

CHAPTER 13

Ways of Exploring

Progress in science depends on new techniques, new discoveries, and new ideas, probably in that order.

Sydney Brenner, 1980

We have repeatedly seen throughout this book how new techniques have led to the discovery of fundamental principles in neurobiology. In this final chapter, we discuss in greater detail some of the key techniques that have advanced our understanding of the nervous system. Studying how these techniques work will enable you to better understand the experiments discussed in the book and to apply these techniques to explore new terrain in neurobiology. I hope that this chapter will also inspire some of you to invent new ways of exploring that will, in turn, bring new discoveries, new ideas, and new principles.

ANIMAL MODELS IN NEUROBIOLOGY RESEARCH

A major goal of neurobiology is to understand how the human brain works. Because the human brain is so complex, and because our ability to perform well-controlled experiments in humans is limited for ethical reasons, most neurobiologists use animal models to conduct their research. As we have seen in previous chapters, many of the principles identified in animal models are generally applicable to all nervous systems, including the human brain. At the same time, the variations observed in different animal models (see Figure 12-2) can be equally informative, as they reveal how the evolution of different nervous systems enabled animals to better adapt to their environmental niches.

What do scientists look for in animal models? According to one scientist, William Quinn, an ideal animal for neurobiology research “should have no more than three genes, a generation time of twelve hours, be able to play the cello or at least recite classical Greek, and learn these tasks with a nervous system containing only 10 large, differently colored, and therefore easily recognizable neurons.” Of course such an ‘ideal’ animal does not exist, but this statement reflects the qualities that neurobiologists look for in an animal model: a simple genome and short generation time to facilitate gene manipulations and genetic studies; complex brain functions and behaviors to extrapolate findings more easily to humans; and large, easily identifiable neurons, the activities of which can be recorded and manipulated individually or together to study the principles of information processing within the neural circuits they constitute.

Before discussing specific techniques, we first have a brief overview of the commonly used animal models, upon which subsequent discussions of specific techniques are based.

13.1 Some invertebrates provide large, identifiable neurons for electrophysiological investigations

Recording the electrical signals from individual neurons and manipulating their activities are essential for investigating the mechanisms by which the nervous system functions (see Sections 13.20–13.25 for more details). The larger the neuron, the more easily researchers can record its activity by placing an electrode

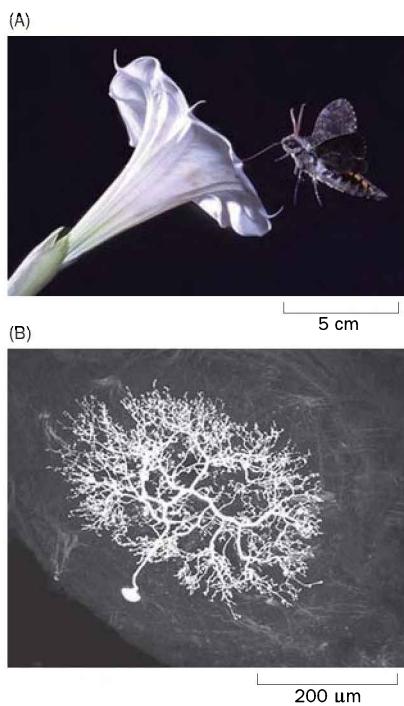


Figure 13–1 Invertebrate animals with large neurons aid neurophysiological investigations. The hawkmoth *Manduca sexta* has been used as a model for studying olfactory and pheromone signaling because it has a superb sense of smell and large olfactory system neurons, from which it is easy to obtain physiological recordings. (A) A nectar-feeding *Manduca*. (B) A local interneuron of *Manduca* arborizes its processes in the antennal lobe, the first olfactory processing center in the insect brain. The neuron has been filled with a fluorescent dye by an intracellular recording electrode. Compare the scale here with that of a similar neuron in *Drosophila* (see Figure 13–23C). (A, courtesy of John G. Hildebrand and Charles Hedgcock, R.B.P; B, from Reisenman CE, Dacks AM & Hildebrand JG [2011] *J Comp Physiol A* 197:653–665. With permission from Springer.)

inside it. A good example is the giant axon of the squid *Loligo*, which was used to discover the ionic basis of the action potential (see Sections 2.9 and 2.10). *Loligo* also offered giant synapses for intracellular recordings from the presynaptic terminals, which validated the role of Ca^{2+} entry in the control of neurotransmitter release (see Section 3.4).

In addition to offering simple preparations for elucidating fundamental principles of neuronal communication, invertebrates have been used to investigate the mechanisms by which neural circuits process and store information. These studies take advantage of the relatively small number of neurons (compared to vertebrate nervous systems), their large size, and their stereotyped arrangement; these properties enable the electrophysiological recording and manipulation of neurons that are individually identifiable, facilitating the comparison of experimental results across different members of the same species. For example, studying the *Aplysia* gill-withdrawal reflex enabled the discovery that changes of synaptic connection strengths underlie behavioral habituation and sensitization (see Section 10.15), and the stomatogastric ganglion of lobsters and crabs has been used to elucidate the mechanisms of central pattern generation that underlie rhythmic movement (see Section 8.5). Many other invertebrate animals such as snails, leeches, locusts, cockroaches, and moths have been used to probe the neural basis of sensation and motor control (for example, Figure 13–1).

13.2 *Drosophila* and *C. elegans* allow sophisticated genetic manipulations

The two invertebrate animals that we have encountered most often in this book are the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*. These species are popular among neurobiologists because researchers can employ efficient genetic tools to manipulate their genes and identifiable neuronal populations with precision (see Sections 13.6–13.12). In contrast to the invertebrate models discussed above, these animals do not offer researchers the benefit of large neuronal size; in fact, *C. elegans* and *D. melanogaster* have the smallest neurons of any animal models commonly used for neurobiology research. Among animals with nervous systems of similar complexity, neuronal size usually correlates with the size of the animal, which is inversely correlated with its generation time. Model organisms for genetic research have been selected for short generation times—about 10 days for *Drosophila* and 3 days for *C. elegans*; hence their small bodies and neurons.

Drosophila has served as a genetic model organism for more than a century. Research first conducted in *Drosophila* laid the foundation for many fundamental concepts in genetics, such as the nature of genes, mutations, chromosomes, and the basis of linkage mapping. *Drosophila* has roughly 10^5 neurons, considerably fewer than the mouse ($\sim 10^8$ neurons) or human ($\sim 10^{11}$ neurons) brain, but a number sufficient to mediate sophisticated neural computation and behavior. Studies in *Drosophila* can also be compared with studies in other insects such as moths, honeybees, ants, locusts, and mosquitoes that act as pollinators or pests in agriculture or as vectors of human diseases; after all, insects constitute the most diverse order in the animal kingdom.

Sydney Brenner, the author of this chapter's epigraph, introduced *C. elegans* in the 1960s for the purpose of studying the nervous system and behavior of a simple organism. *C. elegans* has since been used in many other fields of biological research, contributing to fundamental discoveries such as mechanisms of programmed cell death and RNA interference. Not only is *C. elegans* well suited to genetic manipulation, its transparent body is also advantageous for developmental and imaging studies. *C. elegans* is the only organism for which the entire connectome—that is, the complete set of synaptic connections linking its 302 neurons—has been deciphered using serial electron microscopy (Figure 13–2); this invaluable advance has guided developmental and neural circuits research (for example, see Figure 6–26).

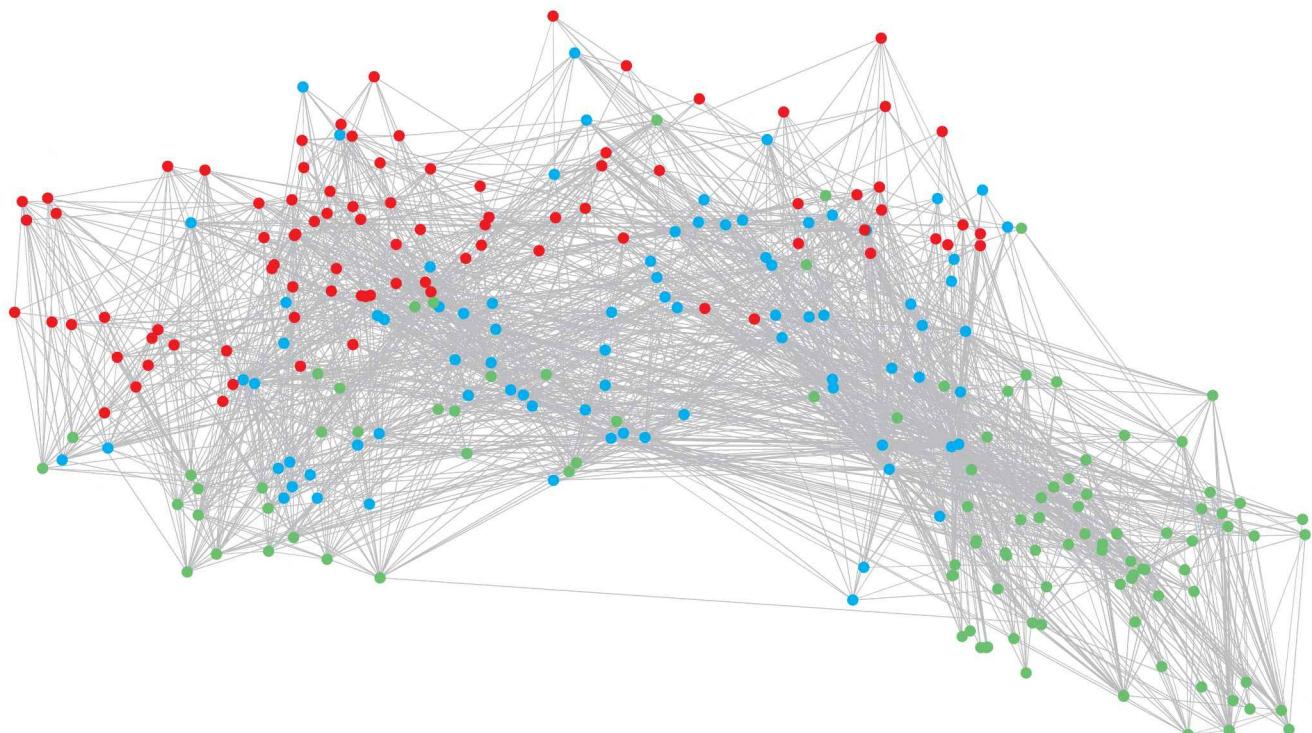


Figure 13–2 Wiring diagram of *C. elegans*. Depicted here are the 279 *C. elegans* somatic neurons (dots) and their synaptic connections (gray lines, 6393 in total), as reconstructed from serial electron microscopic sections. (Of a total of 302 neurons, 20 that constitute the pharyngeal nervous system and 3 that do not make synapses with other neurons are not shown here. Electrical synapses and neuromuscular synapses are also not shown.) Red, sensory neurons;

blue, interneurons; green, motor neurons. The vertical axis represents signal flow (from top to bottom), whereas the horizontal axis represents the connectivity closeness of neurons in the combined chemical and electrical synapse network. (Courtesy of Dmitri Chklovskii. See also White JG, Southgate E, Thomson JN et al. [1986] *Phil Trans R Soc Lond B* 314:1–340; Varshney LR, Chen BL, Paniagua E et al. [2011] *PLoS Comp Biol* 7:e1001066.)

13.3 Diverse vertebrate animals offer technical ease or special faculties

Cold-blooded vertebrate animals including fish, amphibians, and reptiles are useful for producing robust explant preparations in which to study many neurobiological problems. Unlike mammalian tissues, these *in vitro* preparations often do not require constant temperature and oxygenation to maintain tissue integrity. Being vertebrates, their nervous systems share organizational similarities with the human nervous system that are not found in invertebrate models. Studies in amphibian models have contributed to many fundamental discoveries in neurobiology, such as wiring specificity in the retinotectal system (see Sections 5.1 and 5.2) and mechanisms of synaptic transmission (see Sections 3.1 and 3.2). Zebrafish (*Danio rerio*) has in recent years been a popular vertebrate model organism because its body is transparent in the larval stage, which facilitates developmental and imaging studies (Figure 13–3), while its relatively short generation time is well suited for genetic studies.

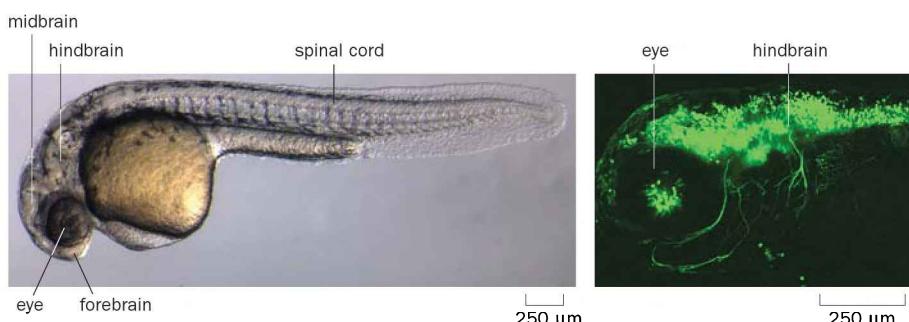


Figure 13–3 The transparency of zebrafish larvae facilitates developmental and imaging analysis. Left, differential interference microscopic image of a living zebrafish embryo at 36 hours post-fertilization. Major nervous system structures are indicated. Right, GFP (green) expression in neurons of a living zebrafish at 3 days post-fertilization. (Left, from Schier AF & Talbot WS [2005] *Annu Rev Genet* 39:561–613. With permission from Annual Reviews; Right, courtesy of Thomas Glenn & William Talbot.)

Whereas some animal models have been selected for technical ease, others have been chosen for their special faculties. One principle of neuroethology is to select a model animal in which the behavior of interest is robustly displayed. For instance, barn owls have been used to study audition because of their superb ability to locate sounds (see Sections 1.3, 6.26, and 10.25). Songbirds have been used to study vocalization and learning because they have advanced vocal communication systems and a sophisticated song-learning process (see Box 9-1). Uncovering the neural mechanisms that underlie a particular property in a species well suited to its study can benefit researchers that investigate the same property in other animals.

13.4 Mice, rats, and nonhuman primates are important models for mammalian neurobiology research

Among mammalian species, rats and mice have been the predominant animal models for many branches of biology, including neurobiology. A major advantage of mice is that they are the only mammals for which the production of transgenic and gene knockout animals is routine; this facilitates genetic manipulation and allows genetically identified neuronal populations to be recorded and manipulated with precision (see Sections 13.6–13.12). Rats have been used longer than mice as models in neurobiology research. Many behavioral paradigms such as operant conditioning (see Figure 10-22) were first developed in rats. Genetic tools first developed in mice are now being expanded to rats, whereas many physiological and behavioral paradigms originated in rats are being adapted for mice.

In addition to studying intact animals, reduced preparations from mice and rats have been widely used in neurobiology research. For instance, neurons can be dissociated and cultured *in vitro* for studying a wide range of topics such as the development of neuronal polarity (see Figure 7-16) and molecular mechanisms of synapse formation (see Figure 7-25) and synaptic transmission (see Figure 3-9). Acute or cultured brain slices have been widely used to study neuronal connectivity (see Figures 3-49 and 4-46), electrical signaling (see Figure 3-44), synaptic transmission (see Figure 3-23), and synaptic plasticity (see Figures 10-11 and 10-18). These *in vitro* preparations offer ease of experimental manipulations, such as performing patch clamp recordings of multiple neurons while controlling the extracellular environment.

Compared with mice and rats, nonhuman primates such as rhesus monkeys have brain structures (see Figure 12-36), gene expression patterns, and physiology that are more similar to those of humans; likewise, their cognitive abilities are superior to those of rodents. Many sophisticated psychophysical and cognitive tests, such as decision-making tasks (see Figure 4-54), were first developed in primate models. The visual system of trichromatic Old World monkeys and apes is very similar to our own (see Figure 4-19). Nonhuman primates are also valuable models for human disease and for drug testing, because, compared with other animals, their physiology is more similar to that of humans.

When working with animals, researchers are obligated to follow certain ethical practices; these include replacing animals with non-animal systems whenever possible, using the smallest number of animals necessary to obtain the desired information, and using all available methods to minimize pain and distress to animals being used for research. These practices apply particularly to vertebrate animals, whose proper use is regulated by governments and research institutions.

13.5 Human studies are facilitated by a long history of medicine and experimental psychology and by the recent genomic revolution

A long history of medicine, which provides many examples of human neurobiology and neuropathology, has contributed uniquely to our understanding of the nervous system. Lesions due to injury in patients provided clues to the existence of language centers in the human brain (see Figure 1-23). Electrophysiological recordings of epilepsy patients elucidated the topographic organization of the

sensory and motor cortices (see Figure 1–25). Studies of amnesic patients such as H.M. revealed different memory systems and their brain localization (see Section 10.1). Likewise, experimental psychology using healthy humans as subjects has contributed substantially to our understanding of perception (see Figure 4–3), cognition, and behavior. Functional brain imaging studies have greatly improved our understanding of normal human brain organization (see Figures 1–24, 10–37, and 10–42), and can be used to monitor disease progression (see Figure 11–11) and therapeutic effect (see Figure 11–21). Genetic variations in humans have helped researchers to identify key genes that are essential for basic neurobiological processes (such as bitter taste; see Section 6.19). Mutations that cause brain disorders are making important contributions to our knowledge of how the normal nervous system develops and functions (see Chapter 11).

With the sequencing of the human genome now complete and the cost of sequencing individual genomes becoming significantly more affordable, we can anticipate a wealth of data correlating genetic variations with many kinds of phenotypes, from brain disorders to personality traits. These data provide fascinating entry points to many new areas of neurobiological investigation.

GENETIC AND MOLECULAR TECHNIQUES

Genes, the basic functional units in the genome, encode the RNAs and proteins that execute all cellular functions. Many biological processes can be viewed as the consequences of a series of actions by individual genes. Thus, by manipulating individual genes, one can dissect complex biological processes into discrete steps. This genetic approach has made fundamental contributions to all branches of biology, including the study of the nervous system. While the gene-centric approach has been more widely used in molecular and cellular neuroscience, the genetic framework has been extended to cell-type-based approach that is becoming instrumental in investigating problems in circuit and systems neuroscience and in animal behavior.

The most fundamental genetic manipulation is to disrupt the function of an individual gene—that is, to create a **loss-of-function mutation** in a gene of interest without affecting any other genes in the genome. Researchers have taken two general approaches to link a gene with its function inferred from loss-of-function phenotypes: forward genetics, which traces an observed phenotype to a gene, and reverse genetics, which follows a gene to its associated phenotype (Figure 13–4).

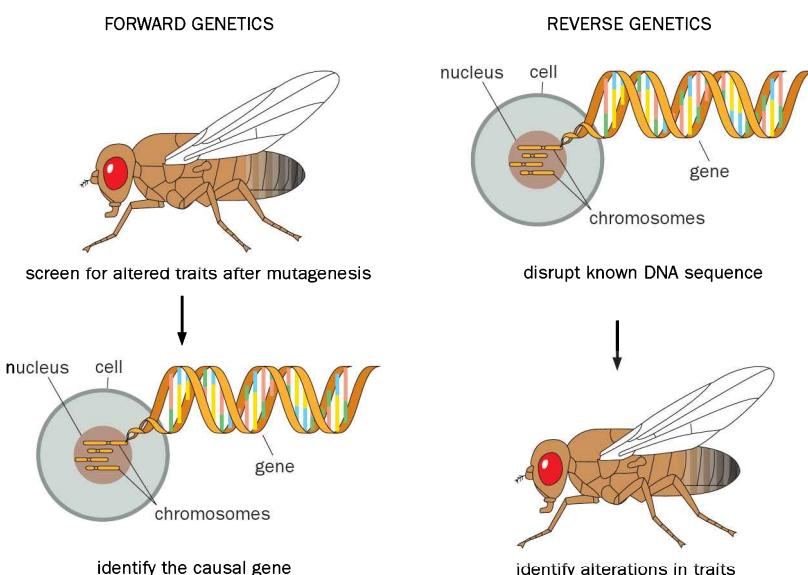


Figure 13–4 Forward and reverse genetics. In forward genetics, researchers start by observing an altered trait (phenotype) to identify the gene responsible for causing the phenotype of interest. In reverse genetics, researchers start with a gene of interest and disrupt the gene function to examine the phenotypic consequences.

13.6 Forward genetic screens use random mutagenesis to identify genes that control complex biological processes

An experimental approach that dominated much of twentieth century genetics, the **forward genetic screen** has provided key insights into many complex biological processes, from cell division and protein secretion to development of multicellular organisms. Forward genetic screens employ a strategy of **random mutagenesis** to identify the genes involved in a biological process of interest. Suppose that a series of unknown genes play essential roles in the process of interest. Researchers can use chemical mutagens, radiation, or transposon insertion (insertion of a transposable DNA element in a gene that disrupts its function) to mutagenize a population of animals, such that each treated animal carries a different set of random mutations in a small number of genes or in a single gene. Researchers can then screen for mutations that disrupt the biological process of interest based on the phenotypes exhibited by the offspring of the mutagenized animals (Figure 13–5).

The mutated gene that causes the phenotype can be traced using a variety of molecular-genetic methods depending on the nature of the mutagen. Mutations caused by transposon insertions can readily be mapped by identifying the DNA sequences that neighbor the insertion sites. Mutations induced by chemicals or radiation can be mapped by molecular-genetic procedures such as **positional cloning**. In this strategy, a large number of meiotic recombinant chromosomes are produced, and the linkage between the mutant phenotype and genetic or molecular markers with known positions in the genome is used to identify where the mutated gene resides; the closer the mutation is to a particular marker, the less frequently the mutation and marker are separated by recombination events. The causal gene can be validated by identifying the disruptive mutation in the candidate gene and by rescue of the mutant phenotype using a wild-type transgene (we will discuss transgenes in Section 13.10). With the development of high-throughput genome sequencing (see Section 13.14), researchers can also compare the whole-genome sequences of mutants and wild-type controls to identify the causal genes.

Forward genetic screens are particularly powerful in tackling problems for which the cellular and molecular pathways are poorly understood. Researchers

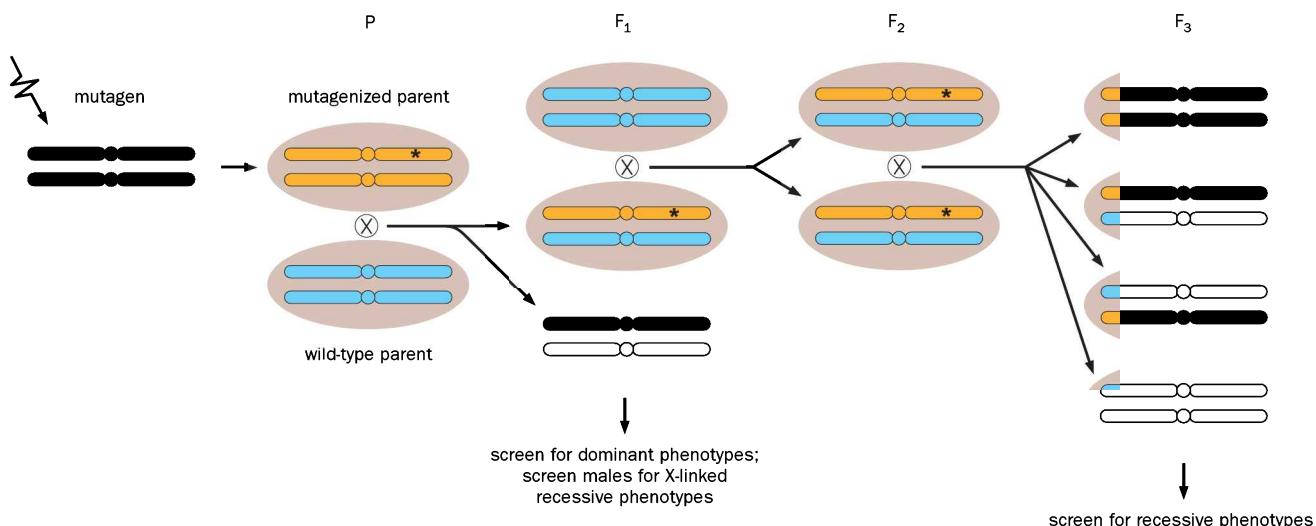


Figure 13–5 A simplified scheme for a forward genetic screen to identify single-gene mutations that cause specific phenotypes. After mutagen treatment, the mutagenized individual (P) is crossed with wild type; the mutation is indicated as * on the chromosome. Individual progeny from the next generation (F_1 , for first filial generation) can be screened directly for mutations that exhibit dominant phenotypes (bottom), or can be crossed with wild type to produce a larger population of progeny (F_2) heterozygous for the

mutation, from which homozygous mutant progeny (F_3) can be bred and screened for recessive phenotypes (top). The simplest recessive mutations to screen for are X-linked alleles in males; because they have only one X chromosome, males can be screened for X-linked recessive traits in F_1 instead of F_3 . Note that for simplicity only a subset of progeny from each cross that is relevant for the progression of mutagenized chromosome is drawn out in the F_1 and F_2 generations.

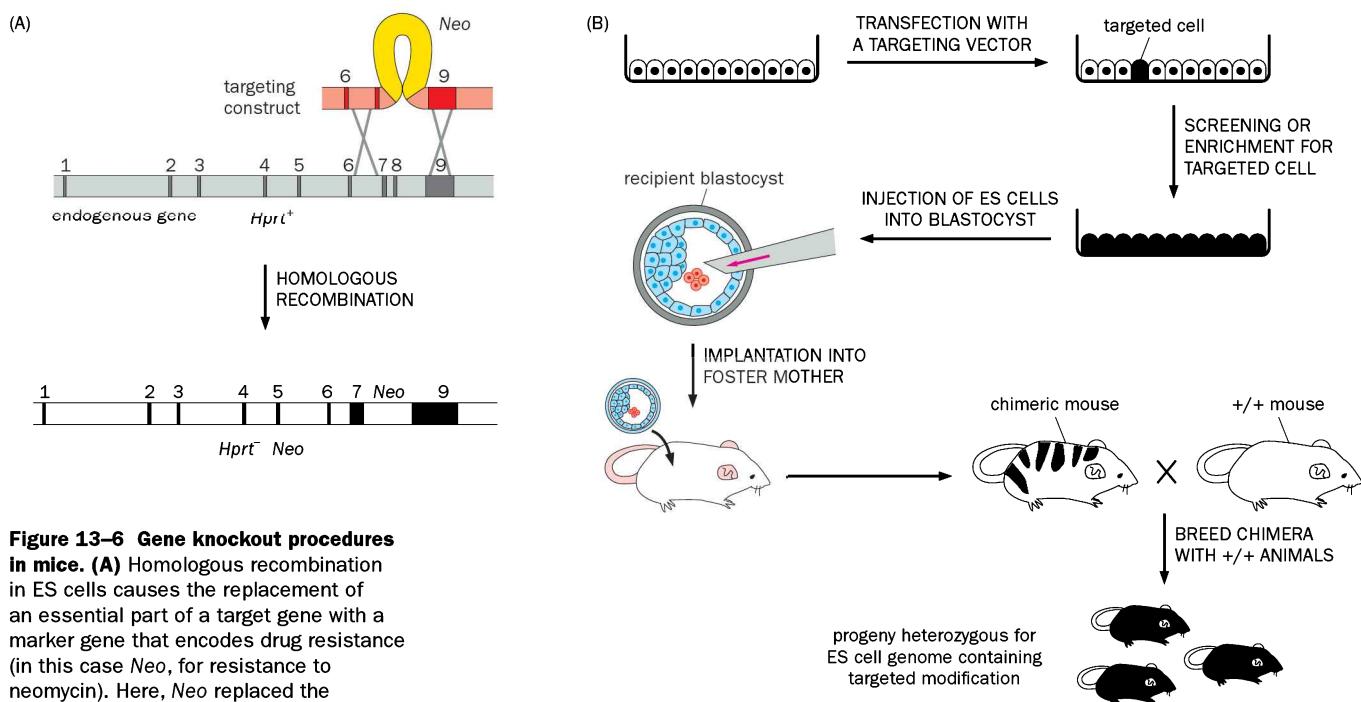
rely on mutant phenotypes to identify genes involved in a particular biological process without any bias or knowledge as to what kinds of genes are expected. The identifications of the *Drosophila Period* gene and mouse *Clock* gene provide striking examples of how forward genetic screens have led to our current understanding of the molecular mechanisms that control circadian rhythms (see Figure 8–45). The same procedures used to identify mutant phenotypes and their causal genes resulting from mutagenesis can also be applied to mutations that arise spontaneously, as in the case of shaking flies (see Section 2.15), obese mice (see Section 8.16), narcoleptic dogs (see Section 8.23), and inherited human disorders (see Chapter 11).

13.7 Reverse genetics disrupts pre-designated genes to assess their functions

We now discuss **reverse genetics**, a term that refers to strategies for disrupting a pre-designated gene (see Figure 13–4, right). Many molecular components of the nervous system were identified by means other than mutant phenotypes arising from forward genetic screens or spontaneous mutations. For example, the Na⁺ channel, synaptotagmin, rhodopsin, and ephrin were identified by biochemical purification of proteins enriched in the electric organ, presynaptic terminals, bovine retina, and developing tectum, respectively (see Sections 2.15, 3.6, 4.3, and 5.4). The TRP channels for sensing temperature were identified from expression cloning (see Section 6.31). Most ion channels and neurotransmitter receptors were first identified based on their sequence homology with known proteins of similar function. With genome sequences completed for most model organisms, researchers can search databases to identify candidate genes that might perform certain functions based on expression patterns and predicted protein sequences. A key approach to test the function of a candidate gene in a suspected biological process is to create loss-of-function mutations and examine the phenotypes of the resulting mutant animals.

The most widely used method for deleting a specific gene of interest is by **homologous recombination**, in which a piece of endogenous DNA essential for the function of a gene is replaced by a piece of *in vitro* engineered DNA, the ends of which have sequences identical (hence the term ‘homologous’) to the endogenous DNA. Homologous recombination is an intrinsic property essential for meiosis in germ-line cells; it also occurs in other cell types, including embryonic stem (ES) cells. Among multicellular animals, the homologous recombination-based gene disruption procedure known as gene **knockout** was first developed in mice and has since become routine in that species (Figure 13–6). The first step is to create an *in vitro* engineered DNA construct that carries a drug-resistance gene flanked on both sides by pieces of DNA (homology arms) derived from the endogenous gene of interest. This construct is then introduced into ES cells, where recombination at both homology arms causes the replacement of an essential part of the gene of interest with the drug-resistance gene (Figure 13–6A). ES cell clones that carry the knockout allele are identified based on their drug resistance, expanded, and injected into blastocyst-stage host embryos. These embryos are subsequently implanted into surrogate mothers, where they develop to produce chimeric pups in which a fraction of germ-line cells derives from the *in vitro* engineered ES cells. (A chimera contains some cells with the genotypes of injected ES cells, and other cells with the genotype of the host embryo.) These chimeras are bred with wild-type animals to generate offspring in which all cells carry the knockout allele (Figure 13–6B), and subsequent breeding of the offspring can yield mice homozygous for the knockout allele.

Since the basic knockout procedure was established in the 1980s, many variations and extensions have been added to make this technique more versatile. For instance, instead of disrupting a gene, single nucleotide changes can be made to test *in vivo* the contribution of specific amino acid residues to protein function (for example, see Figure 3–9B). It is also possible to insert any *in vitro* engineered construct into a predetermined genomic locus. Both procedures are referred to as **knock-in**. Among its many uses, a knock-in mouse can express a marker gene

**Figure 13–6** Gene knockout procedures

in mice. (A) Homologous recombination in ES cells causes the replacement of an essential part of a target gene with a marker gene that encodes drug resistance (in this case Neo, for resistance to neomycin). Here, Neo replaced the sequences that correspond to exon 8 of the *Hprt* gene, which encodes an enzyme for nucleotide biogenesis. Homologous recombination at two crossovers results in deletion of the DNA segment corresponding to exon 8. The resulting recombinant chromosome is deficient for *Hprt* and confers neomycin resistance. Light gray and light red: homologous introns of the *Hprt* gene; dark gray and dark red, homologous exons of the *Hprt* gene. (B) Modified ES cells can be used to create knockout mice by following the steps in this flow chart. Modified ES cells and their derivatives are red, whereas cells in the host blastocyst are blue. Note that the final product in the scheme is heterozygous for the modified ES cell genome, but appears red if the marker for the modified ES cell (for example, a coat color) is dominant. (Adapted from Capecchi MR [1989] Science 244:1288–1292.)

in the spatiotemporal pattern defined by an endogenous gene's promoter (see Figure 6–15); we will discuss many applications of this technology below.

One of the most important extensions of the knockout technique is the production of **conditional knockout** mice. This technique was first developed using the bacteriophage Cre/*loxP* system. **Cre recombinase** is a bacteriophage-derived enzyme that catalyzes recombination between two sequence-specific DNA elements called *loxP* sites. When two *loxP* sites are in the same orientation, a recombination event will delete the intervening sequence. (When the *loxP* sites are in opposite orientations, a recombination event will invert the intervening sequence.) In conditional knockout, two *loxP* sites are inserted in the same orientation by homologous recombination into introns that flank essential exons of a gene of interest. An allele in which essential exons are located between two *loxP* sites is termed a floxed allele (short for “flanked by *loxP*”). In the absence of Cre-mediated recombination, these *loxP*-containing introns are spliced out of RNA transcripts and do not affect gene expression. The gene can be knocked out (that is, the floxed exons can be excised) only in cells where Cre has been active (Figure 13–7). Researchers have generated hundreds of transgenic ‘Cre lines’ with different spatiotemporal patterns of Cre expression, such that gene deletion can be achieved in specific cell types and occurs only after the Cre transgene is first expressed. In addition to the Cre/*loxP* system, other site-specific recombinase systems can be used. For instance, the yeast **FLP recombinase** mediates recombination between two **FRT** (FLP recognition target) sites through a mechanism analogous to Cre-mediated recombination between two *loxP* sites.

An important extension of conditional knockout technology is to engineer Cre so that its activity can be temporally controlled, such as by addition of a drug. One way to achieve such temporal control is to regulate translocation of Cre into the nucleus where recombination takes place. As discussed in Section 9.14, the estrogen receptor normally remains in the cytoplasm but translocates to the nucleus in the presence of estradiol (see Figure 9–24). **CreER** is a fusion of the **Cre recombinase** and the portion of the **estrogen receptor** responsible for cytoplasmic retention. Similar to the endogenous estrogen receptor, CreER remains in the cytoplasm when not bound to its ligand, but translocates to the nucleus in the presence of the estrogen analog **tamoxifen**. (The estrogen-binding site of CreER is modified so that it binds tamoxifen but not endogenous estrogen.) Thus, the CreER-tamoxifen system allows temporal control of recombination of a floxed allele, and hence control of the precise time at which an endogenous gene is deleted.

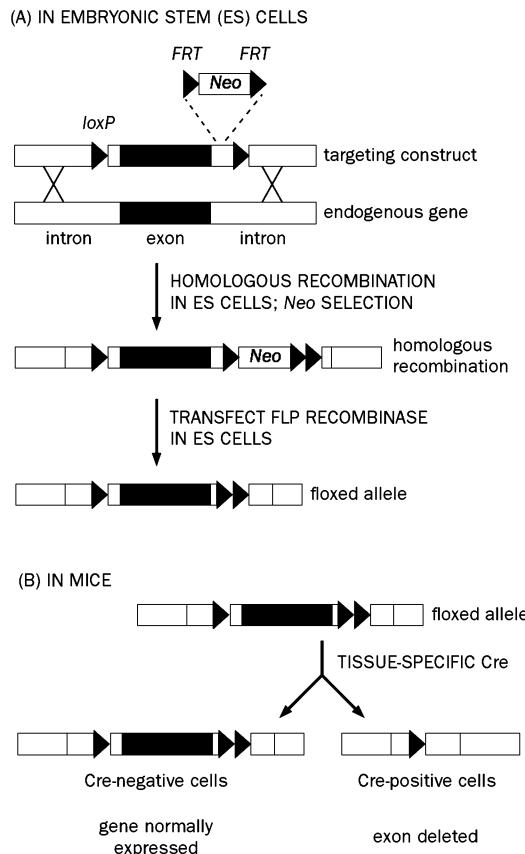


Figure 13-7 Conditional knockout in mice. **(A)** An example of floxed allele production in ES cells. In the targeting construct, a pair of *loxP* sites is inserted into two introns flanking an essential exon of interest. In addition, the *Neo* gene, flanked by a pair of *FRT* sites, is inserted into one of the introns. Recombination at crossovers between homologous sequences produces the desired recombinant after neomycin selection. Subsequent transient expression of the FLP recombinase induces recombination between two *FRT* sites, thus removing the neomycin resistance gene to produce the floxed allele. Because the two *loxP* sites and one *FRT* site are all inserted in the intron, the floxed allele does not affect the expression of the target gene of interest. **(B)** In mice that contain the floxed allele and a transgene expressing Cre recombinase, cells that do not express Cre are unaffected, but the essential exon is removed by *Cre/loxP* mediated recombination in cells in which Cre has been active, thus creating conditional knockout of the gene of interest.

In addition to mice, homologous recombination techniques have also been used successfully for gene deletions in *Drosophila* and rats. The rate-limiting step is to screen for rare recombination event; this has been achieved in mice and rats by developing ES cell culture so that such screens can be performed *in vitro*. In *Drosophila*, the homologous recombination procedure has been sufficiently streamlined so that it is possible to screen recombination events directly *in vivo*. For most model organisms, however, techniques of gene disruption using homologous recombination have not been established. The recent development of genome engineering tools has the potential to enable genetic manipulations, such as the production of knockout and knock-in animals, to be performed in species other than the traditional genetic model organisms (Box 13-1).

Box 13-1: Genome engineering by the CRISPR–Cas9 system

Genome engineering refers to the general process of altering the genome at a predetermined locus, whether by deleting a piece of endogenous DNA, inserting a piece of foreign DNA, or creating a specific base-pair change. The knockout and knock-in procedures discussed in Section 13.7 are genome-engineering procedures that employ homology arms to guide alterations using the homologous recombination system intrinsic to germ-line or embryonic stem cells. An alternative strategy is to induce, at a genomic locus of interest, double-strand DNA breaks that activate endogenous DNA repair systems, and in so doing introduce sequence

alterations. In genome engineering, double-strand breaks are typically induced by DNA-sequence-specific targeting of exogenous nucleases, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or the most recently developed and likely most versatile system for this approach: the CRISPR–Cas9 system.

Discovered in the 2000s, **CRISPR** (clustered regularly interspaced short palindromic repeat) is an adaptive immune system present in many bacteria and archaea. CRISPR is a genomic locus that contains repetitive DNA elements

(Continued)

Box 13-1: Genome engineering by the CRISPR–Cas9 system

derived from the genomes of invading pathogens such as viruses or plasmids. These DNA repeats are then made into small RNA molecules that guide nucleases to degrade the genomes of the invading pathogens through sequence-specific base pairing. Thus, bacteria previously exposed to a pathogen can rapidly defend against future infection by the same pathogen. (Since the modification occurs at the level of genomic DNA, this anti-pathogen trait is inherited by progeny; this constitutes a rare case where directed changes in DNA sequence can contribute to natural selection; see Section 12.3).

Although there are several variants of the CRISPR system, the type II system present in bacteria such as *Streptococcus pyogenes* utilizes a single protein called Cas9 (CRISPR-associated 9) with two nuclease domains that cut both DNA strands to produce a double-strand break (Figure 13-8). Cas9 is brought to a specific site on the target DNA through the action of an engineered guide RNA, which contains sequences that base pair with the DNA target (part of the guide RNA is normally transcribed from the CRISPR locus). Double-strand breaks created by the CRISPR–Cas9 system can be repaired by the **nonhomologous end joining** system in the absence of any homologous DNA sequence to serve as a template. Such repairs usually introduce small deletions or duplications at the break point; if the breaks occur in the coding sequence, repairs have a two-thirds chance of creating a frame-shift mutation that disrupts the protein-coding sequence after the breakpoint. Double-strand

breaks can also be repaired by homologous recombination, which utilizes a donor DNA that shares sequence identity on both sides of the break as a template; homologous recombination-based repair can produce any arbitrary changes to the DNA sequence, from single-base-pair changes to insertions of a *loxP* site or a transgene, at a pre-determined site in the genome (Figure 13-8).

The CRISPR–Cas9 system has been shown to target double-strand DNA breaks and repair events to specific DNA sequences in human cell lines, including induced pluripotent cells (see Box 11-2), as well as to the germ lines of *C. elegans*, *Drosophila*, zebrafish, mouse, and monkey *in vivo*. The efficiency is remarkably high such that multiple guide RNAs can be injected into the same early mouse embryo to create mutations of both copies of multiple genes simultaneously, without requiring ES cell culture, transfection, screening, and injection into blastocysts (see Figure 13-6). The CRISPR–Cas9 system has also been used to create large deletions (between sequences targeted by two guide RNAs) and to insert transgenes such as a fluorescent protein. One limitation is the potential for off-target effects due to the presence of sequences elsewhere in the genome that are similar to the intended target, although techniques have been developed to minimize this effect. Given the rapid development and improvement of the CRISPR–Cas9 system, and the promise it already exhibits, CRISPR–Cas9 is likely to become a major genome-engineering tool.

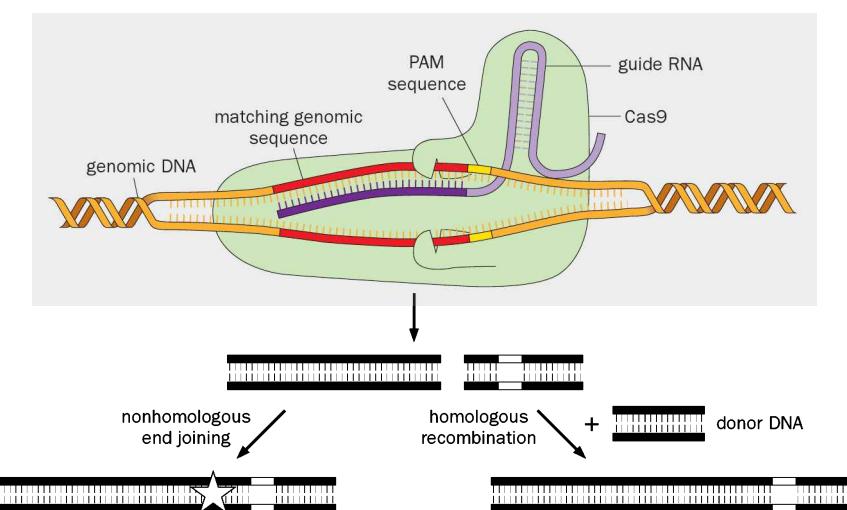


Figure 13-8 The CRISPR–Cas9 system for genome engineering.

Any eukaryotic DNA that contains a PAM sequence (protospacer-associated motif, which is usually two or three nucleotides and thus occurs frequently) can be a target for the CRISPR–Cas9 system illustrated here. (CRISPR stands for clustered regularly interspaced short palindromic repeat, Cas for CRISPR-associated.) A guide RNA that contains sequences complementary to a piece of DNA from the target gene of interest brings the Cas9 enzyme to the target site on the chromosome through DNA–RNA base pairing (purple and red). The two nuclease domains of Cas9 create a double-

strand break in the genomic DNA. This double-strand break can be repaired by the nonhomologous end joining system, through which small deletions or insertions may be created at the repair site (indicated by the star). The double-strand break can also be repaired by the homologous recombination system using a donor DNA as a template, through which specific modifications such as the insertion of a transgene (green) can result. (Adapted from Charpentier E & Doudna JA [2013] *Nature* 495:50–51. With permission from Macmillan Publishers Ltd; see also Ran FA, Hsu PD, Wright J et al. [2013] *Nat Protocol* 8:2281–2308.)

13.8 RNA interference (RNAi)-mediated knockdown can also be used to assess gene function

A genetic technique widely used in recent years to examine loss-of-function phenotypes is **RNA interference (RNAi)**. Stemming from a discovery originally made in *C. elegans* in the late 1990s, RNAi technology takes advantage of a naturally occurring process that efficiently degrades double-stranded RNAs (dsRNAs). RNAi serves as a cellular defense system against invaders such as viruses that produce dsRNAs at least transiently in their life cycle. It utilizes a cascade of RNA-processing enzymes and machinery that is highly conserved in eukaryotic cells for the production and function of endogenous **microRNAs**; these are short, non-coding RNAs (21–26 nucleotides) used to regulate gene expression by triggering the degradation and inhibiting the translation of mRNAs with complementary sequences. As such, RNAi has been exploited by scientists to reduce expression of any gene of interest following the introduction of exogenous dsRNAs.

For experimental gene silencing, dsRNAs can be produced by the base pairing of sense and antisense transcripts with sequences corresponding to a target gene of interest; the dsRNA or genes encoding its components can be delivered to the tissue of interest by microinjection or viral transduction (**Figure 13–9A**). Alternatively, RNAi-mediated gene silencing can be performed by expressing a transgene that encodes the homologous region to the target gene of interest in an inverted repeat (that is, a sequence followed by its reverse complement). Because the two halves of the repeat can base pair with each other, the transgene's RNA product folds into a hairpin, forming a dsRNA substrate for further processing (**Figure 13–9B**). Both approaches make use of the cell's microRNA-production machinery, which cleaves the dsRNA to produce **siRNA**—double-stranded short interfering RNA, with a length similar to microRNA (21–26 nucleotides). The siRNA directs a protein complex to degrade the target mRNA through base pairing.

Since inhibition of gene expression by RNAi tends to be incomplete, the procedure is referred to as causing a knockdown rather than a knockout of the target gene of interest. RNAi, like CRISPR-Cas9 (see Box 13–1), has the potential for off-target effects based on unintended targeting of similar sequences, although RNAi targets homologous RNA instead of DNA. Proper controls are necessary, such as the use of multiple and non-overlapping target sequences or a rescue of RNAi phenotypes by expression of an RNAi-resistant transgene (that is, a transgene that does not contain sequences complementary to those of the dsRNA). The advantage of RNAi over gene knockout is its increased speed and potential for high-throughput screening; this enabled RNAi to be employed not only in reverse genetics but also in genetic screening. Candidate genes that are identified via RNAi screens are often validated subsequently by gene knockout.

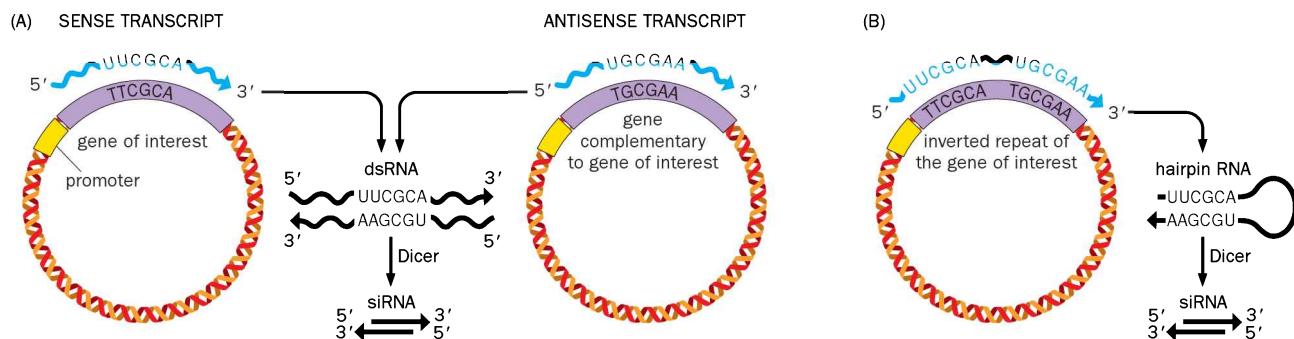


Figure 13–9 Gene knockdown by RNA Interference (RNAi). Double-stranded small interfering RNAs (siRNAs) cause degradation and translation inhibition of target mRNAs bearing the same sequence, and therefore can be used to knockdown the expression of an endogenous gene. **(A)** siRNAs can derive from two genes encoding sense and antisense RNA transcripts from the same DNA sequence. **(B)** siRNAs

can derive from a single gene, the transcript of which contains an inverted repeat (a sequence followed by its reverse complement); the transcript folds back onto itself and the complementary sequences base pair to produce a hairpin. In both cases, double-stranded RNA molecules are cleaved and processed by enzymes in the microRNA-processing pathway (such as Dicer) to produce siRNA.

13.9 Genetic mosaic analysis can pinpoint which cell is critical for mediating gene action

In multicellular organisms, determining which cell requires the function of a gene of interest can provide valuable information about the gene's mechanism of action. The general procedures discussed above for generating loss-of-function mutations, whether produced by random mutagenesis or engineered by gene targeting, result in the breeding of homozygous mutants. Because the gene of interest is disrupted in all cells, these methods do not help researchers identify the specific cell types in which the gene acts to contribute to developmental, cellular, and circuit functions of interest or animal behavior. Conditional knockout using Cre expressed under tissue-specific promoters can narrow this search by revealing the tissue(s) or defined cell populations in which a gene of interest is required. An alternative method is to create genetic mosaics using **mitotic recombination**. In this procedure, DNA recombination occurs between two homologous parental chromosomes in a somatic cell, such that one of the daughter cells can be homozygous for part of one parental chromosome. If an animal is heterozygous for a recessive mutation in a gene of interest and is thus phenotypically normal, one daughter cell (and all its descendants) can be made homozygous for the mutation and thus become phenotypically mutant (Figure 13–10); this creates a **genetic mosaic** animal—that is, an animal that contains cells of more than one genotype.

If cells of distinct genotypes can be differentially labeled, then phenotypic analysis of such mosaic animals can provide information about whether the gene of interest is **cell autonomous** (that is, acts only within the cell that produces the gene product) or **nonautonomous** (that is, acts on cells that do not produce the product) to regulate a given biological process. We have seen examples of genes that act either cell autonomously or nonautonomously in our studies of cell fate determination (see Figure 5–36), wiring specificity (see Figures 5–29, 5–38, 7–41), and mating behavior (see Section 9.8). The rate of mitotic recombination is very low naturally, but can be markedly enhanced by X-ray irradiation or by introducing into the genome a recombinase and its recognition sites, such as the *Cre/loxP* or *FLP/FRT* systems discussed above. When two recombinase recognition sites are at an identical location on two homologous chromosomes in the same orientation, a recombination event between the two sites produces recombination of the two chromosomes (see Figure 13–23 for an illustration). Site-directed recombinases also enable spatiotemporal control of mitotic recombination events via the control of recombinase expression. In addition to determining cell autonomy

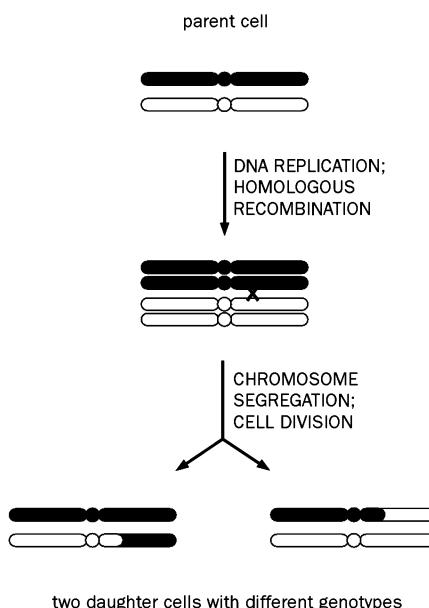


Figure 13–10 Mitotic recombination can create genetic mosaics. A pair of homologous chromosomes is shown. The parent cell is heterozygous for a recessive mutation (*) in a gene of interest. If DNA recombination occurs between the homologous chromosomes (red cross) following DNA replication, chromosomal segregation in the subsequent cell division can create daughter cells that are homozygous for either the mutant (left) or wild-type (right) alleles.

of gene function, genetic mosaics can be used to label single cells, trace cell lineage, and access specific neuronal populations for genetic manipulation (see Section 13.16).

13.10 Transgene expression can be controlled in both space and time in transgenic animals

The ability to introduce an *in vitro* engineered piece of DNA into an organism—that is, to create a **transgenic organism**, has revolutionized biology. A protein-coding **transgene** usually consists of enhancer/promoter elements that direct the spatiotemporal expression pattern, a 5'-untranslated region downstream from the transcription start site, a coding sequence that dictates the production of a specific protein, and a 3'-untranslated region that include the poly-adenylation (poly-A) signal to regulate mRNA stability and nuclear export (Figure 13–11).

Transgenic animals serve two broad purposes in neurobiology research. The first is to examine the function of an endogenous gene *in vivo*. For instance, expression of a transgene that encodes a wild-type protein can be used to rescue a loss-of-function mutant phenotype and thereby confirm a causal relationship between the disruption of a gene and a given phenotype. Expression in defined spatiotemporal patterns of a transgenic hairpin construct that produces RNAi effects against a specific mRNA (see Figure 13–9B) can be used to assess gene knockdown phenotypes. The gene of interest can also be misexpressed at different levels or with different spatiotemporal patterns to test **gain-of-function** effects. Finally, transgenes with specific modifications can be used *in vivo* in both loss- and gain-of-function contexts to assess the structure–function relationships of the gene of interest [that is, which specific domain(s) or amino acid(s) are required for a given function].

The second broad application of transgenes is to express molecular tools, such as cell markers to visualize neuronal morphology and projection patterns, Ca^{2+} or voltage indicators to record neuronal activity, and effectors such as light- or chemical-activated channels to silence or activate neuronal activity (see Sections 13.16, 13.18, and 13.21–13.25).

For both applications, a crucial component is to control the transgene's spatiotemporal expression pattern, which is usually regulated by enhancer sequences surrounding the coding sequence of a gene. Various mechanisms can be employed to mimic the expression pattern of an endogenous gene or to create an artificial expression pattern. One simple method to drive transgene expression in a particular pattern is to use the DNA sequences 5' to the coding sequence of an endogenous gene whose expression pattern is being mimicked (Figure 13–12A). Since regulatory elements can sometimes be distributed far away from the coding sequence, using a **bacterial artificial chromosome** (BAC)—a cloning vector that

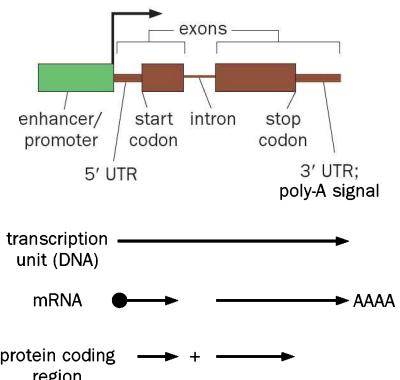


Figure 13–11 Anatomy of a transgene.

A protein-coding transgene typically includes an enhancer/promoter upstream from the transcription unit that directs the spatiotemporal pattern of transcription. The transcription unit shown here comprises a 5' untranslated region (5' UTR); the protein-coding region separated by an intron (or introns) that is spliced out of the transcript during post-transcriptional processing; and a 3' UTR containing a poly-A signal. The protein-coding region constitutes the region of mRNA between the start codon (translation initiation site) and the stop codon (translation termination site). The extents of the transcription unit, mRNA after splicing, and coding region are shown at the bottom. The green dot represents the 5' cap (see also Figure 2–2 and Section 2.1). Sometimes introns, 5' and 3' UTRs, and sequences 3' to the transcription unit may contain additional regulatory elements for gene expression.

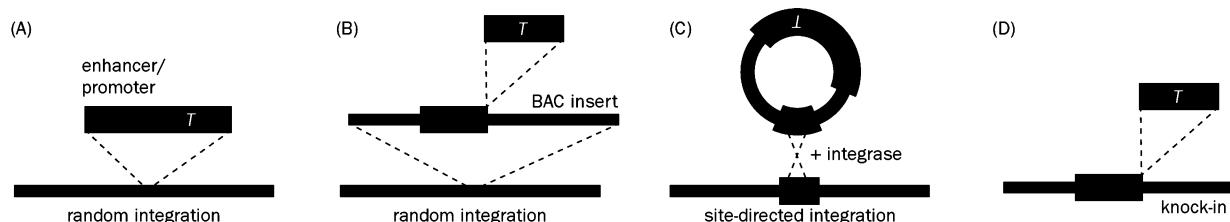


Figure 13–12 Methods of regulating the patterns of transgene expression. In each panel, the genomic DNA from an endogenous gene whose expression pattern is to be mimicked is shown in green. (A) The transcription unit of interest (*T*, brown) is placed under the control of an enhancer/promoter (*E/P*) element, and the resulting transgene is randomly integrated into a host chromosome (gray). (B) *T* is inserted into a large piece of genomic DNA downstream from the enhancer/promoter. The large size of the genomic DNA segment, which is inserted into a bacterial artificial chromosome (BAC) cloning vector capable of integrating several hundred kilobase pairs of DNA,

increases the probability that the segment contains distant regulatory elements. The modified BAC insert is then randomly integrated into the host chromosome. (C) Transcription unit *T*, under the control of an enhancer/promoter, is integrated at a predetermined locus in the host genome by expressing a bacteriophage integrase that catalyzes an irreversible recombination between the *attP* and *attB* sites. (D) *T* is knocked in at the endogenous locus of the gene whose expression pattern is to be mimicked (see Section 13.7). (Adapted from Luo L, Callaway EM & Svoboda K [2008] *Neuron* 57:634–660. With permission from Elsevier Inc.)

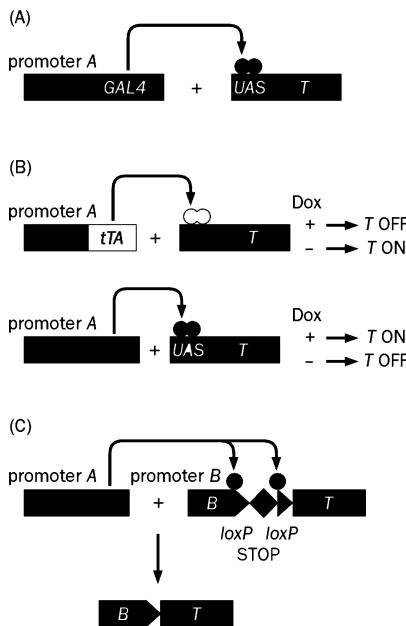


Figure 13–13 Binary expression of transgenes. Circles indicate protein products from the first (driver) transgene that act on the second (responder) transgene to regulate its expression. **(A)** Transcription unit *T* is expressed indirectly under the control of promoter *A* through the GAL4/UAS system using two transgenes. **(B)** Here, *T* is expressed indirectly under the control of promoter *A* through the tTA/TRE or rtTA/TRE systems; exogenous application of doxycycline (Dox), a tetracycline analog, provides an additional mechanism for temporal control. tTA is only active in the absence of Dox, whereas rtTA is only active in the presence of Dox. **(C)** Binary expression can be achieved by using Cre recombinase to excise the transcription and translation stop signals (diamond) between two *loxP* sites (triangles), enabling the expression of *T*. Often a ubiquitous (widely active) promoter is chosen as promoter *B*. (Adapted from Luo L, Callaway EM & Svoboda K [2008] *Neuron* 57:634–660. With permission from Elsevier Inc.)

can accommodate hundreds of kilobases of DNA—to drive transgene expression often improves the likelihood of reproducing the expression pattern of an endogenous gene (Figure 13–12B).

Transgenic animals are usually created by injecting DNA into early embryos (for example, the pronuclei in single-cell embryos of mammals); the injected transgenes then integrate randomly into host chromosomes. Expression of randomly integrated transgenes (usually multiple copies in tandem repeats) can be subject to the influence of endogenous regulatory sequences near the integration sites, causing transgene expression patterns to be variable or unpredictable. Site-specific integration of transgenes, in which an integrase catalyzes the insertion of a single copy of a transgene into a predetermined genomic locus via DNA recombination, offers greater consistency of expression. To facilitate this, one part of the integrase recognition sequence is knocked into the host chromosome at a predetermined locus, and another part of the integrase recognition sequence is inserted into a vector that carries the transgene. Integrase-mediated recombination between the two recognition sequences causes the insertion of the transgene into a predetermined locus (Figure 13–12C). (The integrase itself is either co-injected as an mRNA, or is expressed from a separate transgene.) The most faithful mimicry of endogenous gene expression pattern is achieved by knock-in to insert the transgene at the genomic locus of the endogenous gene (Figure 13–12D). Generating knock-ins is more laborious and currently can be performed only in a limited number of organisms, such as flies and mice. However, the genome-engineering tools discussed in Box 13–1 hold promise for the development of faster procedures that can be used in any organism for which transgenesis is possible.

The regulatory and protein-coding components of a gene of interest can also be expressed separately as two transgenes, a strategy called **binary expression**. For instance, the regulatory elements can be used to drive the yeast transcription factor **GAL4**, and the coding sequence of a gene of interest can be driven by a **UAS** (upstream activating sequence of GAL4). When the two transgenes are present in the same animal, the gene of interest will be expressed in the same cells that express GAL4 (Figure 13–13A). This **GAL4/UAS** binary expression system is widely used in *Drosophila* (for example, see Figure 9–6). A binary expression system often used in mice consists of the transcription factor **tTA** (tetracycline-regulated trans-activator) and its binding sequence **TRE** (tetracycline response element). A gene under the control of a TRE is activated only in cells that express tTA, which is driven by the regulatory elements of interest (Figure 13–13B). In addition, this system can be regulated using a drug: tTA activates TRE only in the *absence* of tetracycline. (A variant of tTA, reverse tTA or **rtTA**, activates TRE only in the *presence* of tetracycline). Since tetracycline and its analog **doxycycline** (Dox) are small molecules that readily diffuse across cells and the blood-brain barrier, drug treatment provides temporal control of transgene expression. Another binary expression system widely used in mice is the **Cre/loxP** system described previously: a gene of interest can be placed after a transcriptional/translational stop sequence flanked by *loxP* sites (called a *loxP-stop-loxP* sequence) following a ubiquitous promoter (a promoter that is strongly active in a wide range of cells, tissues, and developmental stages). In cells that lack Cre activity, transcription and/or translation of the transgene is disrupted by the *stop* sequence such that the transgene is not expressed. Only in Cre-active cells will the transcription and translation stops be excised by recombination so that the transgene can be expressed *under control* of the ubiquitous promoter (Figure 13–13C). As discussed in Section 13.7, CreER can be used instead of Cre to enable temporal control of transgene expression in this system.

The binary systems illustrated in Figure 13–13 have increased flexibility and versatility compared to the single transgene expression systems shown in Figure 13–12. For instance, simple genetic crosses can be employed to combine a given **responder transgene** (under the control of UAS, TRE, or promoter-*loxP-stop-loxP*) with different **driver transgenes** that express GAL4, tTA, or Cre to produce animals in which the responder transgene is expressed in different spatiotemporal patterns. Likewise, the same driver transgene can be combined

with many responder transgenes in different animals. As a specific example, UAS-hairpin RNA transgenes (see Figure 13–9B) have been produced for almost all of the approximately 15,000 protein-coding genes in the *Drosophila* genome, such that researchers can use specific GAL4 drivers to knockdown genes one at a time in cell types of interest. This can facilitate unbiased genetic screening to identify genes that are necessary for any given biological process.

13.11 Transgene expression can also be achieved by viral transduction and other transient methods

Section 13.10 discussed methods of transgene expression that rely on the integration of transgenes into the germ line to produce transgenic animals. In this way, the same expression pattern can be reproduced in different animals across multiple generations. Other methods have been used to express transgenes transiently in somatic cells. Transient methods have the drawback that expression levels and patterns may differ from animal to animal. However, they are simpler and faster, especially in animals that have long generation times or for which germ-line transgenesis techniques have not been established.

One transient method is to directly inject DNA or mRNA encoding a gene of interest, usually into large cells such as those in early embryos. Incorporation of DNA into the host cell genome confers all progeny derived from the cell the potential to express the transgene, whereas mRNA injection is usually limited to studying early development (as mRNAs are diluted with cell division and degraded with time). Another transient method is **electroporation** where DNA containing the transgene is introduced into cells in a specific brain region of a host animal by placing a micropipette containing the DNA near the cells of interest and applying electrical currents to facilitate the transfer of negatively charged DNA molecules into the cells.

A widely used transient method of transgene expression in neurobiology, especially in mammals, is via **viral transduction**. Here, transgenes can be expressed from viral vectors that are used to produce high-titer viruses, which are usually delivered to specific region of interest by **stereotactic injection** (that is, the use of a three-dimensional coordinate system to inject substances such as viruses into a small target region). The most commonly used viruses in neurobiology include **adeno-associated virus (AAV)**, **lentivirus**, or **herpes simplex virus (HSV)**, each of which can be used for specific purposes based on its characteristic properties (Table 13–1). These viral vectors have been engineered to minimize the deleterious effects of transduction and to allow spatiotemporal control of transgene expression using strategies similar to those described above, such as Cre-dependent expression (for example, see Figure 9–37A). Viral vectors are the predominant mechanisms employed to alter gene expression in humans for the purpose of **gene therapy**, that is, the use of DNA as a therapeutic agent to treat disease.

Table 13–1: Properties of commonly used viral vectors for gene expression in the nervous system

Property	Adeno-associated virus (AAV)	Lentivirus	Herpes simplex virus (HSV)
Genetic material	single-strand DNA	RNA	double-strand DNA
Capacity	~ 5 kilobases	~ 8 kilobases	~150 kilobases
Speed of expression	weeks	weeks	days
Duration of expression	years	years	weeks to months
Tropism (cell types susceptible to viral transduction)	from broad to highly preferential depending on the serotype	usually pseudotyped ¹ with coat proteins from other viruses for broad tropism	broad tropism for neurons

¹ To pseudotype a virus, the gene encoding the endogenous coat protein is deleted, and viruses are assembled in cell lines that co-express genes encoding a coat protein from a different virus.

13.12 Accessing specific neuronal types facilitates functional circuit dissection

In complex nervous systems, a cell type rather than an individual cell is often the unit of neural circuit organization. The ability to monitor and manipulate neuronal activities of specific cell types is critical for neural circuit analysis. Thus, establishing genetic access to specific cell types for recording, silencing, or activation has become a fundamental experimental approach (see Sections 13.21–13.26). The most common strategy employed to gain genetic access to a specific cell type is to identify genes that are expressed in that cell type and then use regulatory elements from those genes to drive responder transgenes in the cell type of interest. For example, the promoters of odorant receptors allow genetic access to specific types of olfactory receptor neurons (see Figures 6–15 and 6–28); likewise, the regulatory elements of *fruitless* permit genetic access to many types of neurons that express endogenous *fruitless* (see Figure 9–6).

As the expression pattern of *fruitless* illustrates, a given gene may be expressed in many cell types (see Sections 9.6–9.9). Likewise, most neuronal cell types have no corresponding endogenous genes that are expressed exclusively within those cells and nowhere else. Thus, additional methods have been employed to identify regulatory elements or binary system drivers that are expressed in specific subpopulations of neurons. One approach is to use only a fraction of an endogenous enhancer's elements to drive transgene expression, based on the assumption that distinct, separable regulatory elements control the endogenous gene expression in different cell types. A second approach is to use **intersectional methods**: if promoter A drives gene expression in cell types X and Y, and promoter B drives gene expression in cell types Y and Z, then one can create an AND logic gate (if A and B, then C) for expression only in Y (Figure 13–14A), or a NOT logic gate (if A and not B, then C) for expression only in X (Figure 13–14B). Indeed, the dissection of the *fruitless* circuit for mating behavior has extensively utilized these intersectional approaches (for example, Figure 9–9A). Other methods include the use of the timing of neuronal birth and cell lineage to access specific neuronal populations, with the assumption that specific populations of neurons may be born within a specific developmental window and/or may arise from a common ancestor (see Figure 7–40 and Section 13.16). Yet other methods use activity of neurons to gain genetic access to specific populations by utilizing, for example, properties of immediate early genes (see Figure 10–36).

13.13 Gene expression patterns can be determined by multiple powerful techniques

We have repeatedly referred to recapitulating patterns of endogenous gene expression in previous sections. How is a gene expression pattern revealed in the

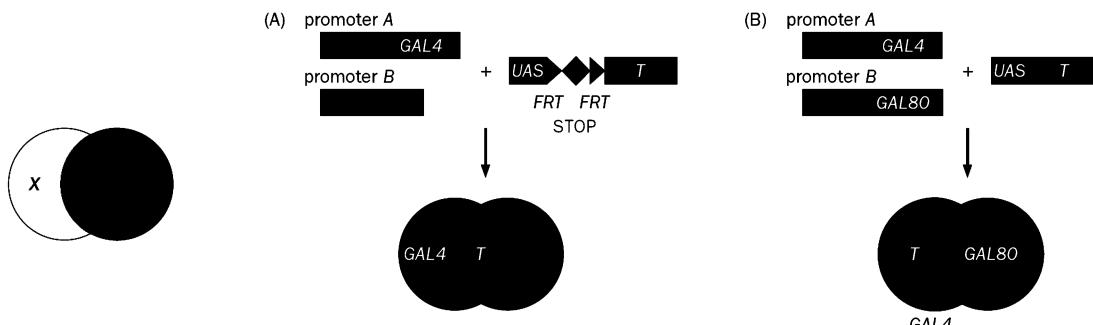


Figure 13–14 Refining transgene expression by intersectional methods. In both examples, promoter A drives gene expression in cell populations X and Y, while promoter B drives gene expression in populations Y and Z. **(A)** An AND logic gate strategy in which target gene T is expressed only in population Y, utilizing a combination of the GAL4/UAS binary expression system and the FLP/FRT recombination system. T can be expressed only in cells in which

GAL4 is available to drive UAS expression and FLP is available to remove the stop signal. **(B)** An NOT logic gate strategy in which target gene T is expressed only in population X. GAL80 is an inhibitor of GAL4; in cells expressing both GAL80 and GAL4, transcription from UAS is repressed. (Adapted from Luo L, Callaway EM & Svoboda K [2008] *Neuron* 57:634–660. With permission from Elsevier Inc.)

first place? Many techniques have been developed for this purpose, depending on whether researchers want to characterize the expression pattern of mRNA or protein, the level of quantitative precision and spatial resolution required, and whether the expression pattern of one gene or many genes is to be characterized.

The first technique involves isolating the mRNAs or proteins from specific tissues, using gel electrophoresis to separate each tissue's mixture of mRNAs or proteins according to physical properties such as molecular weight or net electrical charge, and transferring the separated contents of the gel to a nylon or nitrocellulose membrane for probing. A labeled gene-specific nucleic acid probe can be hybridized to membrane-bound mRNAs to produce a **northern blot** (for example, see Figure 6–9B), whereas probing of membrane-bound proteins to generate a **western blot** is based on antigen recognition by specific labeled antibody probes. (The compass-point names of these and related methods were inspired by **Southern blotting**, a similar technique developed by Edwin Southern, in which DNA immobilized on a membrane hybridizes with sequence-specific DNA probes.) Northern and western blots provide information about the molecular weights and relative abundance of specific mRNAs or proteins, respectively, within a sample, but their spatial resolution is limited to tissue type.

To characterize mRNA distribution in intact tissue, fixed tissue sections can be hybridized with gene-specific probes, a procedure called ***in situ* hybridization** that we have encountered many times (for example, see Figure 5–7). Recently, *in situ* hybridization has been applied systematically to map the expression patterns in the adult mouse brain for all of the estimated 20,000 genes in the mouse genome (Figure 13–15), creating a valuable gene expression database. Protein distribution can likewise be determined by a technique called **immunostaining** that has also been used in numerous experiments described in this book. In this approach, fixed tissues are incubated with **primary antibodies** that bind to specific proteins, followed by **secondary antibodies** that selectively recognize primary antibodies made by specific animal species. The secondary antibodies can be tagged with an enzyme to produce a color substrate (for example, see Figure 7–12A), or with fluorescent molecules that allow simultaneous visualization of multiple proteins (for example, see Figure 2–26B). Although these techniques provide cellular and subcellular resolutions (especially in the case of immunostaining; see also Section 13.17), *in situ* hybridization and immunostaining are not as easily quantified as northern or western blots.

The complete sequencing of whole genomes brought new methods that allow quantitative determination of the expression levels of all genes in specific tissues or cell types. With **DNA microarray** technology, oligonucleotides or gene-specific probes are individually immobilized onto specific spots on a solid substrate; these spots can be arranged densely such that probes corresponding to expressed sequences from the entire genome can be packed onto a single chip (which can contain more than a million probes per cm²). mRNA mixture from a specific tissue can be labeled and hybridized to the chip, and expression levels of all genes can be read out as signal intensities from individual spots approximately proportional to the level of RNA label. Genes with similar expression profiles can then be grouped together for expression pattern analysis (for example, Figure 13–16). A more recent alternative to DNA microarrays is **RNA-seq**, in which mRNA molecules from a given tissue are simply sequenced one by one in a massively parallel fashion using next generation sequencing methods (see Section 13.14). Each

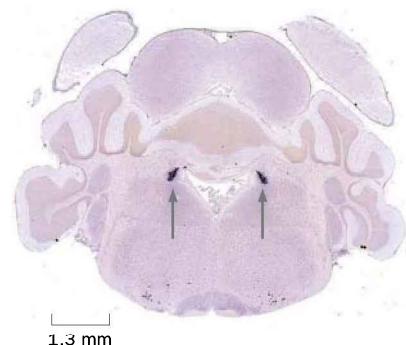


Figure 13–15 Determining gene expression pattern by *in situ* hybridization. The expression pattern of dopamine β -hydroxylase, an enzyme that converts dopamine to norepinephrine, in a coronal section of the mouse brain determined by hybridizing the brain section with a probe that specifically recognizes mRNAs that produce dopamine β -hydroxylase. Arrows point to the bilateral locus coeruleus, where most norepinephrine neurons in the brain reside (see Box 8–1). Expression patterns of all mouse genes in the brain have been determined systematically by *in situ* hybridization. (From Lein ES, Hawrylycz MJ, Ao N et al. [2007] *Nature* 445:168–176. With permission from Macmillan Publishers Ltd; see also <http://mouse.brain-map.org/>.)

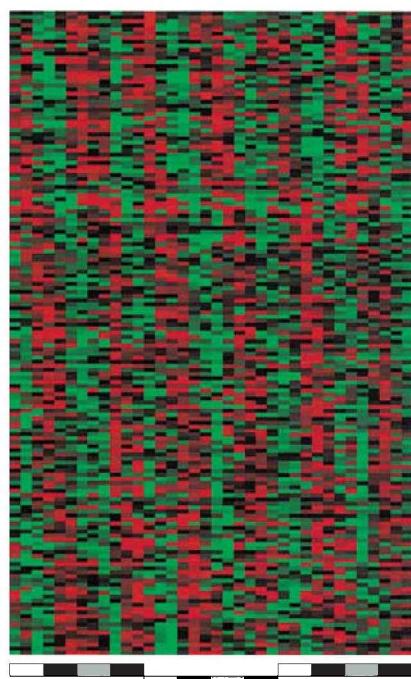


Figure 13–16 Determining gene expression patterns of many genes by microarray analysis. Each row represents a single *Drosophila* gene. Columns represent mRNA levels for given genes during specific phases of the circadian cycle indicated at the bottom (white, day; black, night; gray, subjective day, that is, day according to circadian rhythm under constant darkness condition). *Drosophila* heads were collected every four hours in three two-day time blocks to extract mRNA, from which probes were made for hybridization to the microarray. Green or red represent high or low expression, respectively. These selected genes all exhibit a circadian cycle in their expression, peaking at different phases of the cycle. (From Claridge-Chang A, Wijnen H, Naef F et al. [2001] *Neuron* 32:657–671. With permission from Elsevier Inc.)

mRNA molecule produces a single read of nucleotide sequences that are sufficiently long to identify the gene from which the mRNA is transcribed. The entire set of reads provides qualitative information about which genes are expressed in a given tissue sample and quantitative data regarding the numbers of specific mRNA molecules present in the sample.

Gene expression profiling methods such as microarray and RNA-seq are more informative and powerful when they can be applied at cell-type resolution (that is, to highly purified populations of specific cell types). Many methods have been developed for purifying cell types of interest. For instance, as an extension of traditional physical dissection, laser-capture microdissection allows histologically or fluorescently labeled cells from fixed tissue sections to be cut out with a laser beam for mRNA extraction. Other methods of purifying specific cells include sorting dissociated cells based on fluorescence or cell-surface markers, purifying mRNA specifically from cells that express transgenes encoding a tagged poly-A binding protein or ribosomal subunit, or using a micropipette to select genetically labeled fluorescent cells. The combination of high-fidelity amplification by polymerase chain reaction and high-sensitivity sequencing technology has enabled reliable gene expression profiling from very small populations of cells, even down to a single cell.

13.14 Genome sequencing reveals connections across species and identifies genetic variations that contribute to diseases

Along with recombinant DNA technology, DNA sequencing has transformed modern biology. Since the first development in the 1970s, DNA sequencing technology has seen rapid growth, thanks in large part to the Human Genome Project initiated in the late 1980s. In the subsequent two decades, and in particular with the introduction of many kinds of massively parallel sequencing platforms in the mid-2000s, the cost of sequencing has fallen dramatically while the speed has increased by many orders of magnitude (**Figure 13–17**).

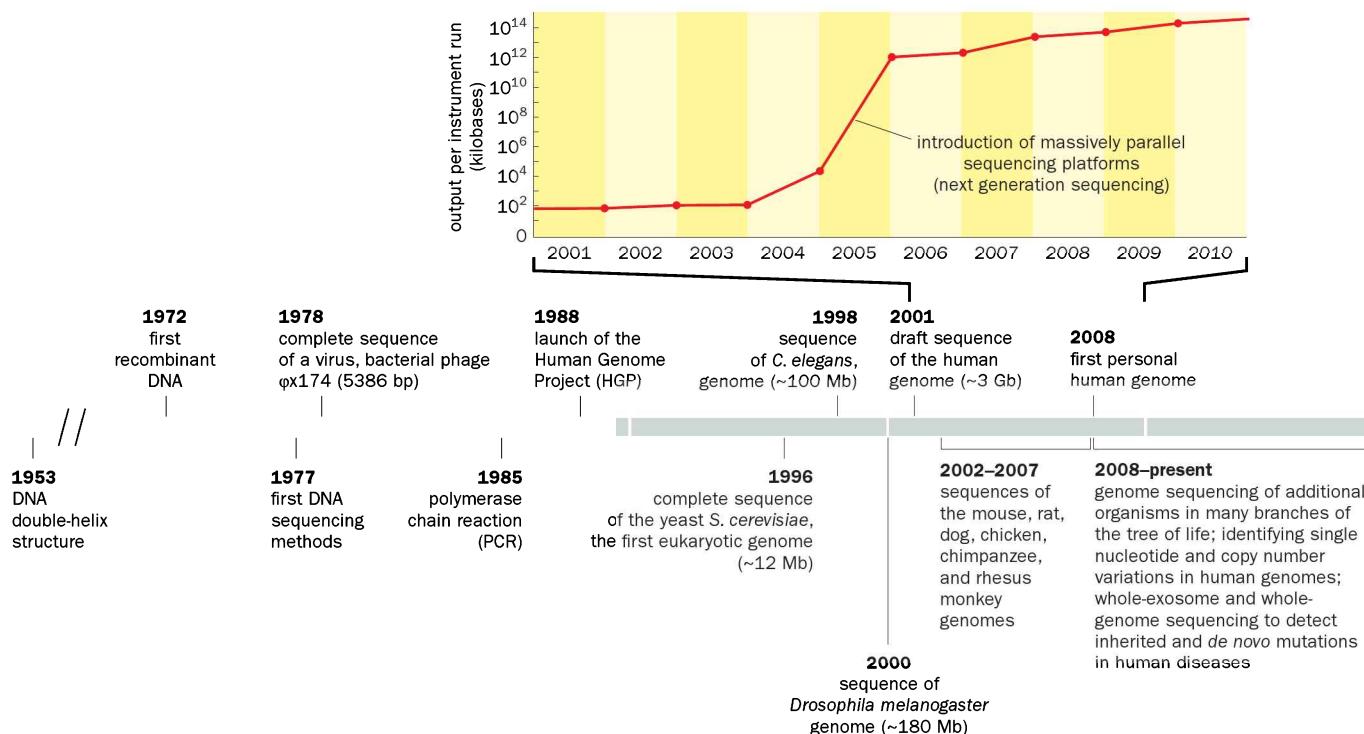


Figure 13–17 Timeline of genome sequencing and related advances. Below the timeline are selected milestones. The graph above illustrates the exponential growth of sequencing technology in the 10 years since the draft human sequences were first published. (The top graph is adapted from Mardis ER [2011] *Nature* 470:198–203. With permission from Macmillan Publishers Ltd.)

In parallel with determining the human genome sequence, the first drafts of which were completed in 2001, whole-genome sequences have been determined for many organisms in all branches of the tree of life. The impact of these data and knowledge on research has been enormous. For example, when researchers identified a gene of interest in a model organism in the 1980s or 1990s, it often took months to years to determine how many similar genes might exist within the same organism or in other organisms and to compare how similar the gene was to its homologs (for example, see Section 4.12). These questions can now be answered definitively in minutes by searching genome sequence databases. Indeed, as we learned in Chapter 12, comparative genomics in different species provides insights into how individual genes arise during evolution and how different organisms are related to each other in the tree of life.

Likewise, comparing the genomes of different individuals within the same species, such as humans, should reveal the genetic contributions to individuality. While its contribution to understanding variation in human traits is still being explored, comparative human genomics has already greatly expanded our understanding of the genetic bases of diseases, including many brain disorders that are inherited or caused by *de novo* mutations (see Box 11–3). It has also launched a new era of personalized medicine, in which treatment strategies are customized based on genetic etiology rather than symptoms; specific treatments may be more successful with patients that share genetic etiologies rather than just similar symptoms.

ANATOMICAL TECHNIQUES

In order to comprehend how the nervous system operates, it is necessary to understand its structure at different levels. In the following sections, we will examine the major anatomical techniques that have advanced our knowledge of nervous system structures. We begin with general histological methods that have provided overviews of nervous system organization. We then review techniques for visualizing individual neurons, the building blocks of the nervous system. We probe further into the fine structures of individual neurons. Lastly, we study methods that determine how neurons connect with each other to construct the wiring diagram of the nervous system.

13.15 Histological analyses reveal the gross organization of the nervous system

The anatomical organization of the nervous system is typically examined in **histological sections**; frozen or chemically fixed tissues are sliced into sections using microtomes, with the thickness of the slices ranging from several to several hundred micrometers, so that the sectioned tissues can be examined under a light microscope. As we introduced in Chapter 1, three commonly used sections are coronal, sagittal, and horizontal, which are perpendicular to the anterior-posterior (rostral-caudal), medial-lateral, and dorsal-ventral axes of the body, respectively (see Figure 1–8C).

Histological sections are typically stained to create contrast and highlight specific structures for microscopic examination. Starting in the nineteenth century, long before molecular techniques such as *in situ* hybridization and immunostaining became available (see Section 13.13), histologists invented staining methods to label cell bodies, axon fibers, or myelin sheaths; this early work revealed the overall organization of the gray and white matters in the CNS, as well as subdivisions within the gray matter. One of the most widely used staining methods for cell bodies is the **Nissl stain**, which utilizes basic (that is, proton-accepting, positively charged) dyes such as cresyl violet that bind to RNA molecules (which are negatively charged) and thereby highlight the rough endoplasmic reticulum in cytoplasm enriched for ribosomal RNAs. When applied to brain sections, Nissl stain provides a comprehensive overview of the density, size, and distribution of neurons and glia, and is commonly used to construct brain atlases and as a

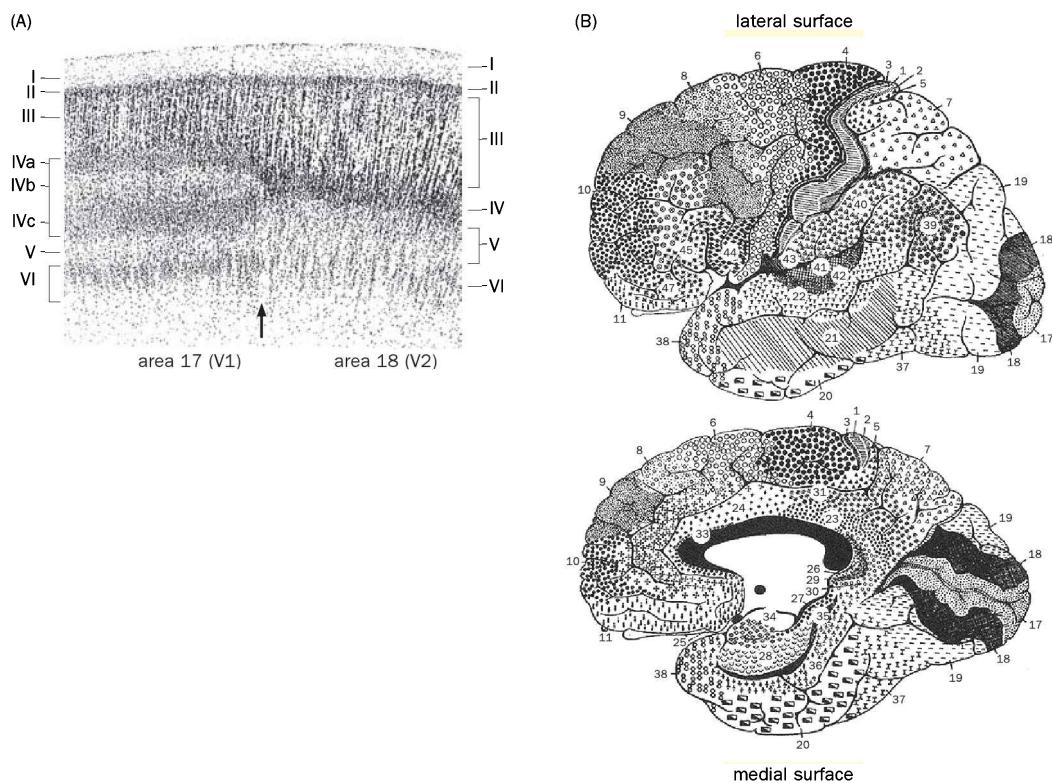


Figure 13–18 Nissl stain and cortical area divisions. (A) Nissl stain of a section across the border of the primary (area 17, or V1) and secondary (area 18, or V2) human visual cortices. Layer IV of V1 is characterized by the presence of three sublayers (IVa, IVb, IVc), whereas V2 has a single layer IV. Arrow indicates the border of V1 and V2. (B) The type of histological staining shown in panel A

has been used to visualize cytoarchitectonic differences that define distinct areas within the cerebral cortex; for example, the human cerebral cortex is divided here into 50 areas, each represented by a specific symbol. (Adapted from Brodmann K [1909] *Vergleichende Localisationslehre der Grosshirnrinde in ihren Prinzipien dargestellt auf Grund des Zellenbaues*. Barth: Leipzig.)

counterstain for *in situ* hybridization, immunostaining, and additional anatomical methods, some of which are discussed below. Other staining methods selectively label cell nuclei using dyes that preferentially bind to DNA. The Nissl and nuclear staining methods enabled many discoveries regarding nervous system organization, such as the layering of the lateral geniculate nucleus and neocortex (see Figures 4–37 and 4–45A), the whisker-barrel pattern in the rodent somatosensory cortex (see Figure 5–27), and the sexual dimorphism of certain mammalian brain nuclei (see Figure 9–28). Indeed, using **cytoarchitectonics**, an approach based on differences in the laminar (layered) organization of cortical neurons as well as the density and thickness of each layer (Figure 13–18A), early twentieth century histologists divided the cerebral cortex into distinct areas (Figure 13–18B); these divisions are still used today.

Histological sections allow ready penetration of staining reagents throughout the sectioned tissues and high-resolution microscopic examination and imaging of neural structures within the sections. However, deciphering large-scale anatomical organization, such as axonal projections from one brain region to another (see Section 13.18), requires the reconstruction of three-dimensional volumes from individual two-dimensional sections. By contrast, **whole-mount** preparations enable investigators to examine the nervous system as a whole, whether in a dissected specimen or an intact organism. High-resolution fluorescence imaging of whole-mount tissues can be obtained by using **confocal fluorescence microscopy** (which is often shortened to confocal microscopy; confocal means ‘having a common focus’). In confocal microscopy, a laser beam is focused on a spot with a volume on the order of one cubic micrometer and a pinhole is employed near the detector to ensure that fluorescence is collected only from this spot (Figure 13–19A). By scanning the laser across a plane to image many focal spots, confocal microscopy can produce thin optical sections of thick tissues, with

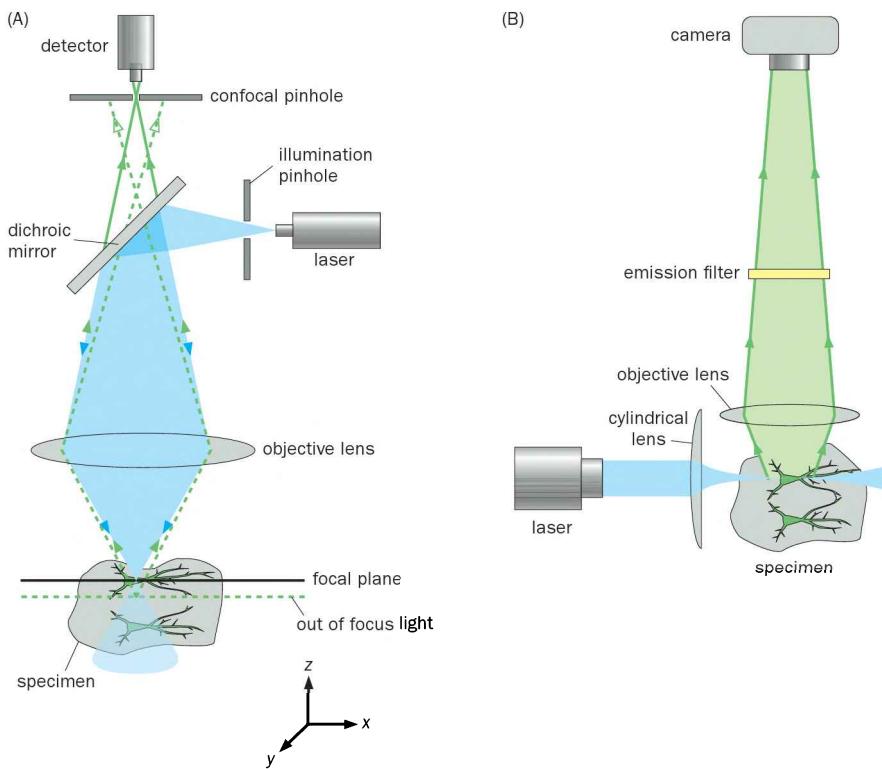
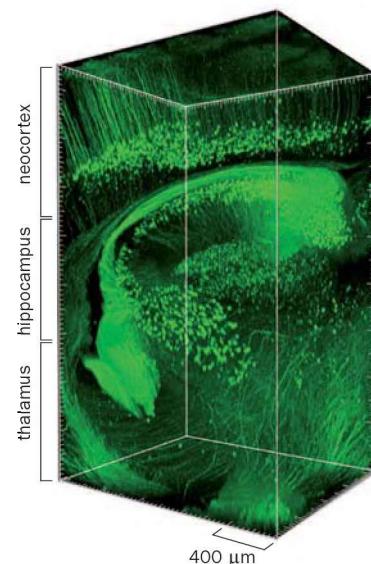


Figure 13–19 Confocal and light-sheet fluorescence microscopy. These schemes are simplified to highlight the unique features of each system. **(A)** In confocal microscopy, a small pinhole is placed in front of a detector such that only fluorescence emission from the focal plane (solid green lines and arrows), but not emission from out-of-focus planes (for example, dashed green lines and open arrows), is collected by the detector. The dichroic mirror reflects the short-wavelength excitation light (blue) but transmits the long-wavelength emission light (green). In a typical imaging experiment, the laser beam scans across different x - y positions on the focal plane such that the detector can reconstruct a two-dimensional image for that focal plane. Then the focal plane is adjusted along the z axis to produce two-dimensional images of other focal planes, eventually producing a three-dimensional confocal stack representing the three-dimensional volume of the specimen. For details, see Conchello JA & Lichtman JW (2005) *Nat Methods* 2:920–931. **(B)** In light-sheet microscopy, the laser illuminates the specimen from the side and, through the cylindrical lens, produces a thin sheet of excitation light at the focal plane of the objective lens. Only tissues in the focal plane are exposed to the excitation light, and all fluorescence emission in the focal plane is collected simultaneously by a detector (camera) to form a two-dimensional image without scanning. By systematically moving the focal plane and excitation beam along the z axis, a three-dimensional stack can be produced to represent the three-dimensional volume of the specimen. For details, see Keller PJ & Dodt HU (2012) *Curr Opin Neurobiol* 22:138–143.

the thickness of the optical section ranging from a fraction of a micrometer to several micrometers. Three-dimensional structures can be reconstructed from a series of such optical sections obtained at consecutive x - y planes along the z axis (the axis perpendicular to the imaging plane). Many images shown in this book are the products of confocal microscopy of whole-mount brains (for example, see Figure 6–33). In addition to imaging of whole-mount preparations, confocal microscopy is also widely used to obtain thin optical sections from thicker physically sectioned tissues.

In the past, the use of whole-mount preparations has typically been restricted to tissues that are less than a few hundred micrometers thick, limited by penetration of staining reagents and the opacity of tissues due to light scattering. Common whole-mount preparations have included intact *C. elegans*, dissected brain and ventral nerve cord of *Drosophila*, or nervous systems from organisms at early stages of development, such as a zebrafish larva or a mouse embryo. More recently, the development of a number of tissue-clearing methods has enabled high-resolution fluorescence imaging of larger pieces of intact tissues, up to several millimeters in each dimension (for example, Figure 13–20; Movie 13–1). **Light-sheet fluorescence microscopy**, which illuminates only the focal plane with a thin sheet of laser beam from the side (Figure 13–19B), offers more rapid imaging (because no scanning is required) and less photobleaching of fluorescent probes (because out-of-focus planes are not illuminated) compared with confocal microscopy. Because of these properties, light-sheet microscopy is particularly

Figure 13–20 CLARITY-based tissue clearing for fluorescence imaging. In this method, intact tissue is fixed in the presence of hydrogel monomers that covalently link DNA, RNA, and proteins into a mesh during subsequent polymerization. Lipids, which are the major source of opacity for fluorescence imaging, are not covalently linked, and are removed during the subsequent clearing process by passive diffusion or electrophoresis in the presence of detergent. The resulting tissue is nearly transparent for fluorescence imaging across several millimeters in each dimension. Shown here is a piece of tissue from a Thy1-Gfp transgenic mouse (see Figure 13–22 for details) imaged with confocal microscopy, showing labeled neurons and their axonal fibers across the neocortex, hippocampus, and thalamus. (From Chung K, Wallace J, Kim SY et al. [2013] *Nature* 497:332–337. With permission from Macmillan Publishers Ltd.)



advantageous for fluorescence imaging of large blocks of tissues and live tissues. As discussed below, these whole-mount imaging methods can facilitate the study of many aspects of the nervous system's anatomical organization.

13.16 Visualizing individual neurons opens new vistas in understanding the nervous system

As we learned in Chapter 1, development of the Golgi staining method for visualizing the morphologies of individual neurons was a milestone in neurobiology. This method was used by Ramón y Cajal and his contemporaries to establish individual neurons as the building blocks of the nervous system and provided important insights into how information flows within and between individual neurons. Methods for visualizing individual neurons are still important in modern neurobiology, since they allow researchers to correlate the morphology and projection patterns of individual neurons with their cell identity, development, physiological properties, and function in neural circuits.

Because of its simplicity, the Golgi staining method is still used today to characterize the neuronal compositions in a given brain region and to analyze the effects of gene mutations on neuronal morphology (see Figure 11-44A). However, Golgi staining has a number of limitations. It does not reliably stain long-distance axonal projections and fine terminal processes; it cannot be used to visualize neurons in live brains; and it cannot specifically stain an intended cell type because of its random nature. A number of methods that overcome these limitations have been developed.

Individual neurons can be labeled by injecting dyes during intracellular recording experiments (see Section 13.22). Injection of small molecules such as Lucifer yellow (a fluorescent dye) or biocytin allows neurons to be visualized either in live brains as neurons are being recorded or by *post hoc* staining. Dye-filling methods allow a cell's electrophysiological properties to be correlated with its morphology. They also permit tracing of long-distance projections of individual neurons, since no other neurons in the brain are labeled to interfere with such tracing (for example, Figure 13-21).

The use of green fluorescent protein (GFP) from jellyfish as a gene expression marker in living cells has revolutionized many fields of biology. Following the initial introduction of GFP as a marker, fluorescent proteins of different colors

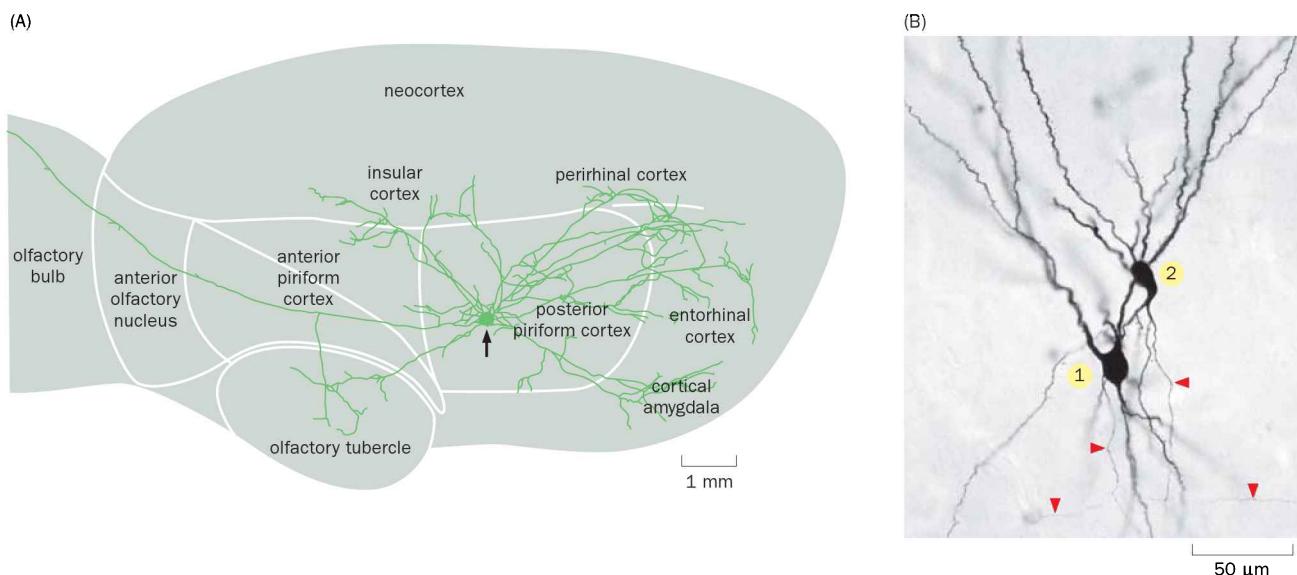


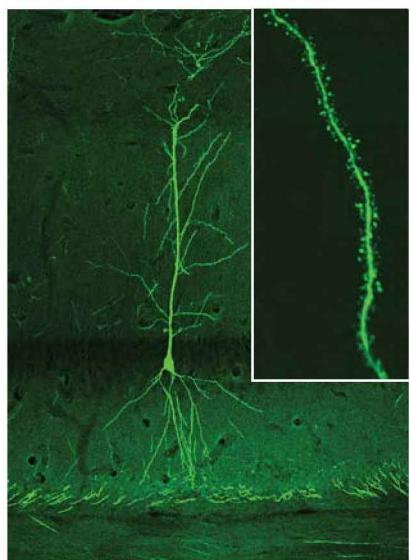
Figure 13-21 Using intracellular dye fill to trace the axonal projection of a single neuron. **(A)** The axonal arborization of a single pyramidal neuron (arrow points to the cell body) in the rat posterior piriform cortex ramifies across the olfactory cortex and other adjacent areas. The reconstruction was performed after intracellular injection of

a tracer *in vivo* followed by several days of recovery before histology. **(B)** A photomicrograph of two numbered cells that have been injected with tracer and stained afterwards. Arrowheads, axons. (Adapted from Johnson DMG, Illig KR, Behan M et al. [2000] *J Neurosci* 20:6974–6982. With permission from the Society for Neuroscience.)

Figure 13–22 Labeling of individual neurons in *Thy1-Gfp* mice. Green fluorescent protein (GFP) expression is driven by the promoter of the *Thy1* gene, which is preferentially expressed in excitatory neurons. Due to random integration of transgenes (see Figure 13–12A), this particular transgenic line labels a very small subset of isolated hippocampal pyramidal neurons, allowing visualization of their dendritic trees and spines (inset). (From Feng G, Mellor RH, Bernstein M et al. [2000] *Neuron* 28:41–51. With permission from Elsevier Inc.)

were discovered and engineered. In conjunction with a variety of genetic methods, these fluorescent proteins enable visualization of individually labeled neurons in live brains. The simplest genetic strategy for visualizing single neurons is one that uses a promoter to drive fluorescent protein expression in sparsely distributed neurons such that only one neuron is visualized in a given region. While such specific promoters are extremely rare in the central nervous system, integration of transgenes into random locations in the genome, for reasons that are still unclear, can result in the fluorescence reporter being expressed in isolated neurons (Figure 13–22). Along with the two-photon microscopy (to be introduced in Section 13.22), this sparse labeling has allowed individual neurons to be visualized in live brains over long periods of time to trace the dynamics and stability of dendritic branches and spines (see Figure 10–48). Individual neurons can also be labeled by sparse activation of a recombinase in animals that express a marker gene in a recombination-dependent manner (see Figure 13–13C). In addition to being able to label live neurons, these genetic methods can specify the type of neurons being labeled through the use of specific regulatory elements to drive expression of the marker gene (see Section 13.10).

Individual neurons can also be labeled by genetic mosaic methods based on mitotic recombination between the homologous chromosomes of somatic cells (see Figure 13–10). The MARCM method (for mosaic analysis with a repressible cell marker; Figure 13–23) has been widely used in *Drosophila* to label individual neurons (see Figure 1–15E) or groups of neurons that share the same lineage (see Figure 7–40). In addition to allowing individual neurons to be labeled, MARCM enables simultaneous deletion of endogenous genes or expression of transgenes in the labeled neurons. An analogous mitotic recombination-based method called MADM (for mosaic analysis with double markers) can achieve similar purposes in mice. Labeling can be restricted to specific cell types by using tissue-specific promoters to drive the recombinase, and can be produced at a desired frequency by controlling the expression level or activity of the recombinase. Because of their versatility, these genetic mosaic methods have been used not only to label individual neurons of a specific type, but also to examine the relationship between lineage and wiring properties (see Figure 7–40) and to determine cell-autonomous gene functions in neuronal morphogenesis and wiring specificity (see Figures 5–29, 5–38, and 7–41).



13.17 Fine structure studies can identify key facets of molecular organization within neurons

Most observations about nervous system structure have been made using light microscopy, which can resolve structures as small as 200 nm, a value that is defined by the diffraction limit of visible light. This resolution is sufficient for visualizing neuronal cell bodies (which have diameters ranging from 3 μm for small neurons in *Drosophila* and *C. elegans* to 10–20 μm for typical vertebrate neurons to roughly 1 mm for giant neurons in *Aplysia*) as well as most dendritic and axonal processes (which are hundreds of nanometers to a few micrometers in diameter). However, resolving fine subcellular structures of neurons, including individual synapses in densely labeled tissues, requires higher resolution techniques.

Electron microscopy (EM) has served neurobiology well since its first use in the 1950s. EM was instrumental in the discovery of the synaptic cleft, the observation of synaptic vesicle fusion with presynaptic membranes, and the finding that glial membranes wrap axons (see Figures 3–3, 3–4, and 2–26). EM has also been used to study many aspects of neuronal cell biology, including cytoskeletal organization, intracellular trafficking (see Figure 2–6), membrane compartment

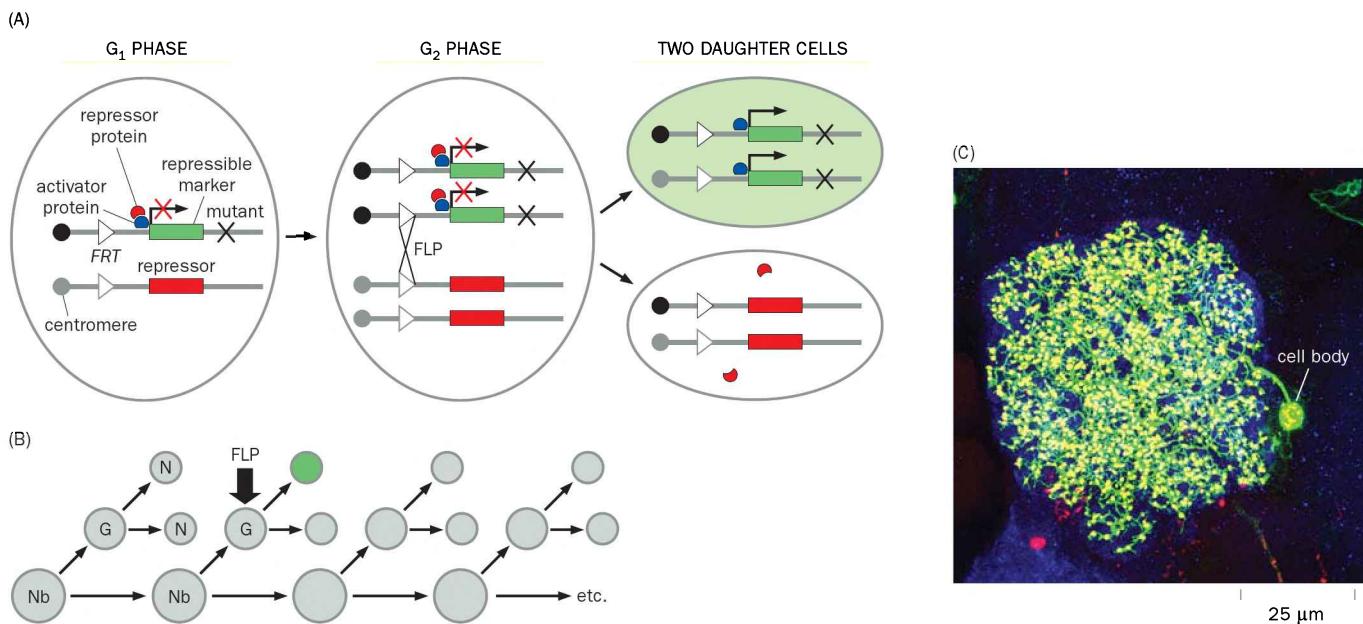


Figure 13–23 Single neuron labeling and genetic manipulation by the MARCM method. (A) In MARCM (mosaic analysis with a repressible cell marker), a cell marker gene (green bar) is under the control of a repressible promoter—it is activated by the activator protein (blue blob) only in the absence of a repressor protein (red blob). The schematic shows a pair of homologous chromosomes in a cell at three cell-cycle stages: before DNA replication (G_1 phase), after DNA replication (G_2 phase), and after cell division that produces two daughter cells. The transgene expressing the repressor protein (red bar) is located on the chromosome *in trans* to a mutation of interest (x). FLP/FRT-mediated chromosomal recombination (cross in the middle panel) followed by cell division results in the loss of the repressor transgene in one of the two daughter cells, thus allowing the marker to be expressed only in that cell, which is also homozygous mutant for a gene of interest. In the original version, the marker is expressed by the GAL4/UAS binary expression system and the repressor is GAL80 (see Figures 13–13 and 13–14). Other

repressible binary expression systems have since been developed. (B) A widely occurring cell division pattern that produces neurons in the insect central nervous system. The neuroblast (Nb) undergoes asymmetric division to produce another neuroblast and a ganglion mother cell (G), which divides once more to produce two post-mitotic neurons (N). If FLP/FRT-mediated recombination is induced in G, then a single neuron is labeled. (C) An example of a MARCM-labeled single *Drosophila* olfactory local interneuron that ramifies throughout the entire antennal lobe. The cell is labeled in green with a membrane-targeted GFP to visualize the neuronal morphology, and in red with an epitope-tagged synaptotagmin that labels its presynaptic terminals, which appear yellow because the red synaptotagmin is expressed within the confine of a green cell. Blue is neuropil staining. (A & B, adapted from Lee T & Luo L [1999] *Neuron* 22:451–461. With permission from Elsevier Inc.; C from Chou YH, Spletter ML, Yaksi E et al. [2010] *Nat Neurosci* 13:439–449. With permission from Macmillan Publishers Ltd.)

organization, and pre- and postsynaptic terminal structures. Most EM images in the book have been taken using **transmission electron microscopy** in which high-voltage electron beams transmit through ultra-thin sections of biological specimen (typically under 100 nm) to create images. **Scanning electron microscopy** produces images by scanning the surface of a biological specimen, collecting information regarding the interaction of the electron beam with the surface areas (for example, see Figure 12–34).

As discussed in Section 13.13, light microscopy can be combined with antibody staining to study the tissue-level and subcellular distributions of individual proteins. Likewise, EM can be combined with antibody staining in a procedure called **immuno-EM** to visualize individual proteins at an ultra-structural level. This method can provide important clues about the actions of individual molecules (for example, Figure 13–24). High-resolution EM has also been used to analyze the atomic structures of proteins such as the nicotinic acetylcholine receptor (see Figure 3–20).

While EM has become the gold standard for ultra-structural analysis, the recent development of a number of **super-resolution fluorescence microscopy** techniques has provided impressive views of the fine structures of neurons at a resolution beyond the diffraction limit of conventional light microscopy. In the variants called **STORM** (stochastic optical reconstruction microscopy) and **PALM** (photoactivated localization microscopy), spatial precision is achieved by photoactivating a random small subset of photo-switchable fluorophores at any one time, such that the position of each fluorophore can be localized to a

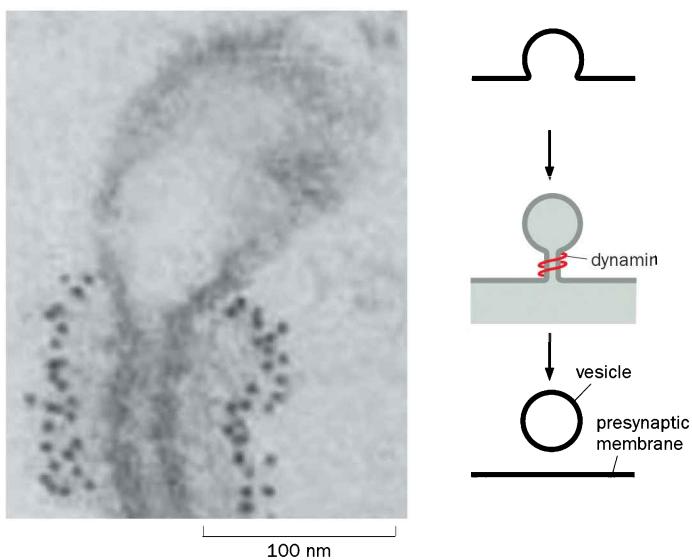


Figure 13–24 Immuno-EM localization of dynamin. Dynamin molecules coat tubular membrane invaginations, visualized here using gold particles in an *in vitro* preparation of nerve terminal membrane. (In this procedure, primary anti-dynamin antibody binds dynamin, and a gold particle-conjugated secondary antibody binds to the primary antibody.) This suggests a function of dynamin in the fission of synaptic vesicles from the plasma membrane during endocytosis, as shown in the schematic at right (red, dynamin localization). This role for dynamin is consistent with the protein's loss-of-function phenotypes (see Figure 3–14). (Adapted from Takei K, McPherson PS, Schmid S et al. [1995] *Nature* 374:186–190. With permission from Macmillan Publishers Ltd.)

precision much finer than the resolution limits set by diffraction. The fluorophores are subsequently deactivated (bleached), and a different subset of fluorophores is photoactivated to allow a second round of localization. Repeated rounds of localization enable the reconstruction of the entire imaging field, but with a resolution that can be <20 nm in the *x*-*y* plane and <50 nm in the *z* axis in brain sections, far superior than the resolution of confocal microscopy (Figure 13–25A). As a result, for example, STORM can be used to determine the distances of different synaptic molecules from the synaptic cleft and the orientations of certain synaptic proteins with respect to the synaptic cleft (Figure 13–25B, C). Likewise, application of **STED** (**s**timulated **e**mision **d**epletion **m**icroscopy), which achieves super resolution by depleting fluorescence from surrounding regions while leaving a central focal spot of the sample active to emit fluorescence, enabled the spatial distribution of **synaptic molecules** at the fly neuromuscular junction to be reconstructed (see Figure 3–11). Compared with immuno-EM for localizing specific molecules with fine resolution, super-resolution fluorescence microscopy is relatively easy to perform, and is especially well suited for imaging multiple proteins that are differentially labeled in the same sample. Super-resolution microscopy can also be used for live imaging to study protein dynamics whereas EM is limited to fixed tissues.

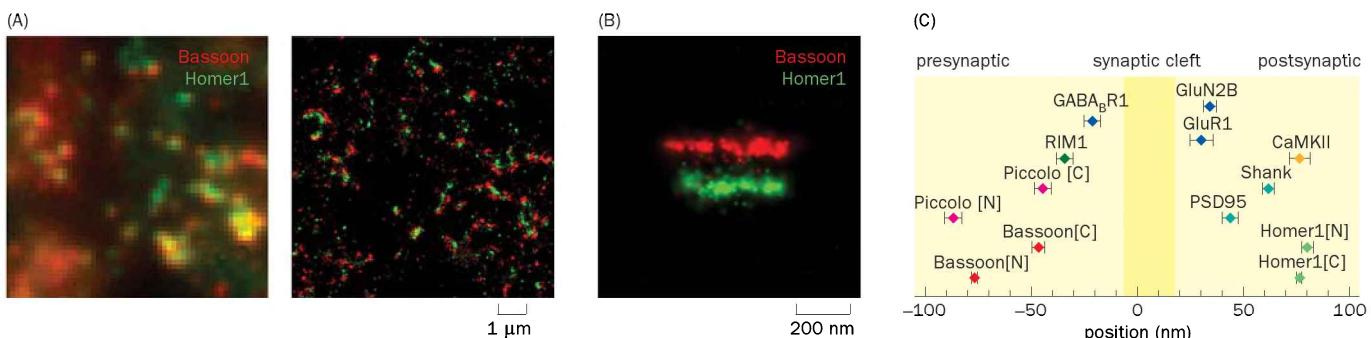


Figure 13–25 Application of super-resolution fluorescence microscopy to map synaptic protein organization. (A) Double labeling of the presynaptic scaffold protein Bassoon (red) and the postsynaptic scaffold protein Homer1 (green) in a section of mouse olfactory bulb's glomerular layer, imaged using confocal fluorescence microscopy (left) and stochastic optical reconstruction microscopy (STORM), a super-resolution microscopic technique (right). Whereas red and green signals exhibit considerably overlap and appear fuzzy at this magnification in the confocal image, they appear distinct in the STORM image. (B) High-magnification view of STORM imaging, which resolves clearly

the distributions of Bassoon and Homer1 across the synaptic cleft. (C) Estimate of the distribution of different synaptic proteins relative to the synaptic cleft, based on the distribution of antibodies against these proteins relative to each other as visualized by STORM. Colored dots are average positions of individual proteins with respect to the synaptic cleft, and vertical bars are standard deviations of the means. To determine the orientation of the molecules with respect to the synaptic cleft, different antibodies were used against N-termini [N] and C-termini [C]. (Adapted from Dani A, Huang B, Bergan J et al. [2010] *Neuron* 68:843. With permission from Elsevier Inc.)

Conventional light microscopy, super-resolution fluorescence microscopy, and EM can be used to systematically study the locations of individual molecules with respect to each other and to the subcellular compartments of neurons. It is conceivable that such investigations might someday provide a realistic model of single neurons at the level of molecular complexes, from the soma to the axonal and dendritic terminals. These molecular neuroanatomical techniques can also probe how protein complexes change with neuronal activity and reveal what goes wrong in brain disorders.

13.18 Mapping neuronal projections allows the tracking of information flow across different brain regions

While fine structural analyses elucidate how molecular complexes are involved in the functioning of individual neurons, deciphering a nervous system's wiring diagram—that is, the representation of how individual neurons connect with each other to form a complex nervous system—poses a great challenge in neurobiology today. All neurons can be classified into one of two categories: **projection neurons** send information from one region of the nervous system to another, whereas the **axonal projections of local neurons** (also referred to as **interneurons**; see Section 1.9) are confined within a given region. Wiring diagrams are being studied at different scales with varying resolutions. Below we discuss methods that are used to map the long-distance connections of projection neurons. In the next section, we study how projection neurons and local neurons form synaptic connections within a local region.

At a global scale, **diffusion tensor imaging** (DTI) is a magnetic resonance imaging technique that allows noninvasive imaging of fiber bundles that connect different structures. The basic idea is that water diffusion in the white matter occurs primarily along the path of axons, whereas water diffusion in the gray matter occurs almost equally in all directions. By acquiring a series of images, each sensitive to diffusion in a specific direction, DTI can determine the motion of water at any given volume in the white matter, which is then used to estimate the trajectories of the axonal fibers passing through that volume. Information obtained across the white matter can then be used to reconstruct the flow lines that approximate the trajectories of axon bundles (Figure 13–26). The resolution of DTI is on the scale of a few millimeters, so it can only depict major pathways in the white matter in large brains such as the human brain; the method is less effective in resolving the trajectories of axons within the gray matter. Still, DTI has already been used to map gross brain connectivity in healthy subjects and abnormalities in patients with neurological disorders.

A widely used method to determine connections between different brain regions in experimental animals is following the trajectory of tracers stereotactically injected at a specific region. **Anterograde tracers** are taken up primarily by



Figure 13–26 Diffusion tensor imaging.
In this sagittal view of the human brain, axon bundles that run mostly along the medial-lateral axis are colored in red, axon bundles that run mostly along the anterior-posterior axis are colored in green, and axon bundles that run through the brainstem are colored in blue. (Courtesy of the Laboratory of Neuro Imaging and Martinos Center for Biomedical Imaging, Consortium of the Human Connectome Project, www.humanconnectomeproject.org)

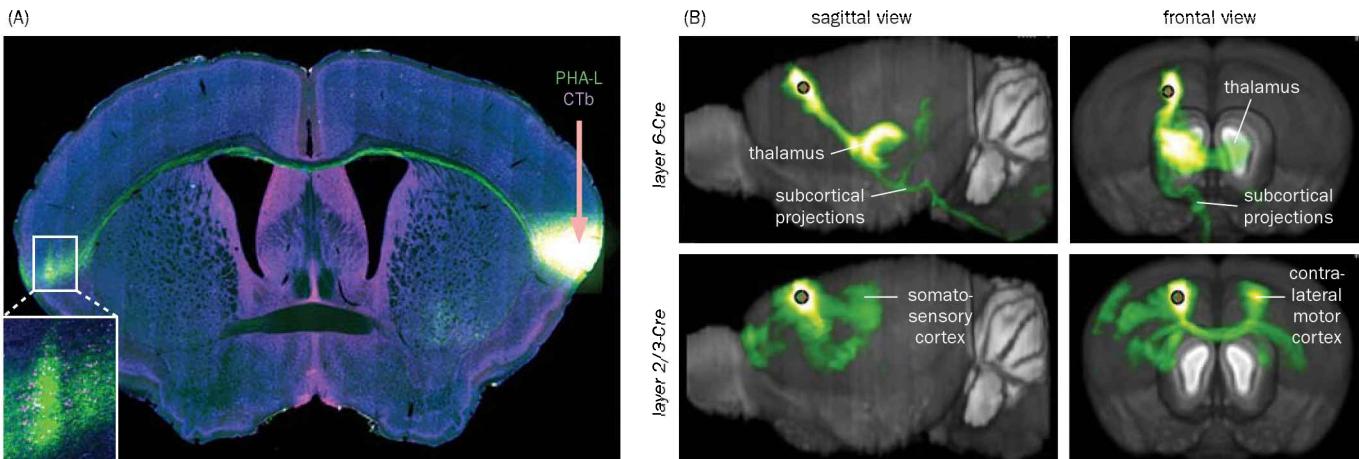


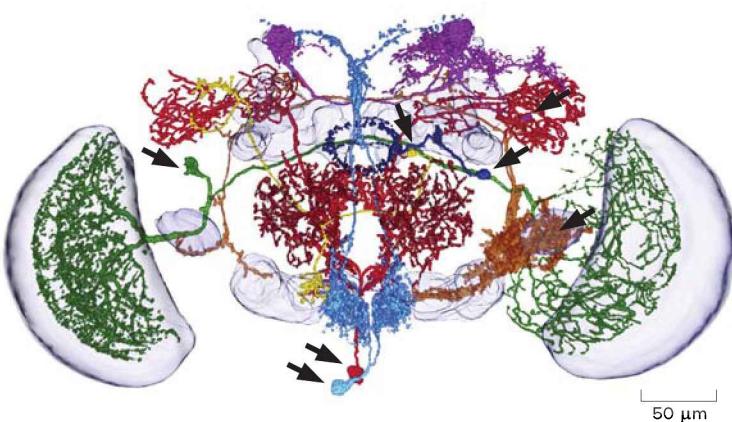
Figure 13–27 Examples of methods that trace long-distance neuronal projections. (A) A mix of phytohemagglutinin (PHA-L, green), an anterograde tracer, and cholera toxin subunit b (CTb, magenta), a retrograde tracer, was injected into the right insular cortex (arrow) of a mouse brain. This coronal section is stained in blue with a fluorescent Nissl stain that labels all cell bodies. PHA-L-labeled axons project to the left (contralateral) insular cortex, where CTb-labeled cell bodies (magenta dots in inset magnified rectangle) represent retrogradely labeled insular cortical cells in the left hemisphere that project to the injection site. Green or magenta areas elsewhere represent projection sites from or to the right insular cortical neurons, respectively. (B) Adeno-associated viruses that express Cre-dependent

GFP (see Figure 13–13C) were injected into the motor cortices of mice expressing Cre recombinase in layer 6 or layer 2/3 neurons. The projection patterns of layer 6 (top) and layer 2/3 (bottom) cortical neurons are shown in sagittal views at left and frontal (coronal) views at right. Circles represent the injection sites. Cre-expressing layer 6 neurons project primarily to the ipsilateral thalamus (with some contralateral thalamic projections) and subcortical regions. Cre-expressing layer 2/3 neurons project mostly within the neocortex. (A, courtesy of Hongwei Dong. See also <http://www.mouseconnectome.org/> and Zeng B, Hintiryan H, Gou L et al. [2014] *Cell* 156:1096–1111; B, courtesy of Honghui Zeng. See also <http://connectivity.brain-map.org/> and Oh SW, Harris JA, Ng L et al. [2014] *Nature* 508:207–214.)

neuronal cell bodies and dendrites, and travel down the axons to label their projection sites. Classic anterograde tracers include radioactively labeled amino acids (which can also be released at the axon terminal and taken up by postsynaptic neurons; see Figure 4–43) and phytohemagglutinin (PHA-L), a lectin from the red kidney bean *Phaseolus vulgaris* (Figure 13–27A). By contrast, **retrograde tracers** are mostly taken up by axon terminals and transported back to the cell bodies. Classic retrograde tracers include horseradish peroxidase and cholera toxin subunit b (CTb; Figure 13–27A). Whether a tracer travels in an anterograde or retrograde fashion is mostly determined empirically. Retrograde tracers likely bind to receptors that are selectively enriched in axon terminals, are taken up by the axon terminals via endocytosis, and utilize the endogenous retrograde axonal transport system to reach cell bodies (see Section 2.3). Anterograde tracers are likely taken up either selectively by receptors in the cell body or nonselectively, with the much greater cell body volume favoring greater uptake by cell bodies than by axon terminals. Anterograde and retrograde tracing are usually performed in conjunction with *in situ* hybridization or more often immunostaining, as well as Nissl stain; the combination of location, axonal projection, and gene/protein expression are often used in conjunction to define neuronal types. Much of our knowledge about the connections between different regions in the mammalian brain has been obtained through experiments utilizing these tracers.

A limitation of classic tracers is that all cells at the injection site take up the tracer, and therefore the projection patterns revealed by these methods represent contributions from a combination of different cell types. Ample data indicate that different cell types within the same region can have distinct projection patterns. For instance, at least 20 different types of retinal ganglion cells are intermingled in the retina with projections to distinct brain targets (for example, only ipRGCs project to the suprachiasmatic nucleus; see Box 4–2); in addition, those types that project to the same target region can have distinct connection patterns within that target region. Tracing the connections of neurons not only from a particular location but also of a particular cell type can thus provide higher resolution in mapping projection patterns. In the mouse, cell-type-specific anterograde tracing can be implemented by injecting an AAV (see Table 13–1) that expresses a marker in a Cre-dependent manner into a transgenic line that expresses Cre recombinase

Figure 13–28 Visualizing many single neurons in the same brain using image registration. Seven neurons, one from each of seven *Drosophila* brains, were individually labeled by the MARCM method using seven different GAL4 lines (representing six different neurotransmitter types); the neurons' projection patterns were determined in whole mount using confocal microscopy. The seven brains were then individually registered to a standard brain using a presynaptic marker counterstain (not shown), and the transformation used to register the counterstain was applied to individually labeled neurons to yield the image. Arrows indicate the seven cell bodies. (From Chiang AS, Lin CY, Chuang CC et al. [2011] *Curr Biol* 21:1–11. With permission from Elsevier.)



in a specific cell type (for example, Figure 13–27B). Since many cell-type-specific Cre lines are available, axonal projections from different cell types within a given region can be studied independently.

Projection patterns of individual neurons can be further deciphered by a number of strategies. For example, a genetic strategy called 'brainbow' has been developed in mice to label individual neurons with different colors using stochastic Cre-dependent expression of different levels of three fluorescent proteins; the labeling of many neurons in different colors can allow multiple neurons to be visualized and traced within the same brain (see Figure 1–12C). An alternative strategy is to generate many singly labeled neurons, each from a different brain, and then use image registration methods to align those images in a common standard brain, such that the projection patterns of individual neurons can be compared (for example, Figure 13–28; see also Section 6.16). While its success is dependent on the accuracy of the brain image registration, this latter strategy has the advantage that individual neurons can be labeled using markers from transgenes with different regulatory elements representing different cell types. The relationships between different cell types, including potential pre- and postsynaptic partners, can thus be examined. However, the overlap of the axons of neuron A with the dendrites of neuron B is necessary but not sufficient for them to be synaptic partners. To reconstruct wiring diagrams with synaptic resolution, it is necessary to use higher resolution anatomical methods discussed in the next section and the physiological methods discussed in Section 13.26.

13.19 Mapping synaptic connections reveals neural circuitry

The most reliable anatomical method to determine whether or not two neurons form synaptic connections is EM, because synaptic connections can be directly visualized in EM. In principle, reconstructions of serial EM sections can establish a neuronal wiring diagram at the synaptic level (also called a **connectome**); indeed, this has been completed for the entire nervous system of *C. elegans* (see Figure 13–2). Serial EM reconstruction of a connectome involves making thin sections (50 nm or less) of the tissue of interest, imaging the sections, segmenting individual images into cell bodies and axonal and dendritic profiles that belong to individual neurons, aligning consecutive images to create a three-dimensional volume, and reconstructing individual segments across image stacks to create three-dimensional volumes of individual neurons (Figure 13–29; Movie 13–2). Synaptic connections between different profiles in EM sections can then be assigned to specific neurons. For larger nervous systems this procedure is not only labor-intensive but also demands a high level of technical precision at each step. Even a very small error rate in a single section may propagate with the tracing of axonal and dendritic profiles across many thousands of sections and compromise the quality of the reconstructed connectome.

Intensive efforts are currently focused on tackling these challenges. For example, in a method called serial block-face scanning electron microscopy, a microtome is integrated with a scanning electron microscope; the cut face of a tissue block is imaged, and then each imaged section is sliced away to allow the next

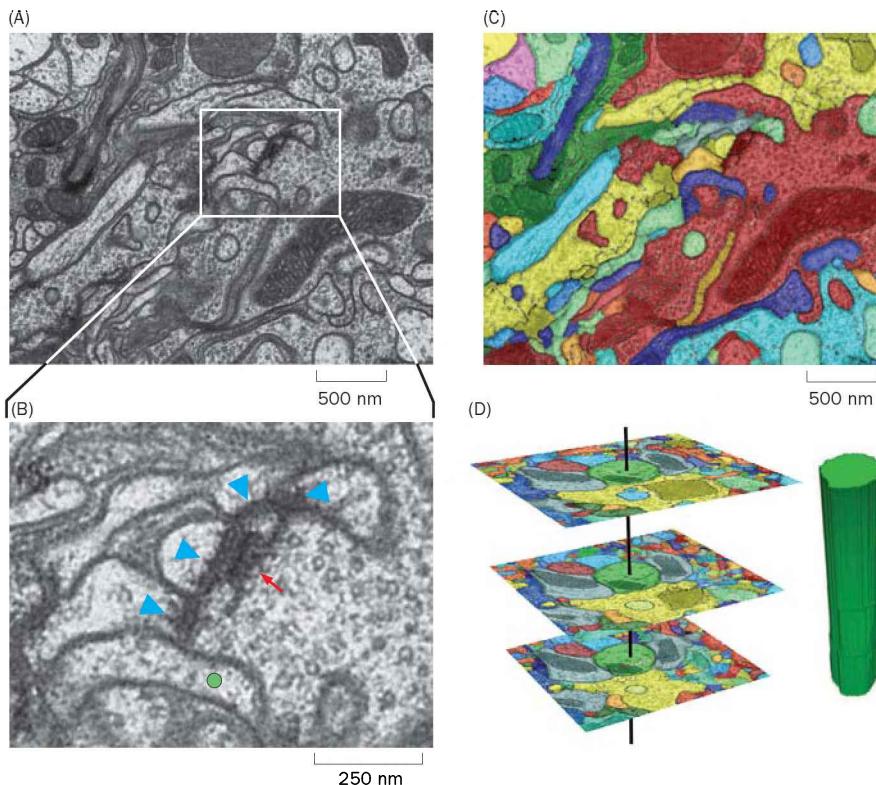


Figure 13-29 Constructing a wiring diagram using serial electron microscopy.

(A) A representative EM micrograph of the *Drosophila* medulla in the optic lobe (see Figure 5–35 for a light microscopic image and a schematic). **(B)** High-magnification view of the box in panel A, showing a presynaptic terminal (red arrow) in contact with four postsynaptic profiles (blue arrowheads). Another profile (green dot) is in contact with the presynaptic terminal but lacks a postsynaptic density in this and adjacent sections, so it was not scored as a synaptic partner. **(C)** The micrograph in panel A is segmented into profiles of neurites (axons and dendrites) by different colors. **(D)** Neurites are reconstructed by linking profiles in thousands of consecutive sections (of which three are shown here) to construct the three-dimensional object shown on the right (see Movie 13–2). (From Takemura S, Bharioke A, Lu Z et al. [2013] *Nature* 500:175–181. With permission from Macmillan Publishers Ltd.)

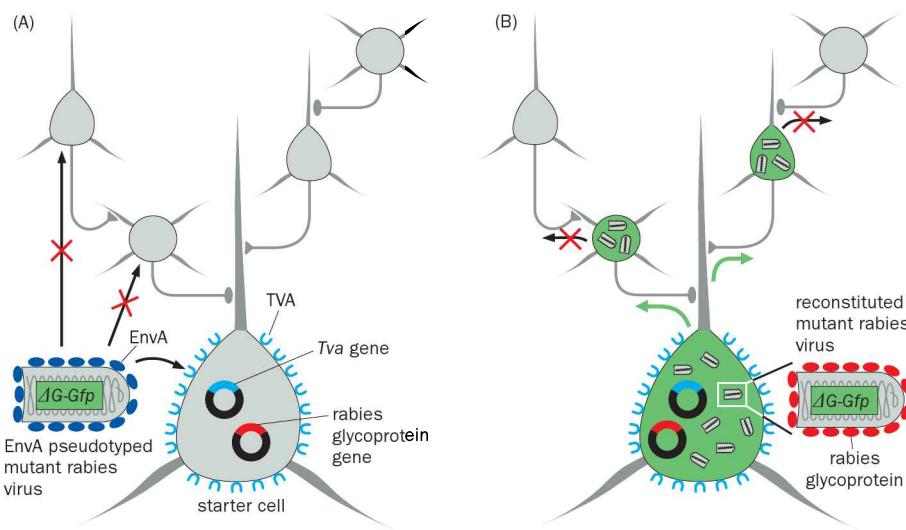
section in the tissue block to be imaged. Because successive images are acquired from the same tissue block, images are automatically aligned. This and other methods have enabled reconstruction of synaptic connections from many hundreds of cells in the retina and primary visual cortex of the mouse, as well as from the fly optic medulla (Figure 13–29). These studies have provided new insights into the mechanisms by which retinal neurons acquire their direction-selective responses (see Figure 4–32), for example.

While serial EM reconstruction provides the ultimate anatomical resolution for constructing synaptic connections within a local region, it is not yet feasible to reconstruct connections between brain regions in large nervous systems such as those of mammals. In 2014, the largest reconstruction volume was about $10^6 \mu\text{m}^3$, a volume equivalent to a cube in which the length of each side is roughly equal to the diameter of a human hair. The mouse or human brain contains roughly 10^5 or 10^9 of that volume, respectively. Other genetic and viral tracing methods have been invented to identify synaptic connections between neurons separated by large distances. A powerful strategy to determine both local and long-distance connections is **trans-synaptic tracing**. An ideal trans-synaptic tracer should have the following properties: if the tracer is expressed from a given neuron (a **starter cell**), a retrograde tracer should be received only by the complete set of neurons that are presynaptic to the starter cell, whereas an anterograde tracer should be received only by the complete set of neurons that are postsynaptic to it. Neurons, axons, or dendrites that are near the starter cell but do not form synaptic connections with the starter cell should not receive the tracer.

Trans-synaptic tracing strategies are still being developed and refined. The most efficient tracers thus far derive from neurotropic viruses such as **rabies virus** and herpes simplex virus, which spread within the nervous systems of their hosts naturally by crossing synapses. Usually, once a virus infects a neuron, it spreads not only to that neuron's direct synaptic partners, but also to the synaptic partners of subsequently infected neurons, making it difficult to distinguish whether two neurons are connected directly or through intermediate neurons. A strategy has been developed to prevent the retrograde trans-synaptic rabies virus from crossing more than one synapse, thereby restricting infection to those cells that are directly presynaptic to the starter cell (Figure 13–30). To achieve this, the viral

Figure 13–30 Strategy for limiting rabies virus spread to monosynaptic partners.

(A) The gene encoding the rabies glycoprotein (G), which is essential for viral recognition of host cells and for viral spread, is replaced by a gene encoding GFP in the rabies genome, such that this mutant rabies virus ($\Delta G\text{-}Gfp$, bottom left) can no longer recognize and transduce normal mammalian neurons (red cross). This mutant rabies virus was assembled in a cell line (not shown) that supplies the EnvA coat protein (blue) from an avian virus, such that the pseudotyped rabies virus can transduce mammalian neurons (starter cells) that express the EnvA receptor TVA (cyan) from a transgene. A transgene that supplies the rabies glycoprotein is also expressed in the starter cells. Circles inside the starter cell symbolize these transgenes. (B) When the mutant rabies virus enters the starter cell via the EnvA–TVA interaction, rabies glycoproteins produced by the starter cell complement the deficiency, producing $\Delta G\text{-}Gfp$ rabies virus with its own glycoproteins (bottom right) that can spread to the starter cell's presynaptic partners. Because the presynaptic partners do not express the rabies glycoprotein, $\Delta G\text{-}Gfp$ rabies virus cannot spread further. Both the starter cell and its presynaptic partners are labeled by GFP; an additional marker is typically introduced into the starter cells (not shown), which distinguishes them from their presynaptic partners. (Adapted from Wickersham IR, Lyon DC, Barnard RJ et al. [2007] *Neuron* 53:639–647. With permission from Elsevier Inc.)



gene encoding the glycoprotein (a coat protein of the virus), which is essential for viral entry into a host cell, is replaced with a gene encoding GFP in the viral genome. This mutant rabies virus is pseudotyped (see Table 13–1) with the envelope protein (EnvA) from an avian virus that cannot transduce mammalian cells. However, if transgenes encoding TVA (the receptor for EnvA) and rabies glycoprotein are expressed in a specific neuron (Figure 13–30A), that neuron can be transduced by the pseudotyped rabies virus and turned into a starter cell. The rabies glycoprotein transgene complements the glycoprotein gene deleted from the mutant viral genome to produce functional rabies viruses, which spread to the starter cell's presynaptic partners. However, since the presynaptic partners do not express rabies glycoproteins, mutant rabies viruses cannot spread further (Figure 13–30B). This monosynaptic strategy for tracing the presynaptic partners of specific starter cell types has been applied to map synaptic connections in many parts of the mammalian nervous system (see Figures 6–21 and 8–10).

In summary, many methods have been developed to decipher the wiring diagrams of nervous systems at different scales with various resolutions. These methods range from noninvasive human brain imaging to the complete reconstruction of synaptic connections in *C. elegans*. However, many technical challenges must be overcome in order to comprehensively map the synaptic connections of larger neural systems. Mapping of electrical synapses poses an additional challenge as they are not as readily identifiable as chemical synapses, even in electron micrographs. Furthermore, a wiring diagram based on anatomical connections alone is just a first step toward understanding a neural circuit. To decipher how a neural circuit operates, investigators must also assess whether synapses are excitatory or inhibitory, how strong they are, and how they are influenced by the actions of modulatory neurotransmitters and neuropeptides (for example, see Section 6.12). Understanding neural circuit function requires research tools that can measure and manipulate the activities of neurons in the wiring diagram in the context of animal behavior; we will discuss these methods in the next part of the chapter.

RECORDING AND MANIPULATING NEURONAL ACTIVITY

Signals in the nervous system spread predominantly by membrane potential changes. Thus, the ability to record membrane potential as a means of measuring neuronal activity is critical to our understanding of nervous system function. In this part of the chapter, we discuss the principal methods for recording neuronal activity.

While observations and measurements are the foundations of discovery, well-designed perturbation experiments are necessary to elucidate the underlying mechanisms. We will also discuss the loss- and gain-of-function approaches investigators have employed to silence and excite neurons of interest and thereby