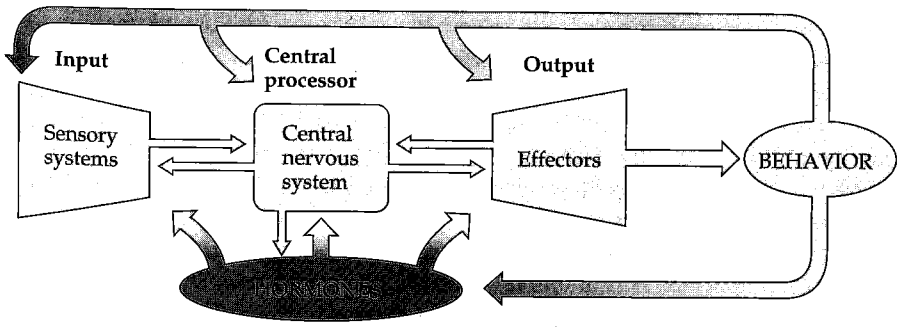
1.5 Stages of behavioral research. From the complete range of an organism's behaviors, the behavioral scientist must first determine which behaviors are relevant to the question under consideration, a process that is inherently prone to undue abstraction or bias. Descriptions of relevant behaviors may focus on the actions themselves (description of action) or on their environmental effects (description of consequence). Examination of the causes of behaviors may proceed at any of four levels of analysis that address either proximate ("how") or ultimate ("why") questions.

the causes of bird song, then, we would want to study both how birds sing and why they sing (Figure 1.5). What developmental and physiological processes occur before and during singing? What is the evolutionary history of bird song? When, phylogenetically (during evolutionary history), did singing appear among birds? What adaptive advantages do singers enjoy relative to nonsingers?

Researchers in different disciplines tend to favor particular types of questions and classes of explanations. For example, physiologists work almost exclusively at the level of immediate causation, whereas behavioral ecologists specialize in evolutionary and adaptive explanations of behavior. Behavioral endocrinologists who focus on physiology and neuroscience tend to work in laboratories, whereas behavioral endocrinologists who focus on behavioral ecology tend to work in the field. In general, laboratory data are more reliable (i.e., repeatable) than field data because the experimental conditions can be tightly controlled. However, field data tend to be more valid (i.e., more ecologically relevant) than laboratory data because they are collected in the setting where the behavior and physiology of animals evolved. The types of explanations that individual scientists pursue in conducting their research reflect their tastes and their training, but their combined efforts allow us to gain the most comprehensive understanding of animal behavior.

How Might Hormones Affect Behavior?

In terms of their behavior, one can think of animals as being made up of three interacting components: (1) input systems (sensory systems), (2) integrators (the central nervous system), and (3) output systems, or effectors (e.g., muscles) (Figure 1.6). Again, hormones do not cause behavioral changes. Rather, hormones influence these three systems so that specific stimuli are more likely to elicit certain responses in the appropriate behavioral or social context. In other words, hormones change the probability that a particular behavior will be emitted in the



1.6 How hormones may affect behavior. Behaving animals may be thought of as being made up of three interacting components: input systems (sensory systems), central processing systems (the central nervous system), and output systems (effectors, such as muscles). Hormones may affect any or all of these three components when influencing behavior. Note that there is a bidirectional causal relationship between hormones and behavior in that an animal's behavior (or the behavior of conspecifics or predators) may affect its endocrine state.

appropriate situation. This is a critical distinction that can affect how we think of hormone–behavior relationships.

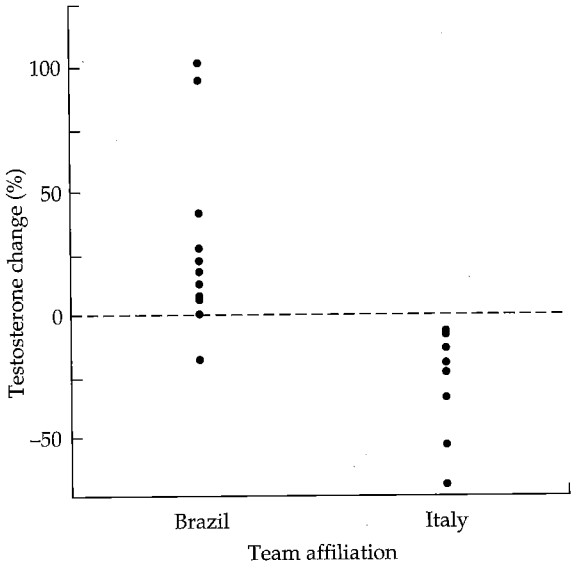
We can apply this three-component behavioral scheme by returning to our example of singing behavior in zebra finches. As previously noted, only the male zebra finch sings in nature. If the testes of adult male finches are removed, the birds stop singing, but castrated finches resume singing if the testes are reimplanted, or if they are provided with the primary testicular hormone, testosterone. Singing behavior is most frequent when blood testosterone concentrations are high. Although it is clear from these observations that testosterone is somehow involved in singing, how might the three-component framework just introduced help us to formulate hypotheses to explore testosterone's role in this behavior? By examining input systems, we could determine whether testosterone alters the birds' sensory capabilities, making the environmental cues that normally elicit singing more salient. If this were the case, females or territorial intruders might be more easily seen or heard. Testosterone also could influence the central nervous system. Neuronal architecture or the speed of neural processing could change in the presence of testosterone. Higher neural processes (e.g., motivation, attention, or perception) also might be influenced. Finally, the effector organs, muscles in this case, could be affected by the presence of testosterone. Blood testosterone concentrations might somehow affect the muscles of a songbird's syrinx (the avian vocal organ). Testosterone, therefore, could affect bird song by influencing the sensory capabilities, central nervous system, or effector organs of an individual bird. We do not understand completely how testosterone influences bird song, but in most cases, hormones can be considered to affect behavior by influencing one, two, or all three of these components, and our three-part framework can aid in the design of hypotheses and experiments to explore these issues. This conceptual scheme will provide the major organization for this book.

How Might Behavior Affect Hormones?

The zebra finch example demonstrates how hormones can affect behavior, but, as noted previously, the reciprocal relation also occurs; that is, behavior can affect hormone concentrations. For example, the sight of a territorial intruder may elevate blood testosterone concentrations in the resident male and thereby stimulate singing or fighting behavior (Wingfield, 1988). Similarly, male mice (Ginsberg and Allee, 1942) or rhesus monkeys (Rose et al., 1971) that lose a fight show reduced circulating testosterone concentrations for several days or even weeks afterward. Similar results have also been reported in humans. Testosterone concentrations are affected not only in humans involved in physical combat, but also in those involved in simulated battles. For example, testosterone concentrations were elevated in winners and reduced in losers of regional chess tournaments (Mazur et al., 1992).

People do not even have to be directly involved in a contest to have their hormones affected by the outcome. For instance, male fans of both the Brazilian and Italian soccer teams were recruited to provide saliva samples to be assayed for

testosterone before and after the final game of the World Cup soccer match in 1994. Brazil and Italy were tied going into the final game, which was hard fought and tied until the final seconds, but Brazil won on a penalty kick at the last possible moment. The Brazilian fans were elated and the Italian fans were crestfallen. When the samples were assayed, 11 of 12 Brazilian fans had increased testosterone concentrations, and 9 of 9 Italian fans had decreased testosterone concentrations, compared with pre-game baseline values (Dabbs, 2000) (Figure 1.7).



1.7 Change in the testosterone concentrations of sports fans. Testosterone concentrations of male Brazilian and Italian fans were measured in saliva samples obtained before and immediately after the final soccer match of the 1994 World Cup, which Brazil won. The graph shows the change in testosterone concentration between the two samples. After Dabbs, 2000.

Even anticipation of behavior may influence hormone concentrations. An anonymous contribution to the journal *Nature* (1970) provided an account by a gentleman whose work caused him to live in isolation on an island for days at a time. Occasionally, he returned to the mainland to pick up his mail and visit his fiancée. This man possessed a strong curiosity, and his isolated lifestyle presumably eliminated many of the usual distractions of modern life. In this peaceful milieu, he noted, while shaving, that his beard seemed thicker immediately prior to his visits to his fiancée. He began weighing the shavings, and determined that his beard thickened on days when he had sex with his fiancée. The rate of beard growth is correlated with blood concentrations of testosterone; high levels of testosterone increase the rate of beard growth, whereas low testosterone concentrations are associated with slow beard growth. Our anonymous colleague suggested that his sexual behavior, as well as his *anticipation* of sexual behavior, caused an elevation in testosterone, which in turn increased beard growth.

You probably recognize that this is not a very robust demonstration of a hormone–behavior interaction because (1) only one experimental subject was used, (2) the experimental subject was aware of the experimental conditions, which, consciously or unconsciously, may have caused him to misread the scale on critical days, and (3) other conditions, such as the drying effects of the air during his travels home, could have accounted for some of the changes in beard growth. In other words, this letter described **anecdotal evidence** of a hormone–behavior interaction—evidence that is not compelling. More recently, testosterone concentrations were measured in four heterosexual couples over a total of 22 evenings (Dabbs and Mohammed, 1992). There were two different types of evenings. On

11 evenings, the samples were obtained before and after sexual intercourse; on the remaining 11 evenings, two samples were obtained during the evening, but there was no sexual intercourse. To avoid the logistic complications of drawing blood samples, testosterone was measured in the saliva of the participants. Engaging in sexual intercourse caused testosterone concentrations to increase in both men and women. The early evening saliva samples revealed no difference in testosterone concentrations between evenings when sexual intercourse took place and evenings when it did not. These results suggest that in humans, sexual behavior increases testosterone concentrations more than high testosterone concentrations cause sexual activity (Dabbs and Mohammed, 1992). Although this is certainly a reasonable conclusion, there are alternative explanations for the results of this study. For example, perhaps physical exercise alone increases testosterone concentrations. To rule out this possibility, additional studies are required in which the level of exercise is similar between experimental groups, but differs in sexual content.

Classes of Evidence for Determining Hormone–Behavior Interactions

What sort of evidence *would* be sufficient to establish that a particular hormone affected a specific behavior or that a specific behavior changed hormone concentrations? Experiments to test hypotheses about the effects of hormones on behavior must be carefully designed, and, generally, three conditions must be satisfied by the experimental results to establish a causal link between hormones and behavior (Silver, 1978):

1. A hormonally dependent behavior should disappear when the source of the hormone is removed or the actions of the hormone are blocked.
2. After the behavior stops, restoration of the missing hormonal source or its hormone should reinstate the absent behavior.
3. Finally, hormone concentrations and the behavior in question should be covariant; that is, the behavior should be observed only when hormone concentrations are relatively high and never or rarely observed when hormone concentrations are low.

The third class of evidence has proved difficult to obtain because hormones may have a long latency of action, and because many hormones are released in a pulsatile manner. For example, if a pulse of hormone is released into the blood, and then no more is released for an hour or so, a single blood sample will not provide an accurate picture of the endocrine status of the animal under study. We might come to completely different conclusions about the effect of a hormone on behavior if we measure hormone concentrations when they are at their peak rather than when they are at their nadir. This problem can be overcome by obtaining measures in several animals and averaging across peaks and valleys, or by taking several sequential blood samples from the same animal. Another problem is that biologically effective amounts of hormones are vanishingly small and difficult to

measure accurately. Effective concentrations of hormones are usually measured in micrograms (μg, 10^{-6} g), nanograms (ng, 10^{-9} g), or picograms (pg, 10^{-12} g); they are sometimes expressed as a mass percentage relative to 100 ml of blood plasma or serum (10 μg% = 10 μg/100 ml = 0.1 μg/ml). The development of techniques such as the radioimmunoassay (see next section), has increased the precision with which hormone concentrations can be measured. However, because of the difficulties associated with obtaining reliable covariant hormone–behavior measures, obtaining the first two classes of evidence usually has been considered sufficient to establish a causal link in hormone–behavior relations.

As we will see, the unique conditions of the laboratory environment may themselves cause changes in an animal's hormone concentrations and behavior that may confound the results of experiments; thus, it has become apparent that hormone–behavior relationships established in the laboratory should be verified in natural environments. The verification of hormone–behavior relationships in natural environments is not yet common, but it is a useful procedure for differentiating laboratory artifacts from true phenomena.

Common Techniques in Behavioral Endocrinology

How do we gather the evidence needed to establish hormone–behavior relationships? Because we cannot directly observe the interactions between hormones and their receptors or their intracellular consequences, we must use various indirect tools to explore these phenomena. This section describes some of the primary methods and techniques used in behavioral endocrinology. Much of the recent progress in the field has resulted from technical advances in the tools that allow us to detect, measure, and probe the functions of hormones. Therefore, familiarity with these techniques will help you to understand and assess the research to be discussed in subsequent chapters.

Ablation and Replacement

The **ablation** (removal or extirpation) of the suspected source of a hormone to determine its function is a classic technique in endocrinology. Recall that this was the method Berthold used to establish the role of the testes in the development of rooster behavior. There are four steps to this time-honored procedure:

1. A gland that is suspected to be the source of a hormone affecting a behavior is surgically removed
2. The effects of removal are observed
3. The hormone is replaced by reimplanting the removed gland, by injecting a homogenate or extract from the gland, or by injecting a purified hormone
4. A determination is made whether the observed consequences of ablation have been reversed by the replacement therapy

This technique is commonly used in endocrine research today. A traditional complementary approach to the ablation–replacement technique is the observation of behavior in individuals with diseased or congenitally dysfunctional endocrine

organs. When ablation occurs in the brain, either through the actions of a researcher or through disease, the result is often called a **lesion**. Modern complementary approaches include the administration of drugs to block hormone synthesis or hormone receptor activity. More recent technologies include manipulation of genes to block hormone production or hormone receptor function (see the section on genetic manipulation).

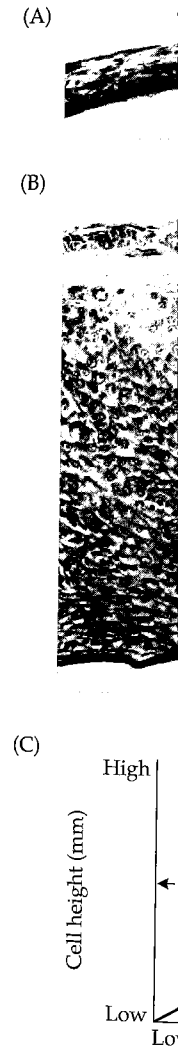
The replacement component of this technique has been improved by technological advances, especially new recombinant DNA methods, that have made highly purified hormones readily available. Access to this virtually pure material has allowed researchers to rule out contaminants as a cause of the physiological or behavioral effects of a particular hormone. In addition, recent studies have emphasized the importance of replacing hormones in patterns and doses (physiological doses) similar to those found in nature, rather than using a single pharmacological dose (usually resulting in hormone concentrations higher than physiological levels). This has been made possible by the availability of implantable timed-release hormone capsules and minipumps that provide precisely timed infusions of purified hormones.

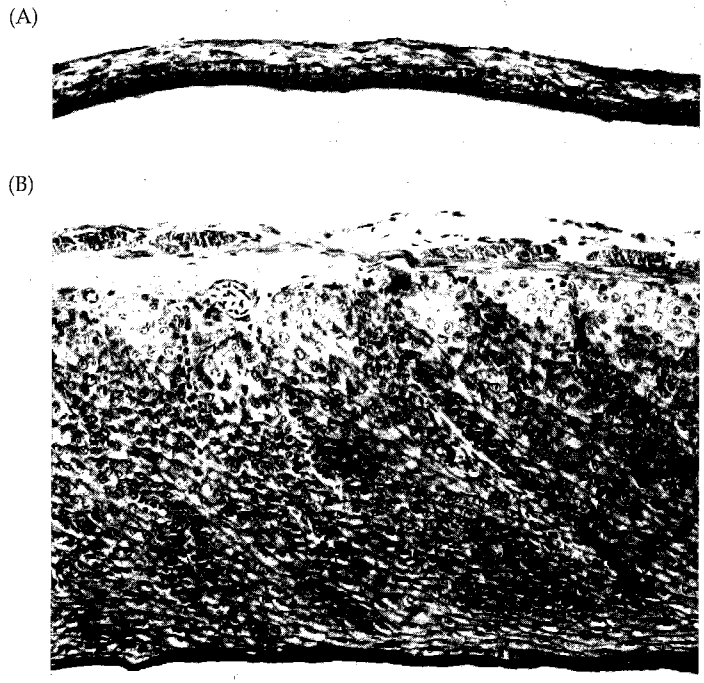
Bioassays

Once the existence of a hormone has been established, the next step is to identify the chemical processes involved in its actions. Classically, this has required a **bioassay**: a test of the effects of the hormone on a living animal. A living animal can serve as a reliable, quantifiable response system on which to test extracts and chemical fractions for biological activity.

A bioassay need not be conducted on the same species from which the hormone was obtained. For example, the crop sac of a pigeon (a structure that produces the "crop milk" fed to young pigeons; see Figure 7.6) can be used to measure levels of a hormone called prolactin in a rat. In incubating pigeons, prolactin stimulates growth of the epithelial cells of the crop sac to prepare it for the feeding of hatchlings; the height of the cells is correlated with the amount of prolactin in the pigeon's blood. To conduct a bioassay, researchers can inject different, known amounts of purified prolactin into pigeons, measure the resulting heights of the crop sac epithelial cells, and generate a standard dose–response curve (Figure 1.8). To measure the unknown prolactin levels of a rat, an extract from a tissue or blood sample obtained from the rat can be injected into a pigeon, and the resulting height of the epithelial cells measured and compared with the dose–response curve.

Probably the most famous endocrine bioassay was the so-called "rabbit test" (or Friedman test) for pregnancy. The rabbit test was developed by Maurice Friedman in 1929 and was the most commonly used pregnancy test in North America until the late 1950s. This procedure tested for the presence of human chorionic gonadotropin (hCG; a hormone released from the implantation site of a blastocyst that prevents menstruation) in the urine of women. The urine was injected into a rabbit, and if hCG was present in the urine, the rabbit's ovaries would form corpora lutea (ovarian endocrine structures formed following ovulation; see Chapter 2) within 48 hours. The rabbit test had several advantages over





1.8 A bioassay for prolactin. (A) Photomicrograph of an untreated pigeon crop sac. (B) Prolactin injection causes a marked increase in the height of crop sac epithelial cells. (C) Different known amounts of purified prolactin can be injected into pigeons to generate a dose–response curve (solid line). The levels of prolactin in pituitary extracts from some other animal (e.g., a rat) can then be measured by injecting a pigeon with an extract sample from the animal and comparing any resulting change in epithelial cell height with the dose–response curve (dashed line). Photomicrographs courtesy of C. S. Nicoll.

the previously used Aschheim–Zondek mouse test (developed in 1928), which required six or more mice and at least 96 hours to complete. However, the rabbit test also had disadvantages. False positives could be obtained if the rabbit used was unduly stressed by the test procedure because stress can cause spontaneous corpora luteal formation in the absence of hCG.

Because biological systems are inherently subject to fluctuations induced by environmental conditions, it is essential that the conditions under which bioassays are conducted be rigidly defined. For example, in the Galli–Mainini

or "frog test" for pregnancy, urine from a woman was injected into a male frog or toad. If the woman was pregnant and producing hCG, the animal would begin to produce sperm. The test provided results in 2–4 hours, and frogs were cheaper to maintain than mammals. However, there were variations in the sensitivity of the frogs: they had a greater tendency to yield "false negatives" during the summer.

The potential for contamination presents a problem in bioassays. Because the concentrations of hormones circulating in the bloodstream are so small, it is quite possible for contaminants in the tissue samples to interfere with the results. Furthermore, a bioassay is obviously only as good as the purified "standard" hormone used to calibrate the dose–response curve. For example, if the "purified" prolactin injected into pigeons in the crop sac bioassay had been contaminated by some other agent that suppressed cell growth, the resulting dose–response curve would not be accurate. Indeed, many early reports of endocrine effects on behavior and physiology were inaccurate because of contamination by hormones other than the so-called "pure" hormones. In recent years, with the advent of molecular biology tools to detect and measure biological products, the bioassay technique has been used less frequently.

BEHAVIORAL BIOASSAYS Several behavioral bioassays for hormones have been developed and used. The "water drive" of newts after injection of prolactin is one example. Prolactin causes newts to seek water; high levels of prolactin in a test sample cause a faster trip to water than low prolactin levels. A more common behavioral bioassay, which measured the behavioral effects of estrogens on the mating posture of female guinea pigs, was used for many years as the most sensitive assay for measuring circulating levels of these hormones. Again, bioassays require rigorous standardization of test conditions for accuracy and reliability. Even minute amounts of estrogen-like plant compounds (phytoestrogens) in their food are sufficient to induce female guinea pigs to display a mating posture. Consequently, laboratory personnel who used guinea pigs for estrogen behavioral bioassays had to make certain that the animals' chow did not contain alfalfa or other phytoestrogen-rich ingredient(s).

Immunoassays

Bioassays were useful because they measured a biological response to the hormone in question. In some cases, they allowed the determination of the presence or absence of a substance (as in the rabbit test), and in others they allowed quantitative measurement of specific hormones (as in the pigeon crop sac test for prolactin). However, bioassays usually required a great deal of time, labor, and the sacrifice of many animals for every assay conducted. The development of the **radioimmunoassay (RIA)** technique reduced these problems and increased the precision with which hormone concentrations could be measured. The ability to measure hormones precisely was such an important scientific advancement that one of the developers of this technique, Rosalyn Yalow, won the 1977 Nobel Prize

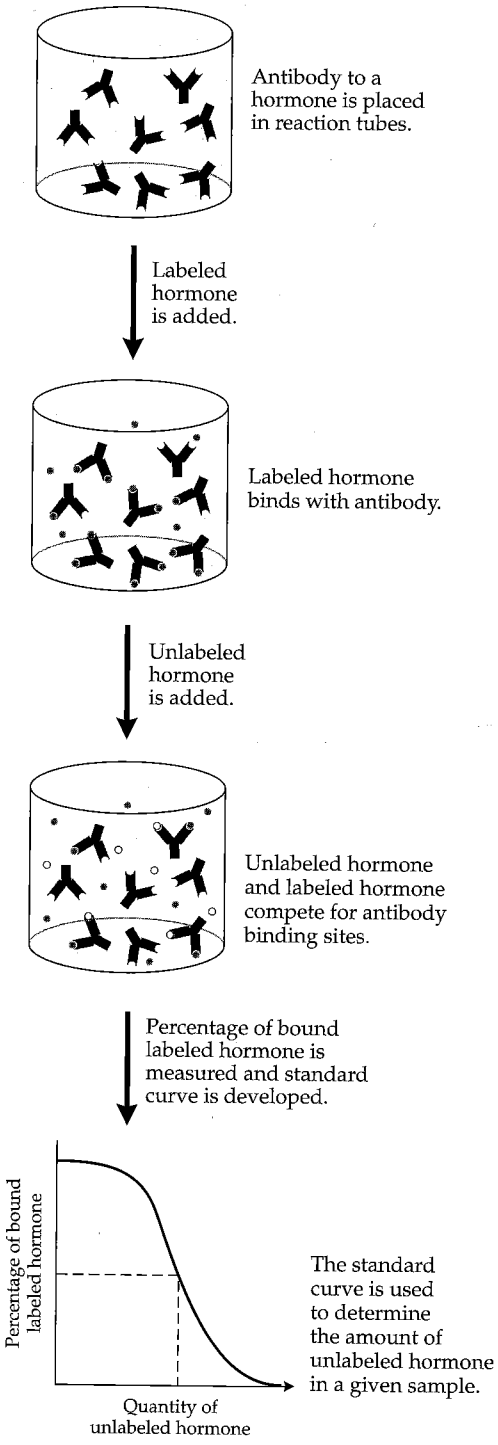
in Physiology or Medicine. (Her close collaborator, Solomon A. Berson, died in 1972, and Nobel Prizes are not awarded posthumously.)

The concept of RIA is based on the principle of competitive binding of an antibody to its antigen. An antibody produced in response to any antigen, in this case a hormone, has a binding site that is specific for that antigen. A given amount of antibody possesses a given number of binding sites for its antigen. Antigen molecules can be "labeled" with radioactivity, and an antibody cannot discriminate between an antigen that has been radiolabeled (or "hot") and a normal, nonradioactive ("cold") antigen.

The first step in a radioimmunoassay is to inject the hormone of interest into an animal (usually a rabbit) to raise antibody; the antibody is then collected from the animal's blood and purified. To develop a standard curve, several reaction tubes are set up, each containing the same measured amount of antibody, the same measured amount of radiolabeled hormone, and different amounts of cold purified hormone of known concentration. The radiolabeled hormone and cold hormone compete for binding sites on the antibody, so the more cold hormone that is present in the tube, the less hot hormone will bind to the antibody. The quantity of hot hormone that was bound can be determined by precipitating the antibody and measuring the associated radioactivity resulting from the radiolabeled hormone that remains bound. The concentration of hormone in a sample can then be determined by subjecting it to the same procedure and comparing the results with the standard curve (Figure 1.9).

As is the case with other techniques, there are limitations to this method. First of all, RIAs require a source of highly purified hormone in order to prepare a highly specific antibody against it, so contamination of the hormone used to generate antibodies is a potential problem. In addition, because many hormones have similar chemical structures, RIAs must be tested for specificity to rule out the possibility that the antibody recognizes other antigens in addition to the one of interest. There is also the possibility that the antibody may bind not only to the intact hormone molecule, but also to a fragment of the hormone molecule that lacks biological activity. For these reasons, when the results of an RIA do not agree with those of a bioassay, the bioassay may be considered more valid even if it is less precise.

The **enzymoimmunoassay** (**EIA**), like the RIA, works on the principle of competitive binding of an antibody to its antigen. The major difference between the RIA and EIA techniques is that EIAs do not require radioactive tags. Instead, the antibody is tagged with a chromogenic compound, which changes optical density (color) in response to its binding with antigen. The home pregnancy test is a familiar example of an EIA. This test, like the rabbit test, is designed to give a yes-or-no answer. However, most EIAs are developed to provide quantitative information. A standard curve is generated, as for RIAs, so that different known amounts of the hormone in question provide a gradient of color that can be read on a spectrometer. The unknown sample is then added, and the amount of hormone is interpolated by the standard curve. A similar technique is called the **enzyme-linked immunosorbent assay** (**ELISA**).



Immunocytochemistry

Immunocytochemistry (**ICC**) techniques use antibodies to determine the location of a hormone or hormone receptors in the body. Antibodies linked to marker molecules, such as those of a fluorescent dye (see Polak and Van Noorden, 1997), are usually introduced into dissected tissue from an animal, where they bind with the hormone or neurotransmitter of interest. For example, if a thin slice of brain tissue is immersed in a solution of antibodies to a hormone protein linked to a fluorescent dye, and the tissue is then examined under a fluorescent microscope, concentrated spots of fluorescence will appear, indicating where the protein hormone is located (Figure 1.10). Fluorescent dyes used for ICC include fluorescein and rhodamine. Other commonly used markers are the enzyme horseradish peroxidase, for bright-field or electron microscopy; the enzyme alkaline phosphatase, for biochemical detection; and the iron-containing protein ferritin, for electron microscopy.

A common alternative ICC technique involves raising a second antibody against

1.9 Radioimmunoassay. Purified hormone (antigen) is injected into an animal to raise antibody, which is collected and purified. Measured amounts of purified, radioactively labeled hormone are added to measured amounts of the collected antibody in several reaction tubes. The antibody binds with the radiolabeled hormone to form reversible hormone–antibody complexes. When different amounts of unlabeled hormone are added to the reaction tubes, this "cold" hormone competes with the "hot" hormone for antibody binding sites and displaces some of it. The antibody is precipitated, and the radioactivity of the bound hormone from each reaction tube is measured. In this way, a standard curve can be developed that expresses the quantity of cold hormone as a decline in radioactivity. The hormone concentration in a blood sample can then be measured by using it as cold hormone in the same procedure and comparing its effect on measured radioactivity with the standard curve.

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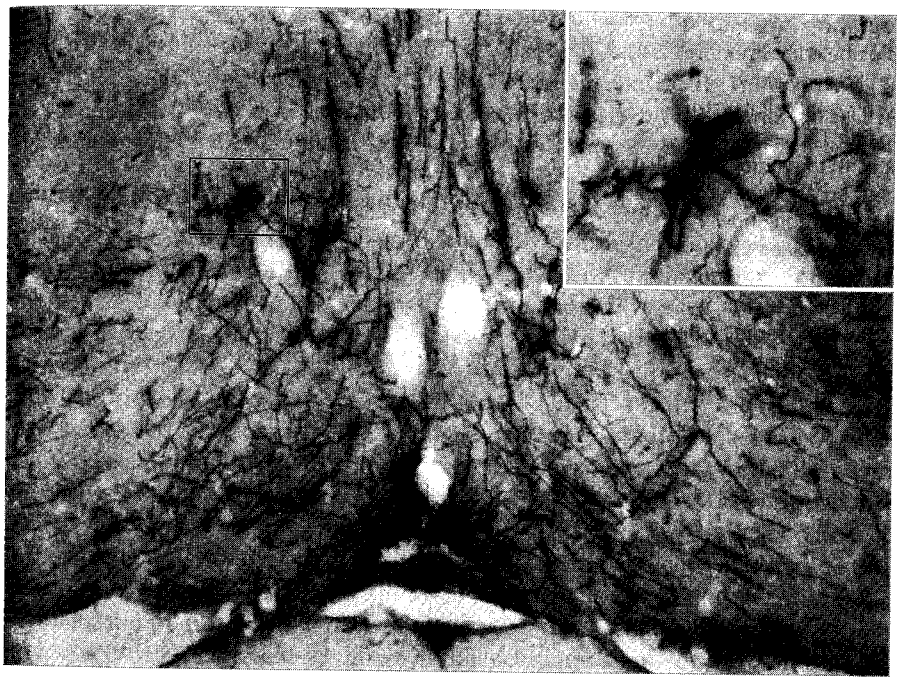
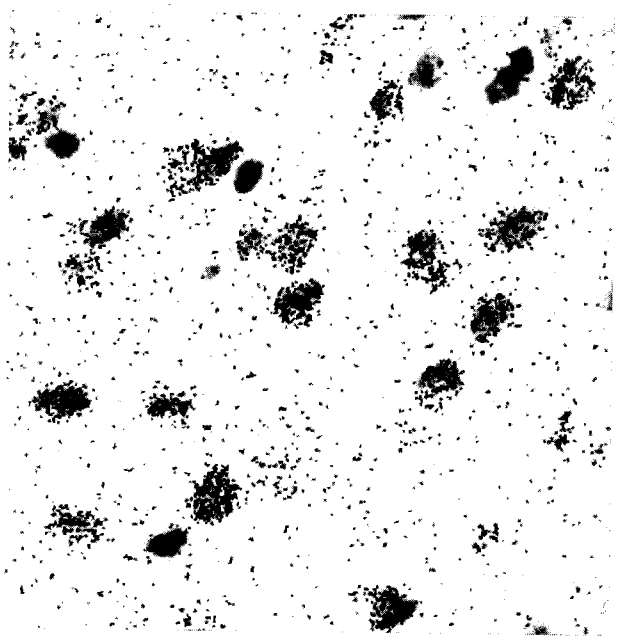


1.10 Immunocytochemistry. Antibodies to a hormone can be linked to a dye and used to determine the location of a hormone in the body. If a slice of tissue is exposed to a solution of antibody linked to such a marker, the binding of the antibody will cause those parts of the tissue containing the hormone to selectively take up the dye, making them more visible under the microscope. This figure is a low-power photomicrograph (×100) of a thin section of a rodent's brain showing immunocytochemically marked cell bodies and fibers that contain gonadotropin-releasing hormone (GnRH). The section is taken from the medial preoptic area (MPOA) at the level of the organum vasculosum of the lamina terminalis (OVLT). Box surrounds a neuron shown at high power (×400) in the inset. Note the appearance of beaded fibers characteristic of neurosecretory cells in the brain. Courtesy of Lance Kriegsfeld.

the primary antibody that recognizes the substance to be measured, then coupling the second antibody to a marker. Sometimes a secondary antibody is linked to biotin, a water-soluble vitamin, which has a strong binding affinity to avidin, a bacterial protein. If avidin is coupled to a marker molecule, it can be used in place of a second antibody. A single biotin–antibody–antigen complex may link to multiple marker molecules, which essentially amplifies the signal indicating the presence of the antigen.

Autoradiography

Many studies have demonstrated that hormone receptors are selectively concentrated in particular target tissues; estrogen receptors, for example, are concentrated in the uterus. **Autoradiography** is typically used to determine hormonal uptake and indicate receptor location. An animal can be injected with a radiola-



1.11 Autoradiography. An animal is injected with a radiolabeled hormone, and adjacent tissue slices are either treated with stains to reveal cellular structures or exposed to film. Those parts of the tissue that bind with the radiolabeled hormone darken the film, and when combined with the stained section, show which cell structures have taken up the hormone in the largest amounts. In this autoradiograph, cell nuclei in a monkey brain that have accumulated radiolabeled estrogen are seen as darker than the background neurons. Courtesy of Bruce McEwen.

beled hormone, or the study can be conducted entirely in vitro. Suspected target tissues are sliced into several very thin sections; adjacent sections are then subjected to different treatments. One section of the suspected target tissue is stained in the usual way to highlight various cellular structures. The next section is placed in contact with photographic film or emulsion for some period of time, and the emission of radiation from the radiolabeled hormone develops an image on the film. The areas of high radioactivity on the film can then be compared with the stained section to determine how the areas of highest hormone concentration correlate with cellular structures (Figure 1.11). This technique has been very useful in determining the sites of hormone action in nervous tissue, and consequently has increased our understanding of hormone–behavior interactions.

Blot Tests

Other techniques allow whether or not a particular protein or nucleic acid is present in a specific tissue. In the so-called **blot tests**, the tissue of interest is homogenized and the cells are lysed with detergent. The resulting homogenate is placed in gel, which is subjected to electrophoresis. **Electrophoresis** refers to the application of an electric current to a matrix or gel, which results in a gradient of molecules separating out along the current on the basis of size (smaller molecules move farther than larger molecules during a set time period). The homogenate is transferred to a membrane or filter, and the filter is then incubated with a labeled substance that can act as a tracer for the protein or nucleic acid of interest: radiolabeled complementary deoxyribonucleic acid (cDNA) for a nucleic acid assay, or an antibody that has been radiolabeled or linked to an enzyme for a protein assay. If radiolabeling is used, the filter is then put over film to locate and measure radioactivity.

In enzyme-linked protein assays, the filter is incubated with chromogenic chemicals, and standard curves reflecting different spectral densities are generated. The test used to assay DNA is called Southern blotting, after its inventor, E. M. Southern; the test used to assay RNA is called Northern blotting, and the test for proteins is called Western blotting.

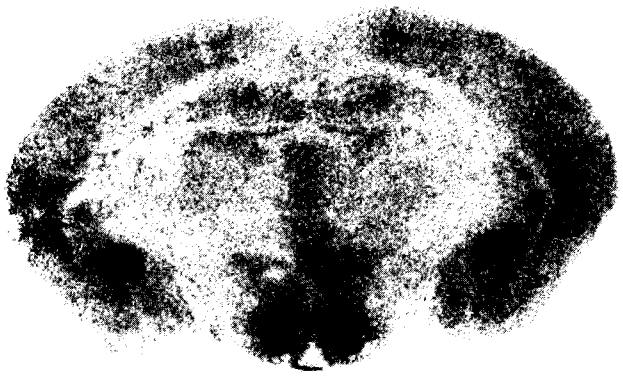
Autoradiography Using In Situ Hybridization

An important tool used at the cellular level to examine gene expression is called **in situ hybridization**. This technique is used to identify cells or tissues in which messenger RNA (mRNA) molecules encoding a specific protein—for example, a hormone or a neurotransmitter—are being produced. The tissue is fixed, sliced very thinly, mounted on slides, and either dipped into emulsion or placed over film and developed with photographic chemicals. Typically, the tissue is also counterstained to identify specific cellular structures. A radiolabeled cDNA probe is introduced into the tissue. If the mRNA of interest is present in the tissue, the cDNA will form a tight association (that is, hybridize) with it. The tightly bound cDNA, and hence the mRNA, will appear as dark spots (Figure 1.12). The techniques previously described, such as blot tests, can typically determine only whether or not a particular substance is present in a specific tissue, but in situ hybridization can be used to determine whether a particular substance is *produced* in a specific tissue. Recent advances in the technique allow for the quantification of the substance being produced. Blot tests cannot match the resolution or sensitivity of in situ hybridization.

Stimulation and Recording

By using **electrical stimulation** to "turn on" specific neurons or brain centers, we can discover the effects of various endocrine treatments on the central nervous system. In this technique, a fine electrode is precisely positioned in the brain, and a weak electric current is used to stimulate neurons. This technique has been used to study the releasing and inhibiting hormones of the hypothalamus (see Chapter 2).

The electrical activity of single neurons can be monitored through the use of **single-unit recording**, which involves the placement of very small electrodes



1.12 In situ audioradiography. The dark spots at the bottom of this slice of a rodent brain represent cells in the ventromedial hypothalamus (VMH) that contain mRNA for an oxytocin receptor. This tissue was treated with a specific cDNA that hybridized with the mRNA that encodes the oxytocin receptor. Courtesy of Thomas Insel and Larry Young.

in or near one neuron to record changes in its activity during and immediately after exposure to hormones. This technique can help to uncover the direct effects of various endocrine products on neural activity. Often, several neurons are recorded simultaneously and an average change in activity in these multiple units is calculated.

Pharmacological Techniques

The development of synthetic **agonists** (mimics) and **antagonists** (blockers) of hormones for medical purposes has taught us a great deal about the functioning of the endocrine system. Some specific chemical agents act to stimulate or inhibit endocrine function by affecting hormonal release; these agents are called general agonists and antagonists, respectively. Other drugs act directly on hormone receptors, either enhancing or negating the effects of the hormone under study; these drugs are referred to as receptor agonists and antagonists, respectively. Cyproterone acetate (CPA), for example, is a powerful anti-androgen (anti-testosterone) that has been used clinically as a treatment for male sex offenders (see Chapter 5). This antagonist binds to testosterone receptors but does not activate them, thereby blocking the effects of testosterone on behavior and physiology. Other examples of hormone agonists and antagonists will be presented throughout the book.

In the technique known as **cannulation**, hollow electrodes or fine tubes (cannulas) are inserted into specific areas of the brain and used to introduce substances into those sites. Davidson (1966a) used this technique to find out where in the brain testosterone acts to influence sexual behavior in rats. In Davidson's study, male rats were castrated, after which they were observed to cease mating. Testosterone was then introduced through cannulas into different areas of the brain in different rats; a control group of rats received cholesterol, the precursor of testosterone. Those animals that received testosterone in one specific location, the preoptic area of the hypothalamus, resumed sexual behavior; the rats that received cholesterol or received testosterone in other brain regions did not respond to the treatment.

Another type of cannulation involves inserting a small hollow tube into the jugular vein, carotid artery, or other blood vessel. In this way, specific hormones or pharmacological agents can later be injected directly into the animal without further disturbance, or blood samples can be obtained to correlate hormone levels with behavior. In a related technique, **anastomosis**, the blood systems of two animals are connected via cannulation tubing to see if the endocrine condition of one animal can cause a behavioral change in the other.

Microdialysis

The **microdialysis** technique is based on the principle of dialysis, in which a semipermeable membrane, which allows passage of water and small molecules, divides two fluid compartments. Developed in the 1980s, microdialysis allows assessment of responses to neurotransmitters, drugs, and hormones in a conscious animal (DeLange et al., 1997). Typically, a cannula that is divided into two compartments by a semipermeable membrane is implanted in the brain region of interest using

stereotactic surgery. The end of one compartment is continuously perfused with a liquid, and molecules are exchanged with the extracellular fluid by diffusion in both directions. Hormones or drugs can be delivered through one part of the cannula while extracellular signaling molecules (e.g., neurotransmitters) can be monitored via the second compartment. Because microdialysis can be performed in awake, freely moving animals, this method is especially well-suited for the study of the interactions of hormones, neurotransmitters, and behavior. The method can both introduce and remove molecules from the brain. It is possible to sample continuously for hours or days. Typically, the samples are analyzed with high-performance liquid chromatography (HPLC) to detect such substances as amino acids, acetylcholine, biogenic amines, choline, glucose, histamine, and purines. Microdialysis is sometimes performed from the human brain for diagnostic purposes.

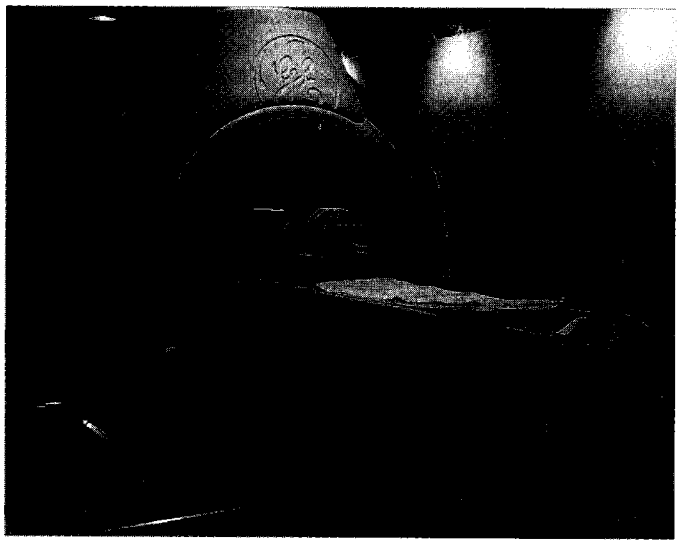
Brain Imaging

Several brain scanning techniques are used in behavioral endocrinology to determine brain structure and function (DeLange et al., 1997; Van Bruggen and Roberts, 2002). Comparisons can be made, for example, between the brains of men and women or among those of individuals in different hormonal conditions. One important scanning technique used to determine regional brain activation is called **positron emission tomography** (**PET**). Unlike a simple X-ray or CT scan, which reveals only anatomical details, PET scanning permits detailed measurements of real-time functioning of specific brain regions of people who are conscious and alert. PET gives a dynamic representation of the brain at work. Prior to the availability of PET scanners, changes in neurotransmitter levels or hormonal activation of specific circuits could only be inferred on the basis of autopsy data.

Before the PET scan begins, a small amount of a radioactively labeled molecule that mimics glucose or a radioactive gas such as oxygen-15 is injected into the individual. When neurons become more active, they use more glucose and oxygen, so the radioactive material is taken up at high rates by the most active neurons. This radioactive material emits positrons. When a positron collides with an electron, the collision produces two gamma rays that leave the body in opposite directions and can be detected by the PET scanner. This information about where glucose is being metabolized or oxygen is being used is then converted into a complex picture of the person's functioning brain by a computer.

A **computer-assisted tomography** (**CT**) scanner shoots fine beams of X-rays into the brain from several directions. The emitted information is fed into a computer that constructs a composite picture of the anatomical details within a "slice" through the brain of the person. **Magnetic resonance imaging** (**MRI**) does much the same thing, but uses non-ionizing radiation formed by the excitation of protons by radio-frequency energy in the presence of large magnetic fields (Van Bruggen and Roberts, 2002). MRI can be used in anatomical studies, and assessing anatomical irregularities is its main function as a medical diagnostic tool. **Functional MRI** (**fMRI**) uses a very high spatial (~1 mm) and temporal resolution to detect changes in brain activity during specific tasks or conditions. Most fMRI studies require the person to lie still in a narrow tunnel (Figure 1.13), so only

1.13 A modern MRI scanner.

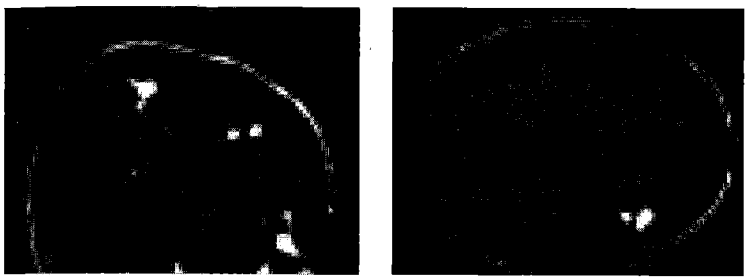


cognitive or affective changes can be monitored. As noted above, when neurons become more active, they use more energy, so they require additional blood flow to deliver glucose and oxygen. The fMRI scanner detects this change in cerebral blood flow by detecting changes in the ratio of oxyhemoglobin and deoxyhemoglobin. Deoxyhemoglobin is paramagnetic (becomes magnetic in magnetic fields), whereas oxyhemoglobin is diamagnetic (does not become magnetic in magnetic fields). Thus, deoxyhemoglobin molecules act like little magnets in the large magnetic field of the MRI and dephase the signal. When brain regions increase their activity, more oxygenated blood is present than before the activation. More oxyhemoglobin results in a net decrease in paramagnetic material (deoxyhemoglobin), which leads to a net increase in signal because of reduced dephasing of the signal. A complex computer program plots all of the phase changes of the signal and applies this picture on top of a structural picture of the brain usually obtained with a CT scan (Figure 1.14).

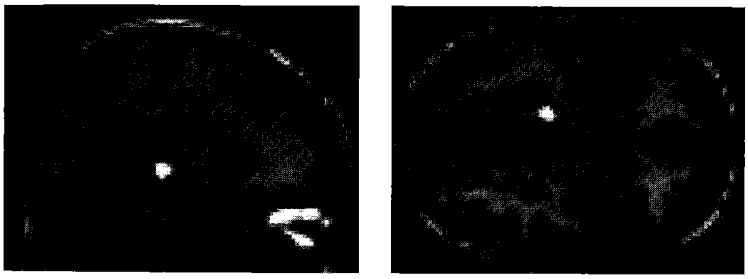
Genetic Manipulations

With new advances in molecular biology, it is possible to perform specific genetic manipulations. In behavioral endocrinology research, common genetic manipulations include the insertion (creating a **transgenic** organism) or removal (**knockout**) of the genetic instructions encoding a hormone or the receptor for a hormone. The genetic instructions for each individual are contained in its DNA, located in the nucleus of nearly every cell. These instructions are encoded in the form of four nucleotides, adenine (A), thymine (T), cytosine (C), and guanine (G). The specific order of these four nucleotides along the "rails" of the DNA double helix forms the genetic instructions for all organisms, from those as simple as slime molds to those as complex as mice and humans. Each **gene** represents the

(A) Women minus men



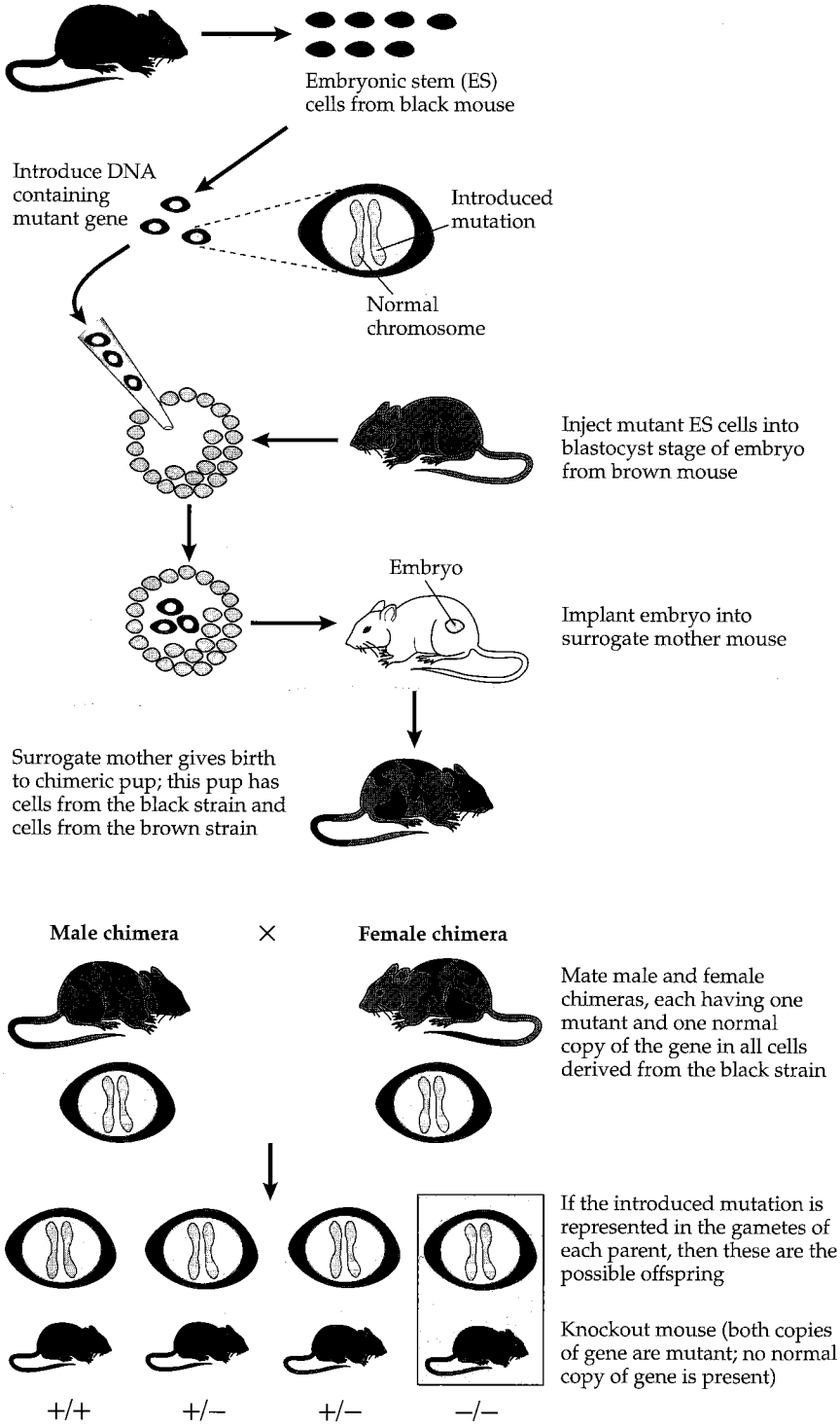
(B) Men minus women



1.14 fMRI. These images are the results of fMRI scans of groups of men's and women's brains that were superimposed on CT scans of the sagittal (left) and transverse (right) planes. The bright areas represent the most active parts of the brain. Men and women were asked to navigate (via keyboard) through a spatial maze. (A) There was more activation in the right inferior parietal lobe in women than in men. (B) There was more activation in the hippocampus in men than in women. The brains of men and women show similar activation throughout the brain; for instance, when you take the female brain activation map and subtract the male brain activation map, you get the difference indicated in (A) and (B). From Grön et al., 2000.

complex instructions for the production of a specific protein in the cell. Thus, nucleotide "syntax" is critical in conveying the instructions encoded in the genes. To inactivate, or knock out, a gene, molecular biologists scramble the order of the nucleotides that make up the gene (Aguzzi et al., 1994; Soriano, 1995).

Cloning a gene is different from cloning an animal. To make Dolly, the famous cloned sheep, the researchers in Scotland first obtained ova from the ovaries of a female sheep and destroyed the cell nucleus of each one. Then a new nucleus was obtained from cells of the adult sheep that was to be cloned. The manipulated ova were stimulated to divide, and one of the resulting embryos was implanted into a surrogate mother. Thus, Dolly was genetically-identical to the individual from which she was derived. To clone a gene, the gene must first be identified, then the specific piece of DNA that contains the gene is placed into a vector. A vector is another DNA molecule (usually from a simple organism such as bacteria or yeast) that can be inserted into a viral host. This produces a new DNA molecule termed recombinant DNA. This recombinant DNA is an impor-



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◀ **1.15 Engineering knockout mice.** DNA that has been engineered to contain a mutant (inactive) copy of the gene of interest is introduced into embryonic stem cells (ES cells) from black mice that are growing in tissue culture. The black coat color serves as a genetic marker. ES cells with one mutant copy of the gene are introduced into an early mouse embryo (blastocyst), which incorporates the cells into the body of the developing mouse. Mice that are born from this manipulation are called "chimeras." These chimeric mice are mated to each other. Only chimeric mice in which the ES cells have been incorporated into the germ line (gametes) will produce offspring carrying the mutation. By simple Mendelian genetics, one in four of those offspring will be knockout mice, which contain two mutant copies of the gene. The entire process takes 6–12 months.

tant tool in understanding the function of the gene in normal and pathological states.

Among vertebrates, the identification of genes has been most successful among laboratory mice (*Mus musculus*). Consequently, mice are currently the most commonly used species in targeted gene deletion studies. Knocking out a specific gene in a mouse is an arduous task that relies on several low-probability events. First, the gene of interest must be identified, targeted, and marked precisely (Figure 1.15). This has been accomplished for an astounding number of murine (mouse) genes during the past decade (Takahashi et al., 1994). Next, a mutated form of the gene is created (i.e., a piece of DNA that contains a genetically engineered, inactive copy of the gene of interest). Mouse embryonic stem cells (ES cells) are harvested and cultured, and copies of the altered gene are introduced into the cultured cells by microinjection (Tonegawa, 1994). A very small number of the altered genes will be incorporated into the DNA of the ES cells through recombination (Bernstein and Breitman, 1989; Sedivy and Sharp, 1989). The mutant ES cells are then inserted into otherwise normal mouse embryos (blastocysts), which are implanted into surrogate mothers (Boggs, 1990; Le Mouellic et al., 1990; Steeghs et al., 1995). The ES cells are equipotential, which means that they may become incorporated into any part of the developing body. All of the cells descended from the mutant ES cells will have the altered gene; the descendants of the original blastocyst cells will have normal genes. Thus, the newborn mice will have some cells that possess a copy of the mutant gene and some cells that possess only the normal (wild-type) gene. This type of animal is called a **chimera**. If the mutated ES cells have been incorporated into the germ line (the cells destined to become the sperm and eggs), then some of the mouse's gametes will contain one heritable copy of the mutant gene. If these chimeric mice are mated to each other, then approximately half of their offspring will be heterozygous for the mutation; that is, they will possess one copy of the mutant gene. Approximately one-fourth of their offspring will be homozygous for (i.e., have two copies of) the mutant gene; the product that the gene typically encodes will be missing from these homozygous (knockout) mice (Sedivy and Sharp, 1989). These homozygous mice can then be interbred to produce pure lines of mice with the gene of interest knocked out (Galli-Taliadoros et al., 1995).

Behavioral performance can then be compared among wild-type (+/+), heterozygous (+/–), and homozygous (–/–) mice, in which the gene product is pro-

duced normally, produced at reduced levels, or completely missing, respectively. The comparison of +/+ and −/− littermates of an F_2 recombinant generation is probably the minimal acceptable control in determining behavioral effects in knockout mice (Morris and Nosten-Bertrand, 1996). The use of new inducible knockouts, in which the timing and tissue-specific placement of the targeted gene disruption can be controlled, promises to be an extremely important tool in future behavioral endocrinology research (Nelson and Chiavegatto, 2001). Similarly, the use of transgenic animals in which there is overexpression of specific genes has become an increasingly common technique in behavioral endocrinology investigations.

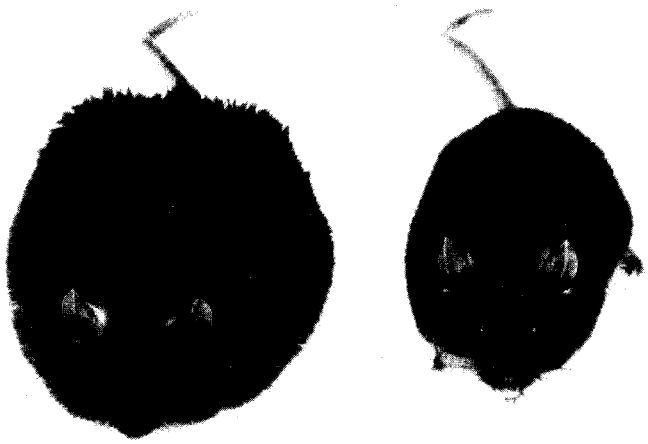
Gene Arrays

Another new technology that has become extremely useful in behavioral endocrinology is the **gene array** or **microarray**, which is a marriage of genomics and computer microprocessor manufacturing. Essentially, a minuscule spot of nucleic acid of known sequence (usually cDNA, although RNA is also used) is attached to a glass slide (or occasionally a nylon matrix) in a precise location, often by high-speed robotics. This identified, attached nucleic acid is called an **oligonucleotide** (Phimister, 1999). Because thousands of oligonucleotides (pieces of RNA or cDNA) can be added to the array, an experiment with a single array can provide researchers with information on thousands of genes simultaneously. The underlying principle of gene arrays is hybridization, the process by which nucleotide bases pair (i.e., A–T and G–C for DNA; A–U and G–C for RNA). A nucleic acid sample to be identified is called a **probe**. By observing which of the probes hybridize with the oligonucleotides on the array, we can identify some of the mRNAs that are present in the sample.

In behavioral endocrinology, gene arrays might be used to determine relative gene expression during the onset of a behavior, or during a developmental stage, or among individuals that vary in the frequency of a given behavior or hormonal state. For example, mRNA may be extracted from brain regions that are thought to regulate aggressive behavior. To see whether specific gene expression differs in this region between intact and castrated rats, the mRNA extracted from the brain tissue would be labeled with fluorescent dyes and added to a cDNA microarray, where it would be available for hybridization to the attached cDNA oligonucleotides. Any differences in hybridization between the samples would be indicated by changes in the color of the fluorescence readout. The relative amount of hybridization as compared to hybridization of a standard "housekeeping gene" would indicate the relative amount of gene expression in the tissue. Of course, the nucleic acid sequences of interest must be attached to the array to be detected; also, because the gene array usually provides only information about gene expression from pooled tissue samples, an additional method, such as quantitative PCR, must be conducted on individual samples.

A Case Study: Effects of Leptin on Behavior

Recently, a novel hormone was discovered that is released from **adipose** (fat) cells. This hormone was named **leptin**, a term derived from the Greek word *leptos*, which means "thin." Studies of the behavioral effects of leptin will be used here to



1.16 Behavioral effects of leptin. Both of these mice have defective *ob* genes. This mutation typically results in a large increase in food intake and subsequent obesity. The mouse on the right was treated daily with the protein encoded by the *ob* gene, called leptin. It weighs about 35 grams; the untreated mouse weighs about 67 grams. A mouse with a normal *ob* gene would weigh about 25 grams at this age. Photograph by John Sholtis.

demonstrate how the various techniques described above are used to understand the biological functions of hormones.

For many years, specific mutations in mice that cause extreme obesity have been recognized. Animals that are homozygous for a mutation in the *ob* gene are hyperphagic (overeat), obese, and reproductively sterile (Figure 1.16). These animals may be considered natural knockouts for the *ob* gene, which normally codes for leptin. Another gene, the *db* gene, encodes a leptin receptor; mice that are homozygous for a mutation in this gene are diabetic. Leptin is released by adipose cells into the bloodstream, where it travels to specific receptors in the central nervous system and elsewhere to regulate feeding and energy balance. Since its identification in 1994 (Zhang et al., 1994), many of the techniques described in the previous sections have been used to understand the behavioral functions of leptin.

For years, the effects of the *ob/ob* mutation on the ability to maintain normal body mass in mice have been known. However, research into the mechanisms underlying this mutation began in earnest only with the identification and sequencing of the leptin protein (Zhang et al., 1994). The *ob* gene was cloned, and a copy of the gene was inserted into bacteria; the resulting transgenic bacteria produced purified leptin. Using this purified leptin, a "replacement study" was conducted in which leptin was provided to *ob/ob* mice to determine whether replacing their missing leptin would ameliorate their hyperphagia, energy impairments, and obesity. It did.

The availability of purified leptin also allowed researchers to produce specific antibodies that could be used in developing assays to determine blood concen-

trations of leptin (e.g., Cohen et al., 1996). An RIA was developed to determine the blood plasma leptin concentrations of obese and diabetic humans to see whether leptin was implicated in human obesity or diabetes (McGregor et al., 1996); in this study, no connection was found. A leptin ELISA was developed for rats and mice, which was used to determine that fasting or exposure to low temperatures caused circulating leptin concentrations to fall (Hardie et al., 1996). Immunocytochemistry techniques revealed that leptin was present in both white and brown adipose tissue (Cinti et al., 1997) as well as other peripheral tissues.

Injections of leptin into the third or lateral ventricles in the brains of mice were found to reduce food intake (Campfield et al., 1995). To determine the site of action of leptin in the brain, purified leptin was radiolabeled and injected into mice. Using autoradiography, dense, specific binding of the radiolabeled leptin was found in the choroid plexus, a brain structure located in the dorsal part of the third ventricle, as well as in the lateral ventricles (Lynn et al., 1996). This tissue was then used to clone a leptin receptor (Tartaglia et al., 1995). In situ hybridization showed that the mRNA for the leptin receptor was expressed in several brain regions, including the hypothalamus (Mercer et al., 1996; Zamorano et al., 1997).

In an effort to "cure" obesity, gene replacement therapy was attempted (Chen et al., 1996; Muzzin et al., 1996). The assumption behind this effort was that mice deficient in the wild-type *ob* gene were obese because of a deficiency of leptin. If the leptin or the *ob* gene were replaced, then the individual should display normal food intake and body mass. When *ob/ob* mice were treated with a recombinant adenovirus containing the mouse *ob* gene, there was a dramatic reduction in both food intake and body mass (Muzzin et al., 1996). Similar findings were reported for rats (Chen et al., 1996). In addition to being obese, *ob/ob* mice are also sterile; treatment of *ob/ob* mice with recombinant leptin reverses the sterility (Chehab et al., 1996; Mounzih et al., 1997).

These exciting findings suggested the possibility of treating obese humans with gene therapy. However, leptin appears to have significant weight-reducing properties only in rodents. Despite screening of thousands of obese humans, only five individuals in two families have been found with mutations in the leptin gene (Montague et al., 1997; Strobel et al., 1998) (i.e., they are like the *ob/ob* mice), and three members of a single family have been found that are homozygous for mutations in the leptin receptor gene (Clement et al., 1998) (i.e., they are like the *db/db* mice). Only moderate obesity is observed among people who are heterozygous (one normal and one mutant copy) for their leptin genes (Farooqi et al., 2003). The effects of leptin treatment on human obesity have been equivocal. Treatment of a 9-year-old girl who is homozygous for a mutation in the leptin gene with daily injections of leptin for a year reduced her body mass from 94.4 kg (208 pounds) (55.9 kg was estimated to be adipose tissue [i.e., fat] to 78 kg (172 pounds) (Farooqi et al., 1999). It was reported that the leptin injections reduced her appetite and food intake. Treatment of obese individuals with normal leptin genes resulted in moderate body mass loss (–7.1 kg versus –1.3 kg for a placebo group). Because obese people tend to have higher levels of leptin than lean people, it has been proposed that obese individuals are "resistant" to leptin. Recently, it has been proposed that obesity is a disease of the blood–brain bar-

rier (BBB). According to this hypothesis, obesity occurs in some individuals because leptin cannot cross the BBB to signal the brain that sufficient fat reserves exist (Banks, 2003). Although leptin has only been identified for about a decade, much has been learned about this hormone; however, additional research is required to sort out the role of leptin in human and nonhuman energy balance.

Taken together, all of these techniques are useful in elucidating hormone–behavior relationships; later in the book, other specific techniques will be introduced as we examine the details of specific hormone–behavior interactions. In the next chapter, many of the fruits of these techniques will be presented in the context of a general introduction to endocrine anatomy, chemistry, and physiology.

Summary

- Behavioral endocrinology is the study of the interaction between hormones and behavior. This interaction is bidirectional: hormones can affect behavior, and behavior can influence hormone concentrations.
- Hormones are chemical messengers that are released from endocrine glands and travel through the bloodstream to target cells, where they induce changes in the rate of cellular function. The endocrine system and the nervous system work together to regulate the physiology and behavior of individuals.
- Behavior is generally thought of as involving movement, but nearly any type of output can be considered behavior. A complete description of behavior is required before researchers can address questions of its causation. All behavioral biologists study a specific version of the general question, "What causes animal A to emit behavior X?"
- The four interacting levels of analysis that can be used for exploring and explaining the causes of behavior are immediate causation, development, evolution, and adaptive function.
- Hormones influence behavior by increasing the probability that a particular behavior will occur in the presence of a particular stimulus. Hormones can influence behavior by affecting an animal's sensory systems, integrators, and/or effectors or peripheral structures. Hormones can also be influenced by behavior.
- Three types of evidence are necessary to establish a causal link between hormones and behavior: (1) a behavior that depends on a particular hormone should diminish when the source of, or actions of, the hormone are removed, (2) the behavior should reappear when the hormone is reintroduced, and (3) hormone levels and the behavior in question should be covariant.
- Several techniques have been useful in advancing research in behavioral endocrinology: ablation and replacement; bioassays; modern assays that utilize the concept of competitive binding of antibodies; autoradiography; immunocytochemistry; electrical stimulation and single-unit recording; pharmacological methods; methods that make use of cannulation, including in vivo microdialysis; and gene arrays and genetic manipulations.

Questions for Discussion

1. What are some of the problems associated with attempting to determine causation in a hormone–behavior interaction? What are the best ways to address these problems?
2. An experimenter is interested in the effect of a particular hormone on aggressive behavior in butterflies. It is hypothesized that butterflies fight because of high levels of this hormone. An alternative hypothesis is that the butterflies that fight to guard territories and potential mates produce more offspring in subsequent generations. If the results of an experiment rule out the hypothesis that the hormone is required for aggressive behavior, does that necessarily mean that the alternative hypothesis is true? Why or why not?
3. Hormones cause changes in the rates of cellular processes or in cellular morphology. Suggest some ways that these hormonally induced cellular changes might theoretically produce profound changes in behavior.
4. In recent years, investigations of hormone–behavior relationships have involved increasingly elaborate and precise methodology for identifying and locating hormones. Discuss the proposition that comparable increases in sophistication, quantification, and so forth are needed on the side of behavior. Give examples of desirable and undesirable approaches.
5. In lesion studies or studies of animals with specific genes deleted, the behavioral tests study the effects of the *missing* brain region or the *missing* gene, respectively and not the effects of the brain region directly. Discuss how these conceptual shortcomings can be overcome in evaluating the results of studies using these types of procedures.

Refer to the accompanying Student CD for additional resources, including Web links, videos, animations, and additional photos.

Suggested Readings

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