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).

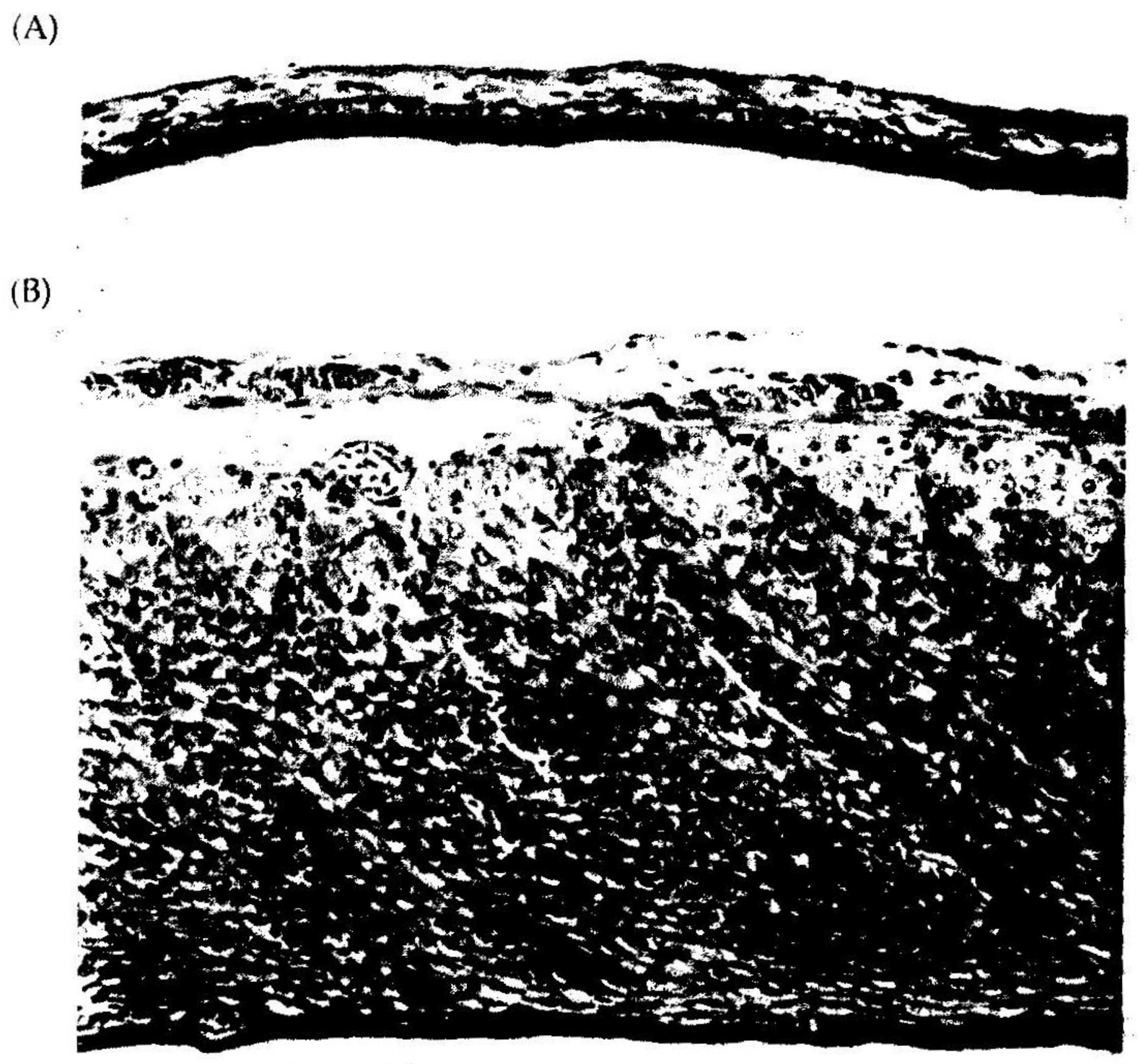
The replacement component of this technique has been improved by technological advances, especially new recombinant DNA methods, that have made highly purified hormones readily available. Access to this virtually pure material has allowed researchers to rule out contaminants as a cause of the physiological 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.

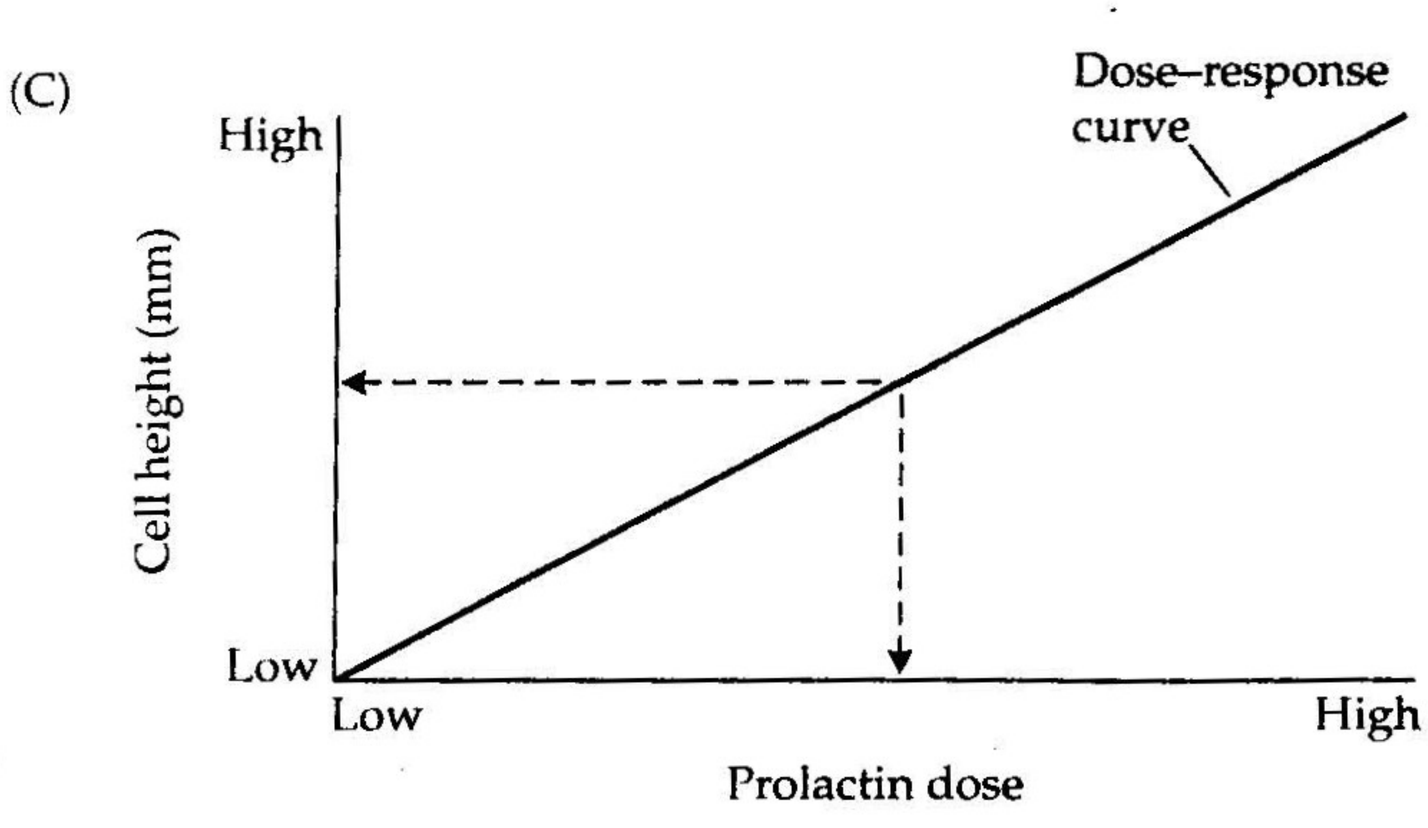
Bioassays

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

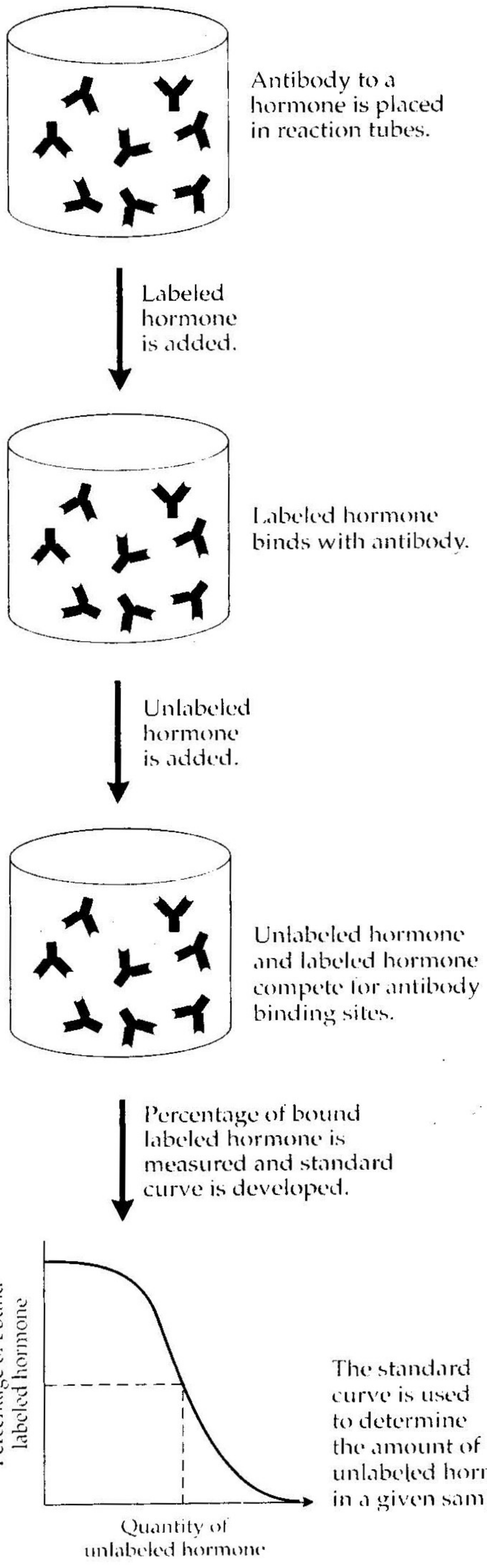
in Physiology or Medicine. (Her close collaborator, Solomon A. Berson, died in 1972, and Nobel Prizes are not awarded posthumously.)

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

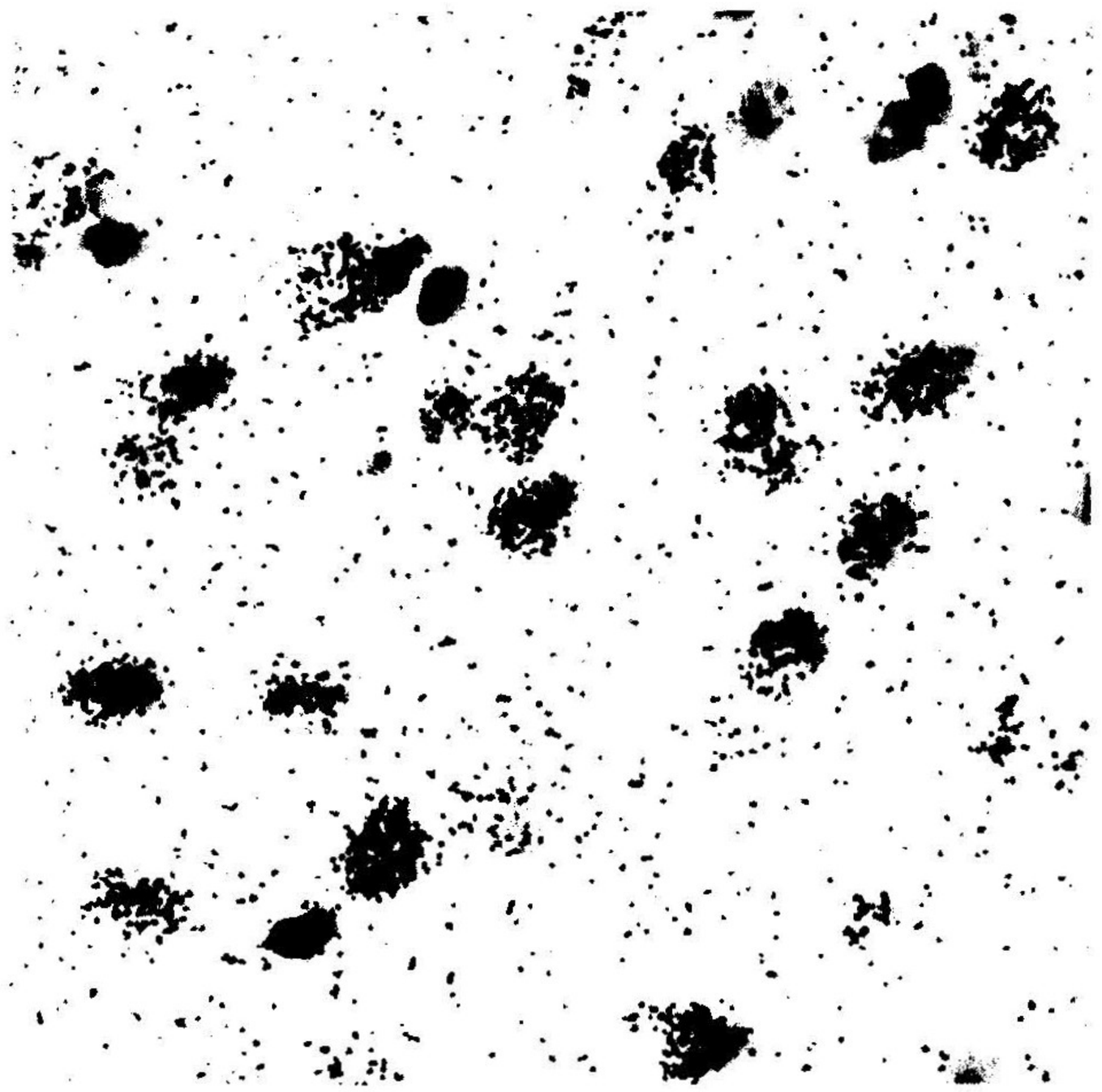
1.9 Radioimmunoassay. Purified hormone (antigen) is injected into an animal to raise antibody, which is collected and purified. Measured amounts of purified, radioactively labeled hormone are added to measured amounts of the collected antibody in several reaction tubes. The antibody binds with the radiolabeled 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.

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.

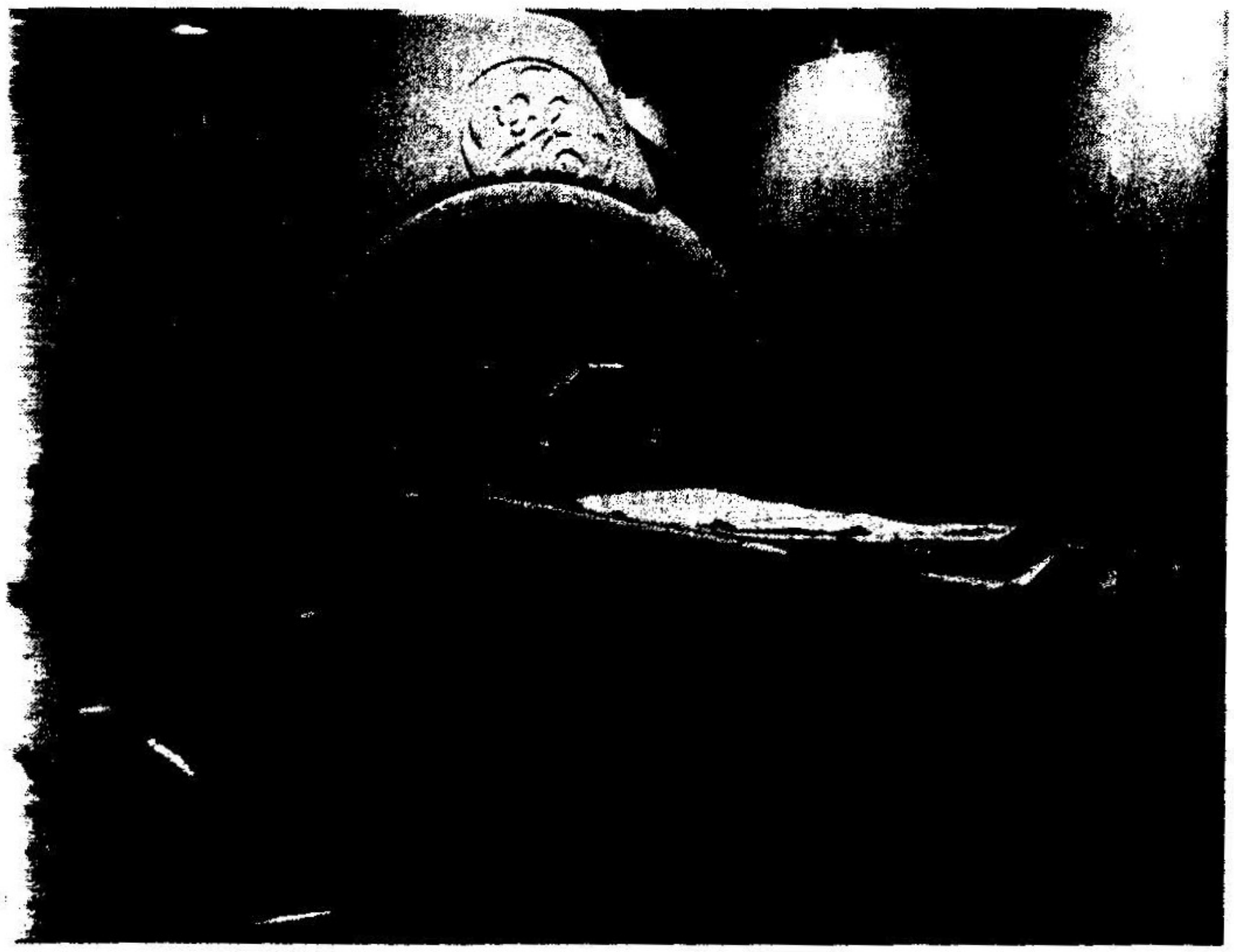
Brain Imaging

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

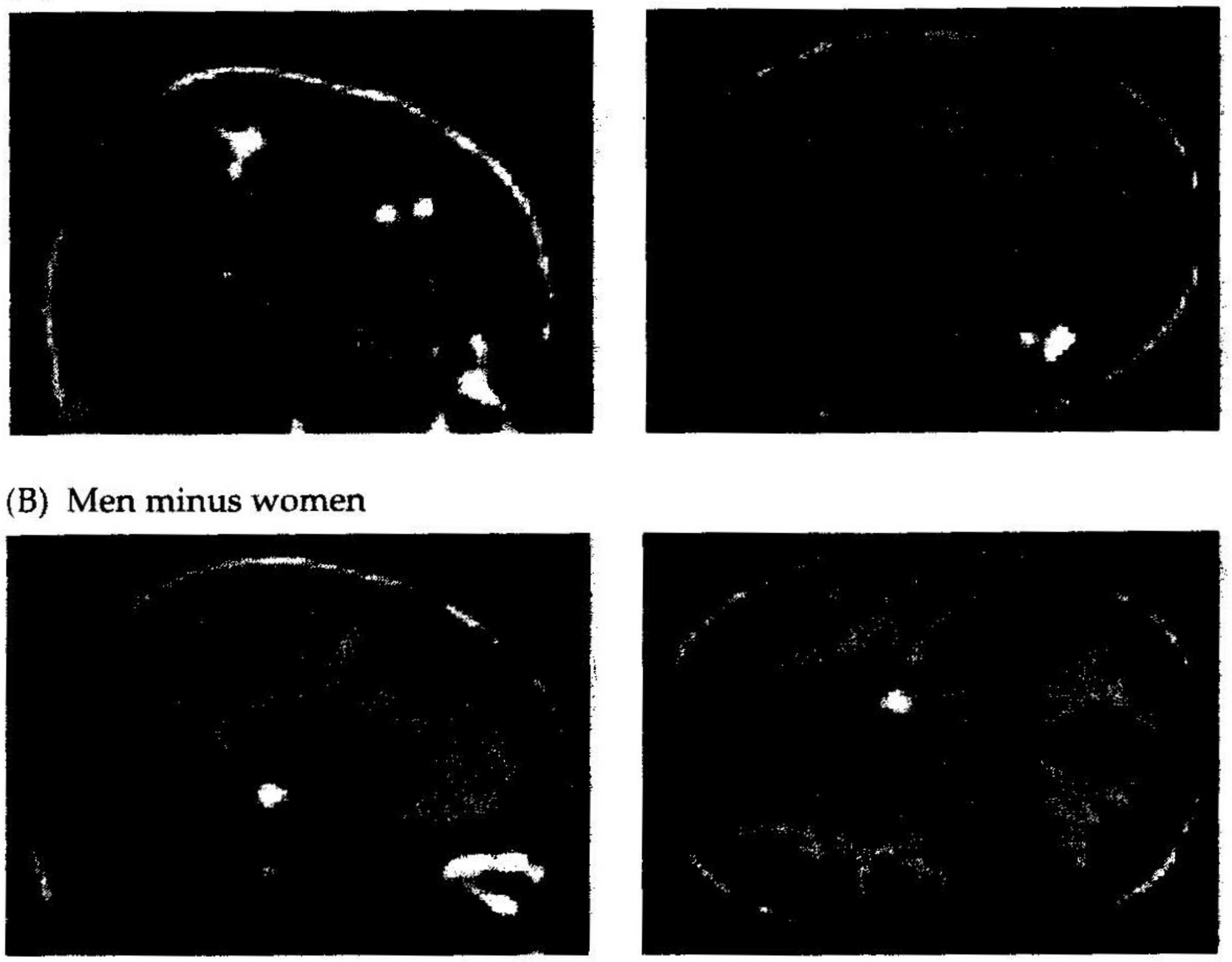
1.13 A modern MRI scanner.



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

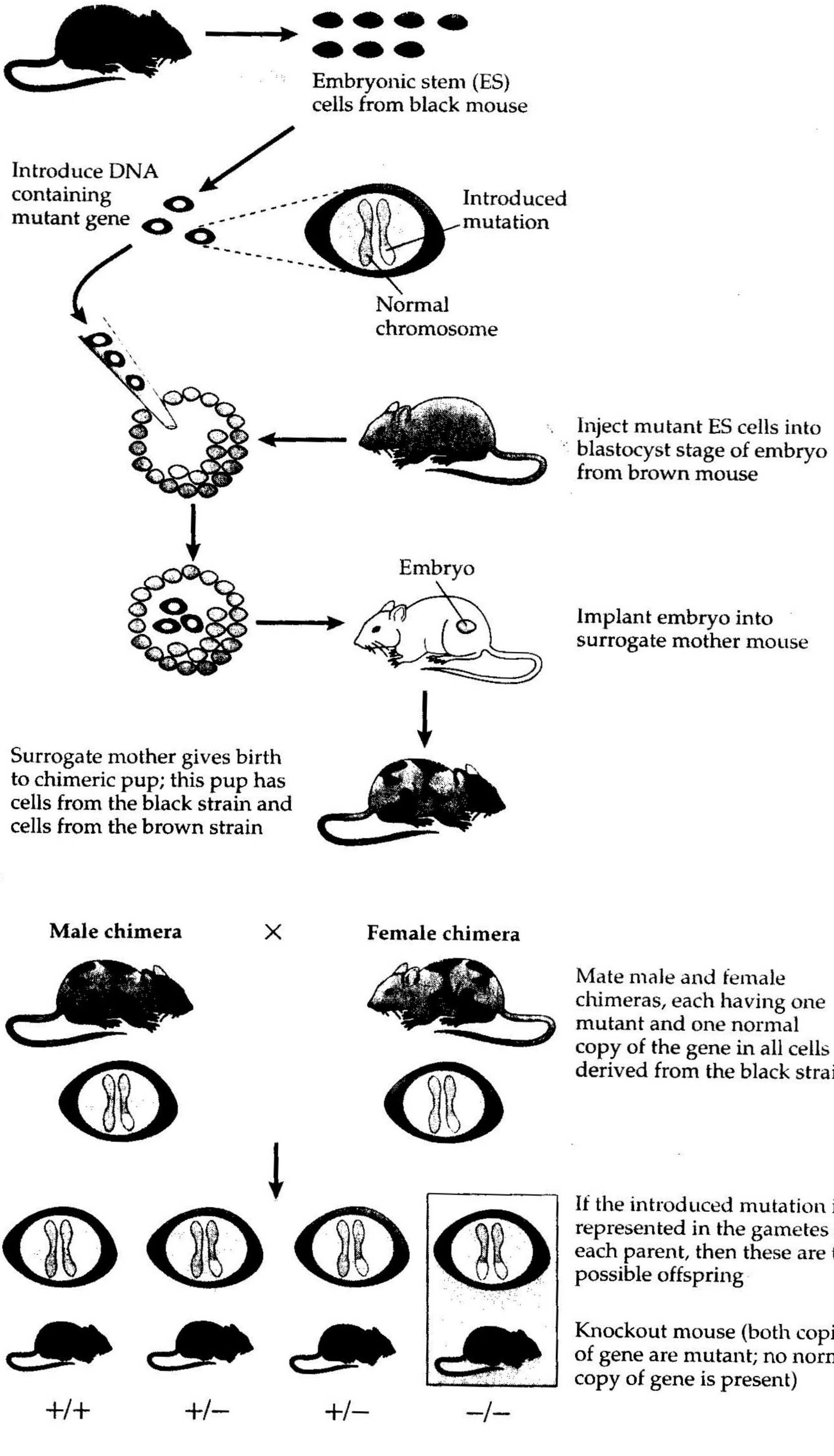
With new advances in molecular biology, it is possible to perform specific genetic manipulations. In behavioral endocrinology research, common genetic manipulations include the insertion (creating a **transgenic** organism) or removal (**knockout**) of the genetic instructions encoding a hormone or the 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



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-


Embryonic stem (ES) cells from black mouse
Introduce DNA containing mutant gene
Introduced mutation
Normal chromosome
Inject mutant ES cells into blastocyst stage of embryo from brown mouse
Embryo
Implant embryo into surrogate mother mouse
Surrogate mother gives birth to chimeric pup; this pup has cells from the black strain and cells from the brown strain
Male chimera
×
Female chimera
Mate male and female chimeras, each having one mutant and one normal copy of the gene in all cells derived from the black strain
If the introduced mutation is represented in the gametes of each parent, then these are the possible offspring
Knockout mouse (both copies of gene are mutant; no normal copy of gene is present)
+/+
+/−
+/−
−/−

◀ **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 *lepto* which means "thin." Studies of the behavioral effects of leptin will be used here t