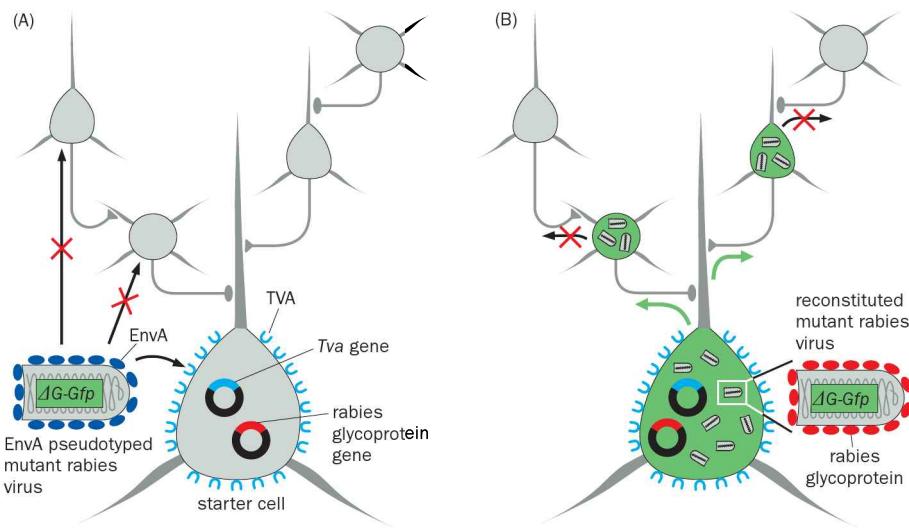


Figure 13–30 Strategy for limiting rabies virus spread to monosynaptic partners.

(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

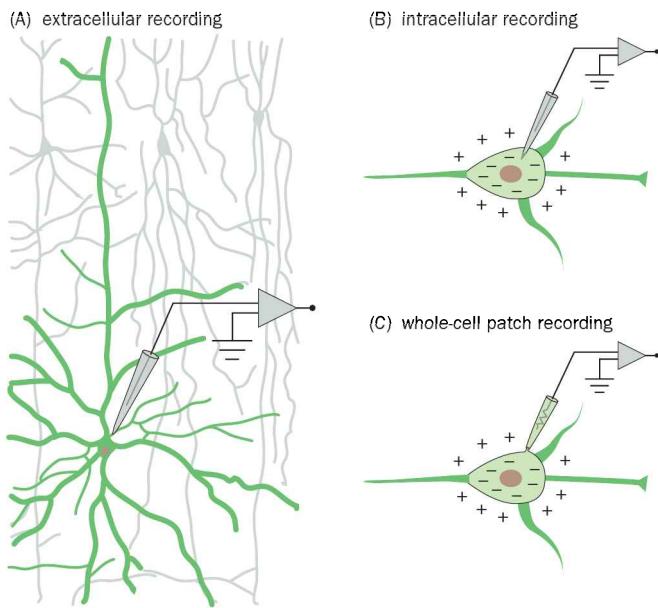


Figure 13–31 Three principal methods of electrophysiological recordings.

(A) In extracellular recording, the tip of an electrode is sufficiently close to the neuronal cell body such that when the neuron fires action potentials, the electrode can detect the extracellular voltage changes. **(B)** In intracellular recording, a sharp electrode penetrates into the cell to measure intracellular membrane potentials directly. **(C)** In whole-cell patch recording, the patch electrode forms a tight seal with the plasma membrane to measure intracellular membrane potentials (see Figure 13–37B for more details).

identify the roles that specific neurons or populations of neurons play in the function of neural circuits and the behavior of animals.

13.20 Extracellular recordings can detect the firing of individual neurons

Three principal electrophysiological methods have been employed to record neuronal activity. In **extracellular recording**, an electrode, often made of metal wire that is insulated except at the tip, is placed at close range outside a neuronal cell body to record voltage changes when the neuron fires an action potential (Figure 13–31A). **Intracellular recording** typically utilizes a sharp electrode, usually made of glass with a very fine, open, solution-filled tip, which penetrates into the cell to directly record the intracellular membrane potential (Figure 13–31B). **Whole-cell patch recording** is a special form of intracellular recording in which the glass electrode forms a tight seal with the plasma membrane of the recorded cell (Figure 13–31C). We discuss extracellular recording in this section, and intracellular and whole-cell patch recording in the following section.

Although the simplest form of recording in electrophysiology, extracellular recording has proven to be a powerful method. When a cell fires an action potential, the ionic flow creates voltage changes not only inside the cell but also in its immediate surroundings. When an electrode tip is close to a neuronal soma (Figure 13–31A), the signal picked up by the electrode predominantly reflects the spiking activity of this single nearby neuron (Figure 13–32A). Action potentials with a specific amplitude and waveform as detected in extracellular recording define a ‘unit;’ thus extracellular recording aimed at detecting the firing patterns of individual neurons is also called **single-unit recording**. Sometimes an extracellular electrode can detect and distinguish action potentials from more than one neuron because of the distinct amplitudes or waveforms of action potentials from neurons at varied distances to the electrode tip. (This effect is amplified by the use of a **tetrode**, an extracellular electrode that contains four independent wires enabling four independent recordings of spiking activities of neurons nearby the electrode. Firing patterns of up to 20 neurons may be resolved by tetrode recording.) Single-unit extracellular recording of neuronal activity has been the key method for making many conceptual advances in neurobiology. As we learned in Chapter 4, for example, extracellular recording at successive stages of the visual processing pathway revealed how the brain’s representation of a visual scene is transformed as the signals travel from the retina to the visual cortex. Extracellular recording is still the predominant method used for recording neuronal activities *in vivo* today.

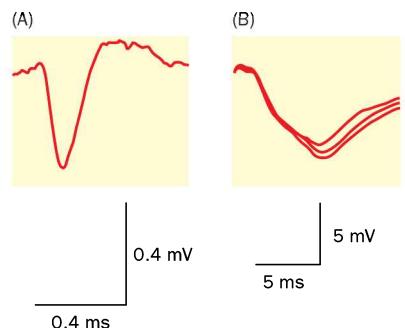


Figure 13–32 Examples of single-unit and field potential recordings. Extracellular electrophysiological recordings have a significant history in neurobiology. Shown here are **(A)** a single ‘spike’ of a cat retinal ganglion cell recorded by an extracellular electrode in response to light stimulation, and **(B)** three superimposed traces of local field excitatory postsynaptic potentials (fEPSPs) in the dendritic layer of the dentate gyrus in response to electrical stimulation of axons in the perforant path. Panel A is from the original recording that described the receptive fields of retinal ganglion cells (see Figure 4–24). Panel B is from the original recordings that led to the discovery of long-term potentiation (see Figure 10–8). Note that in both examples, the voltages recorded from extracellular electrodes become more negative as neurons are activated. This is because positive ions flow into the cell during the rising phase of the action potential (panel A) or when postsynaptic cells are depolarized (panel B). Note also that the time course of the spike in panel A is much faster than the field EPSP in panel B. (A, adapted from Kuffler SW [1953] *J Neurophysiol* 16:37–68; B, adapted from Bliss TVP & Lømo T [1973] *J Physiol* 232:331–356.)

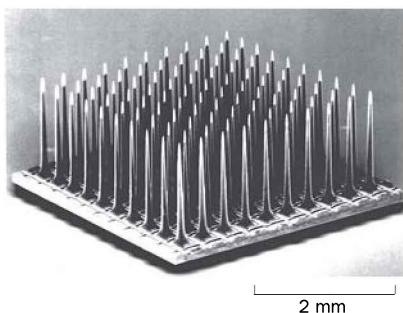


Figure 13–33 Multi-electrode array. Multi-electrode arrays similar to this 10×10 silicon-based prototype have been widely used for recordings of cortical neurons in neural prosthetics (see Section 8.11). (From Campbell PK, Jones KE, Huber RJ et al. [1991] *IEEE Trans Biomed Eng* 38:758–768. With permission from IEEE.)

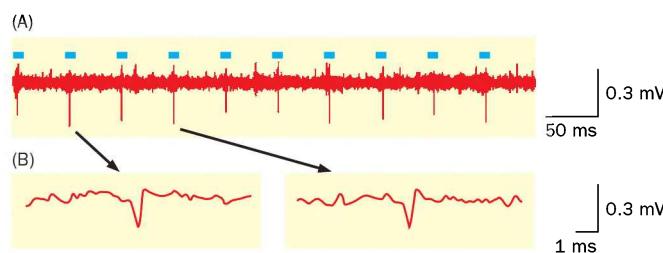
Extracellular recording can also be used to measure **local field potentials**, which are local potential variations measured relative to a distal ground. Local field potentials can be measured with an electrode for single-unit extracellular recording but high-frequency signals such as those produced by action potentials are removed by signal-processing methods such as a low-pass filter. The remaining low-frequency signals reflect collective dendritic and synaptic activities of many neurons near the electrode tip. We have seen the application of field potential recording to studies of hippocampal long-term potentiation *in vivo* (Figure 13–32B; see also Figure 10–8) or in freshly prepared brain slices (acute slices) *in vitro* (see Figure 10–10).

In order to explore how groups of neurons act together to encode and process information, various kinds of **multi-electrode arrays** have been developed that record many neurons at the same time. These consist of many independent electrodes horizontally arranged in grids (for example, Figure 13–33) or vertically along different depths (see Figure 4–47A). Multi-electrode arrays can record tens to hundreds of neurons simultaneously *in vitro*, such as in brain slices or explants; for example, recording simultaneously from many retinal ganglion cells allowed the retinal wave to be characterized (see Figure 5–21A). Multi-electrode arrays can also be implanted *in vivo* to record the activities of many neurons in awake, behaving animals (see Figures 8–26 and 8–29) or humans (see Figure 8–30).

Electroencephalography (EEG) is essentially a noninvasive field potential recording method that usually involves multi-electrode arrays. In a typical EEG setting, the electrodes are attached to the surface of the scalp. Because the distance is greater between EEG electrodes and the recorded neurons compared with conventional extracellular recording, EEG can only detect the synchronized activities of tens of thousands of neurons or more. EEG enabled the discovery of brain waves of various frequencies and is an extremely useful tool for assessing brain states such as different forms of sleep (see Figure 8–51A) or epileptic conditions (see Figure 11–47).

Extracellular recordings are typically performed blind to the type of neuron that is being recorded. They have a bias toward detecting the dominant neuron types and the most active neurons, because extracellular recording procedures often involve the researcher shifting electrode positions until a spike is detected. Spike waveforms and firing properties have been used to classify cell types from extracellular recordings, but different cell types may have overlapping properties in most regions of the brain, making such classification ambiguous. Recently, this limitation has been addressed by a phototagging method, where single-unit recording was performed in a tissue in which neurons of specific types can be stimulated using optogenetics (see Section 13.25). The cell types of the recorded units can be classified based on whether or not they respond to optical stimulation. For example, the ventral tegmental area (VTA) has a mixture of dopamine and GABA neurons. When mice that express Cre only in GABA neurons are injected in the VTA with viral vectors encoding a channelrhodopsin-2 (ChR2) transgene that requires Cre for expression, GABA neurons can be selectively stimulated using light. Likewise, mice that express Cre only in dopamine neurons can be used to selectively activate dopamine neurons using light. To distinguish between dopamine and GABA neurons, recordings can be collected from these two types of transgenic mice in which either dopamine or GABA cells can be optically activated. Researchers were able to determine whether a recorded neuron in a given Cre mouse was GABAergic or dopaminergic based on whether or not it could be depolarized by photostimulation (Figure 13–34). Using this approach, it

Figure 13–34 The use of phototagging to identify cell types in extracellular recordings. Extracellular recording of ventral tegmental area neurons was performed in a transgenic mouse that expressed Cre in dopamine neurons and that was infected with an adeno-associated virus expressing a Cre-dependent channelrhodopsin (ChR2). The top trace shows that each photostimulation (cyan bar) resulted in a spike of the recorded neuron; two spikes are temporally magnified below. These results are consistent with recording of a dopamine neuron that expresses ChR2 channels and produces action potentials in response to depolarizations caused by light-induced opening of those channels. (Adapted from Cohen JY, Haesler S, Vong L et al. [2012] *Nature* 482:85–88. With permission from Macmillan Publishers Ltd.)



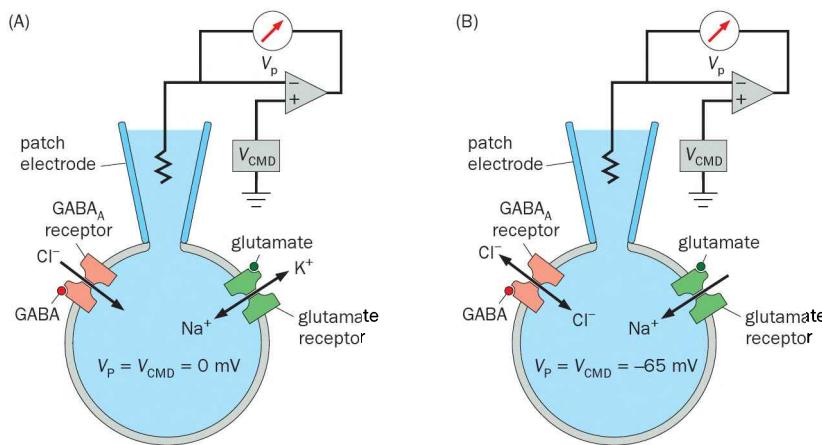


Figure 13–35 Using voltage clamp to dissect inhibitory versus excitatory input. **(A)** When the voltage is clamped at 0 mV in whole-cell recording, at the reversal potential of glutamate receptors (see Section 3.15), even if glutamate receptor channels are open upon glutamate binding, cation influx and efflux balance out and do not contribute to the net current across the cell membrane. The measured current is mostly contributed by Cl^- flow through the GABA_A receptor in response to GABA, thereby reflecting inhibitory input. **(B)** When the voltage is clamped at -65 mV , around the reversal potential of the GABA_A and glycine receptors (that is, the equilibrium potential of Cl^- ; see Section 3.17), the measured current reflects mostly excitatory input from the glutamate receptor channel. V_{CMD} , commanding voltage; V_p , voltage of the patch electrode.

was found that VTA dopamine and GABA neurons exhibit distinct properties with regard to reward presentations (see Section 10.24). A caveat of the phototagging method is that spikes can also be driven indirectly in non-ChR2-expressing cells; extremely short latency between light onset and spike ($\sim 1 \text{ ms}$), or certainty that there is no local excitation, are required for confidence in cell identification.

13.21 Intracellular and whole-cell patch recordings can measure synaptic input in addition to firing patterns

Compared to extracellular recordings, intracellular recordings with a sharp electrode (see Figure 13–31B) offer much higher sensitivity and signal-to-noise ratio for detecting electrical signals. Intracellular recordings can detect not only firing patterns but also sub-threshold membrane potential changes resulting from excitatory or inhibitory input received by the recorded neuron. Many discoveries about neuronal communication we discussed in Chapters 2 and 3, including the ionic basis of the action potential and the mechanisms of synaptic transmission, were made using intracellular recording methods.

Whole-cell patch recording (or simply whole-cell recording; see Figure 13–31C) is one of several variations of the patch clamp method (Box 13–2). Here, the interior of the electrode forms a continuous compartment with the cytoplasm, which allows the electrode to measure the membrane potential with a high sensitivity comparable to intracellular recording with a sharp electrode. The patch electrode affords greater access to the cell—the tip diameter is usually $1 \mu\text{m}$ or so for a patch electrode, an order of magnitude greater than the diameter of an intracellular sharp electrode. As a result, whole-cell patch electrodes not only can record the activity of a neuron but also can pass currents that change a neuron's membrane potential, effectively achieving voltage clamp (see Figure 2–21). In this manner, inhibitory input to a neuron can be recorded in isolation when the neuron is voltage clamped at the reversal potential of the glutamate receptors, such that excitatory input does not make a contribution (Figure 13–35A). Likewise, excitatory input to a neuron can be recorded without interference from inhibitory input when the neuron is voltage clamped at the reversal potential of Cl^- (Figure 13–35B). These techniques are often combined with pharmacological blockers for specific receptors to dissect excitatory and inhibitory inputs to specific neurons in slices or explant preparations (for example, see Figure 4–31). Dissection of inhibitory and excitatory input using the strategies outlined in Figure 13–35 can also be performed *in vivo*.

Because the solution that fills the patch electrode is continuous with the cytoplasm of the cell, macromolecules such as dyes or fluorescent markers can be included in the patch electrode solution to label the recorded cell (Figure 13–36); signaling molecules can also be included to alter the properties of the recorded cell (see Figure 10–13B). The filling of recorded cells during whole-cell recording (and during intracellular recording with a sharp electrode) can be used to



Figure 13–36 Dye fill during whole-cell patch recording from the dendrite and the cell body. In this example, two patch electrodes are used for whole-cell recording at the cell body (bottom) and at the apical dendrite (top) of a cortical pyramidal cell in a rat brain slice. Dual patching of the dendrite and cell body of the same neuron is confirmed by the mixing of two different fluorescent dyes, one from each electrode. This dual patch approach can be used to study synaptic potential propagation from the dendrite to the cell body, and action potential back-propagation from the cell body to the dendrite. (From Stuart GJ & Sakmann B [1994] *Nature* 367:69–72. With permission from Macmillan Publishers Ltd.)

Box 13–2: Patch clamp recordings can serve many purposes

Patch clamp recording requires the formation of a very tight seal (with a resistance in the range of gigaohms, or 10^9 ohms) between a glass patch electrode, also called a patch pipette, and the plasma membrane of a target neuron (Figure 13–37A). Due to this high resistance, the very small currents that pass through individual ion channels in the membrane underneath the patch electrode can be recorded. Indeed, the measurement of single-channel conductances in a defined extracellular environment (the internal solution of the patch electrode) in this **cell-attached recording** mode (Figure 13–37B) was the first application of the patch clamp technique (see Figure 2–30). The patch clamp technique can also be used in a number of additional modes. For example, the membrane patch underneath the electrode can be excised (Figure 13–37C). The **excised patch** can be placed in a defined solution so that both the extracellular and intracellular environments of the ion channels in the patch can be controlled. Excised patch recording is widely used to study the biophysical and biochemical properties of ion channels (for example, see Figure 4–9). In the whole-cell recording mode, a gentle suction applied to a cell-attached patch ruptures the membrane underneath the patch electrode, such that the interior of the patch electrode and the cytoplasm of the recorded cell form a single compartment (Figure 13–37D).

Because the intracellular solution and cytoplasm are continuous in the whole-cell recording mode, the cellular

contents may be diluted with solution from the patch electrode during prolonged recording. A procedure called **perforated patch** can be used to minimize this concern; in this method, the membrane between the patch electrode and the cell is not fully ruptured as in whole-cell mode, but the internal solution of the patch electrode contains chemicals that make small holes in the underlying neuronal membrane, such that the patch electrode can record the current and voltage of the cell with minimal exchange of macromolecules. In another variation called **loose-patch recording**, a patch electrode is placed against the cell membrane without forming a gigaohm seal. Recording in loose-patch mode thus does not affect the cellular contents. However, this approach does not provide sufficient sensitivity to record sub-threshold activity; it can only record action potentials. Loose-patch recording is commonly used for *in vivo* recordings. Compared with conventional extracellular recording, it does not have a bias toward active neurons, as the criterion it uses to assess whether the electrode is approaching a cell is a sudden increase in resistance, rather than the recording of action potentials. Furthermore, DNA can be added to the internal solution of the patch electrode and introduced specifically into the recorded cell by electroporation after the recording. One application of this single-cell electroporation technique is to introduce DNA encoding a fluorescent protein so that the same cell can be located and recorded at a later time, for instance after a specific experience.

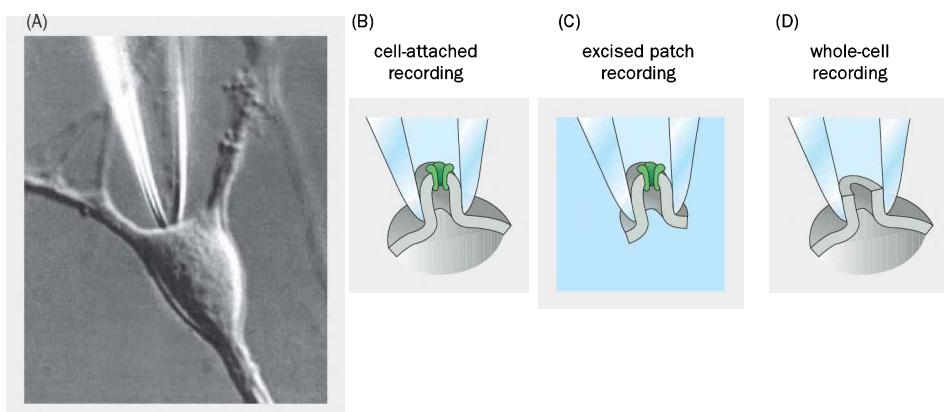


Figure 13–37 Multi-functionality of patch clamp recording. (A) Photomicrograph of a patch electrode in contact with the plasma membrane of a neuron in culture. (B–D) Three patch clamp modes are schematized. In a cell-attached patch (B), the patch electrode forms a tight seal with a neuron's plasma membrane, which allows measurement of ion flow through a single channel in the patch of membrane underneath the electrode. In an excised

patch (C), the piece of membrane underneath the electrode is excised from the cell and can be placed in defined medium to study the properties of the channel. In whole-cell recording (D), the membrane patch under the electrode is ruptured, such that the interior of the electrode and the recorded cell becomes a single compartment. (Adapted from Neher E & Sakmann B [1992] *Sci Am* 266:44–51. With permission from Macmillan Publishers Ltd.)

determine the locations, morphologies, and projection patterns of recorded neurons (see Figures 13–1 and 13–21). Such information is highly valuable in determining how the structures and functions of neurons are correlated (for example, see Figure 4–45).

Another important advantage of intracellular recording is that it allows a genetically defined population of cells to be targeted for recording. A given brain region most often contains a mixture of different neuronal types with varying densities. As discussed in the previous section, blind recording cannot distinguish unequivocally between different cell types, and it may not allow rare cell types to be recorded at all. The job of an electrophysiologist is made easier if the type of neuron targeted for recording is pre-labeled with fluorescent protein using genetic strategies. For example, recording specific pairs of genetically labeled pre- and postsynaptic partner neurons in the *Drosophila* olfactory system enabled investigators to examine how the representation of olfactory information is transformed as signals travel between the presynaptic olfactory receptor neurons and the postsynaptic projection neurons (see Section 6.14). With the advance of genetic technology to access specific neuronal types (see Section 13.12), these targeted electrophysiology experiments have become increasingly powerful in revealing how neural circuits process information.

13.22 Optical imaging can measure the activity of many neurons simultaneously

To appreciate a symphony, it's not enough to hear one instrument at a time; the listener must be able to hear all of the orchestra's musical instruments simultaneously. Likewise, a deep understanding of how neural circuits encode and process information requires that researchers capture the simultaneous activity of many (ideally all) neurons in the circuit. Even with multi-electrode arrays, investigators can only record at most hundreds of neurons at a time. In addition, the spacing of recorded neurons is constrained by the spacing of the electrodes. The only method currently available that can in principle record the activity of all neurons within a region at cellular resolution is **optical imaging**, which uses changes of fluorescence or other optical properties as indicators of neuronal activity.

Since neurons communicate by membrane potential changes, the ideal indicator would be one that reports voltage changes directly. Indeed, many variants of **voltage-sensitive dyes**, which change fluorescence intensity or other optical properties in response to membrane potential changes, have been developed. However, the voltage-sensitive dyes invented thus far have been limited by low signal-to-noise ratios and high levels of phototoxicity to be widely applicable for measuring neuronal activity with cellular resolution *in vivo*. Other optical sensors have been developed to enable imaging of neurotransmitter release and receptor conformation changes as proxies for neuronal activity. By far the most widely used sensors of neuronal activity are **Ca^{2+} indicators**, which translate changes of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) into changes in fluorescence signal. A rise in $[\text{Ca}^{2+}]_i$ usually accompanies neuronal activation due to the activation of postsynaptic neurotransmitter receptors that are permeable to Ca^{2+} and the opening of voltage-gated Ca^{2+} channels in response to depolarization in both cell bodies and presynaptic terminals.

Some of the Ca^{2+} indicators used to record neuronal activity are made from synthetic chemicals, while others are protein based. Chemical indicators typically link a Ca^{2+} -chelating moiety with a fluorophore. As a specific example, binding of Ca^{2+} to the chemical indicator **fura-2** shifts the wavelength of maximal fluorescence excitation about 30 nm shorter (Figure 13–38A). Thus, the ratio of fluorescence intensity measured at the excitation wavelengths of 350 nm and 380 nm can be used as a sensitive measure of $[\text{Ca}^{2+}]_i$. Protein-based Ca^{2+} indicators are also called **genetically encoded Ca^{2+} indicators** because they can be expressed as a transgene in specific cell types. For example, the cameleon indicator reports $[\text{Ca}^{2+}]_i$ utilizing **fluorescence resonance energy transfer (FRET)**, a mechanism of energy transfer between two fluorophores. The efficiency of FRET is inversely proportional to the sixth power of distance and therefore can be used to determine

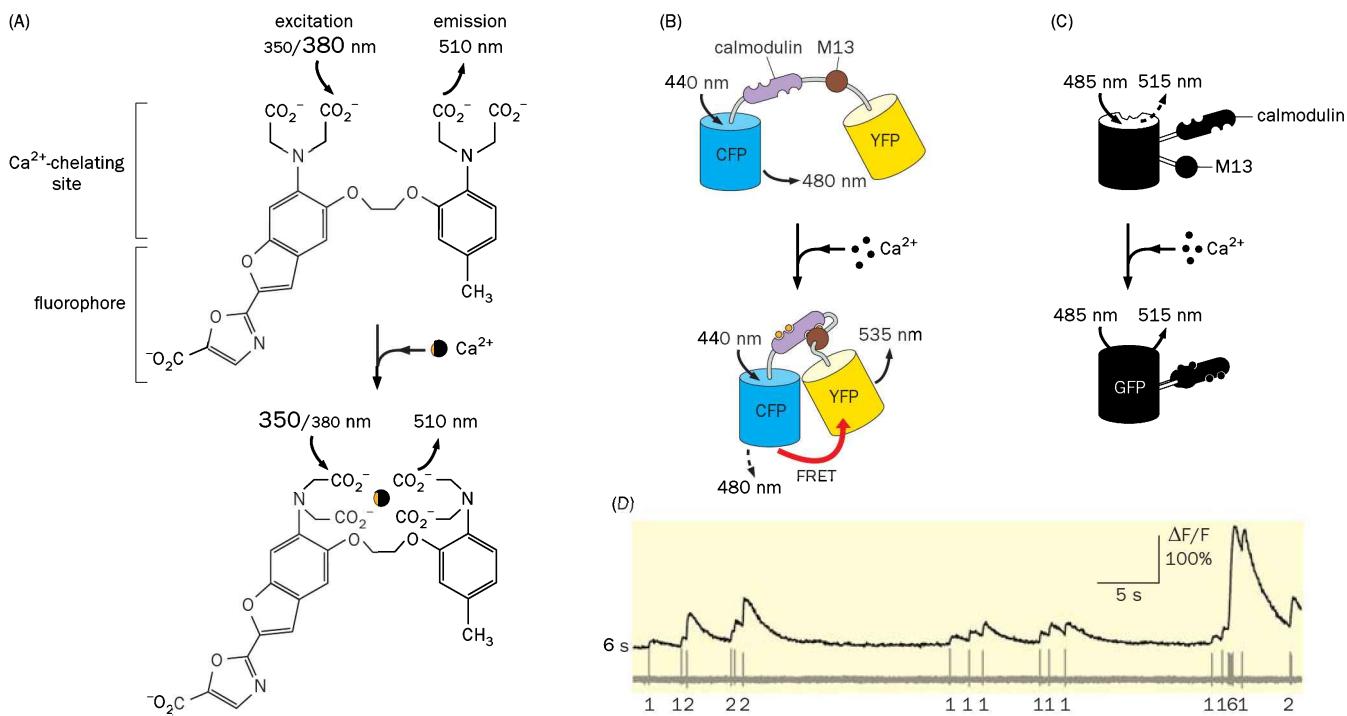


Figure 13–38 Chemical and genetically encoded Ca^{2+} indicators. **(A)** Fura-2 is a chemical Ca^{2+} indicator consisting of a fluorophore fused with a Ca^{2+} -chelating site from the Ca^{2+} buffer EGTA. When $[Ca^{2+}]_i$ is low, excitation at 380 nm produces stronger fluorescence emission than excitation at 350 nm (symbolized by the size of the numbers); when $[Ca^{2+}]_i$ is high, the converse is true. Thus ratiometric imaging at 350/380 excitation wavelengths serves as a sensitive reporter of $[Ca^{2+}]_i$. **(B)** Design principle of cameleon, the first genetically encoded Ca^{2+} indicator. When $[Ca^{2+}]_i$ increases, the binding of calmodulin (CaM) to the CaM-binding peptide M13 brings two fluorescent proteins closer, thus increasing fluorescence resonance energy transfer (FRET) as measured by the ratio of 535 nm emission (reflecting the contribution of FRET) over 480 nm emission (no FRET). **(C)** Design principle of GCaMP. A permuted GFP is restored to its native three-dimensional structure with an associated increase in fluorescence after Ca^{2+} -triggered binding of M13 and CaM, which are

at the ends of GCaMP. The fluorescence intensity of GCaMP can thus be a readout of $[Ca^{2+}]_i$. **(D)** Single action potentials reliably induce fluorescence changes of GCaMP6 in mouse visual cortical neurons *in vivo*, as measured by simultaneous loose-patch recording to identify action potentials (bottom) and GCaMP6 fluorescence intensity change (top). $\Delta F/F$ is the ratio of fluorescence intensity change (ΔF) over basal fluorescence intensity (F). Note that when action potentials occur in rapid succession (indicated by numbers below), individual action potentials cannot be resolved as individual peaks by imaging. (A–C, adapted from Grienberger NL & Konnerth A [2011] *Neuron* 73:862–885. With permission from Elsevier Inc.; D, adapted from Chen TW, Wardill TJ, Sun Y et al. [2013] *Nature* 499:295–300. With permission from Macmillan Publishers Ltd. See also Grynkiewicz G, Poenie M & Tsien RY [1985] *J Biol Chem* 260:3440–3450; Miyawaki A, Llopis J, Heim R et al. [1997] *Nature* 388:882–887; Nakai J, Ohkura M & Imoto K [2001] *Nat Biotech* 19:137–141.)

the distance between two fluorophores. Cameleon is an *in vitro* engineered protein in which calmodulin and its target M13 peptide link two fluorescent proteins, cyan and yellow fluorescent proteins (CFP and YFP). Excitation of CFP can cause emission from CFP, or from YFP via FRET if YFP is sufficiently close. Ca^{2+} -triggered binding of calmodulin and the M13 peptide decreases the distance between CFP and YFP, and hence increases the emission from YFP (Figure 13–38B). Genetically encoded Ca^{2+} indicators can also report $[Ca^{2+}]_i$ by increasing fluorescence intensity. For instance, in an engineered protein called GCaMP, the positions of two halves of the green fluorescent protein GFP are switched, and the swapped halves are linked with calmodulin and M13. Ca^{2+} -triggered binding of calmodulin to M13 restores the original GFP conformation and hence increases the fluorescence intensity (Figure 13–38C).

Chemical indicators have offered superior sensitivities compared to genetically encoded indicators in the past decades and have been widely used in experiments described in this book (see Figures 4–42C and 5–21B). However, protein engineering has produced a new generation of genetically encoded Ca^{2+} indicators, such as GCaMP6, that can detect single action potentials *in vivo* more reliably than chemical indicators (Figure 13–38D), achieving an important milestone in optical imaging. Moreover, selective expression of genetically encoded Ca^{2+} indicators can be specified by cell type and can enable repeated

imaging of the same neuron over the course of months, which is not possible with chemical indicators. These developments will make many new discoveries possible in the coming years.

In addition to activity indicators, optical imaging relies on suitable microscopes for visualizing fluorescence changes in live tissues with high spatial resolution, strong signals, and minimal photodamage to the imaged tissues. Conventional fluorescence microscopes have poor resolution along the *z* axis and are best used with relatively thin tissues such as retinal explants (for example, Figure 5–21B). As described earlier, laser-scanning confocal microscopes have great resolution along the *z* axis because fluorescence emission is collected via a small pinhole to block out-of-focus fluorescence from other planes (see Figure 13–19A). However, tissues above or below the imaging plane are nevertheless exposed to the laser excitation during scanning; the resulting photodamage includes heating of the tissues and photobleaching of the fluorescent indicators. In addition, light scattering in brain tissues limits the depth of effective imaging by confocal microscopy to about 100 μm from the surface.

The most widely used method for *in vivo* optical imaging is **laser-scanning two-photon microscopy**, which relies on the absorption of two long-wavelength photons simultaneously in order to excite a fluorophore (Figure 13–39A). Only at the focal plane is the density of photons high enough to cause substantial fluorescence emission, such that photodamage is limited to the focal plane (Figure 13–39B). Furthermore, the longer excitation wavelength allows light to penetrate more deeply into the light-scattering brain tissue. Lastly, because fluorescence excitation is limited to the focal plane, any emission photons collected by the objectives, including those resulting from scattering, can be used without the need of a pinhole (Figure 13–39C; compared with Figure 13–19A). Thus, two-photon microscopes collect emitted photons more efficiently than confocal microscopes. Many optical imaging studies discussed in early chapters utilized two-photon microscopy, including Ca^{2+} imaging of neuronal activity (see Figure 4–42C) and structural imaging of neuronal morphology (see Figure 10–48). But even two-photon microscopes can effectively image only those tissues that are within about 500 μm from the surface. This restriction prevents imaging of most of the nervous system of even small mammals such as mice. Additional imaging methods, such as fluorescence endoscopy, are being developed to allow imaging of deeper tissues (see Box 13–3 for a comparison of different methods).

In addition to fluorescence-based imaging, other imaging techniques such as **intrinsic signal imaging** (see Figure 4–42B and Figure 6–16) and **functional magnetic resonance imaging (fMRI)** (see Figures 1–24, 4–51, and 10–37) also report neuronal activity. These techniques use blood flow near the excited neurons as an indicator of neuronal activity, as increased neuronal activity is associated with changes of blood flow and oxygenation. These imaging methods have poorer spatial and temporal resolution compared to fluorescence imaging of individual neurons. For instance, the current fMRI resolution is about 2 mm in the linear dimension; 8 mm³ of tissue contains hundreds of thousands of neurons. Nevertheless, an important advantage of fMRI is its noninvasiveness, making it a favored tool for imaging neuronal activities in the human brain.

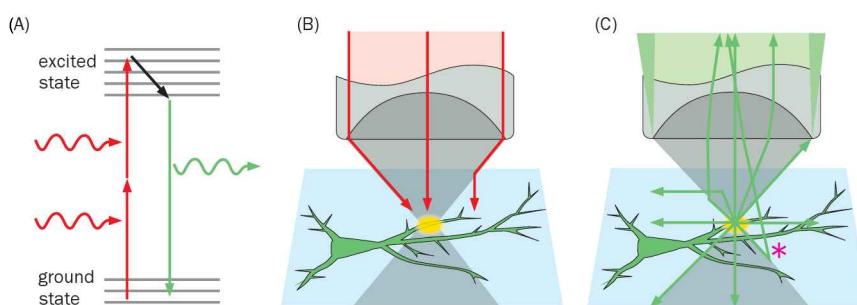


Figure 13–39 Laser-scanning two-photon microscopy. The schematic of a two-photon microscope is similar to that of a confocal microscope (see Figure 13–19A) except that the pinhole next to the detector is omitted. **(A)** Simultaneous absorption of two long-wavelength (lower energy, red) photons brings a fluorescence indicator molecule to the excited state, from which it subsequently relaxes while emitting fluorescence (green). **(B)** Schematic of a microscope objective on top of a brain tissue sample (cyan) focusing on a portion of a dendrite. The near-simultaneous absorption of two infrared photons (the left and middle photon paths) excites fluorophores at the focal plane; the excitation light outside the focal plane due to scattering (the photon path on the right) is insufficiently intense to allow efficient two-photon excitation, so excitation is effectively restricted to the imaging spot at the focal plane (highlighted in yellow). **(C)** All emitted photons (green paths) collected by the objective, including those resulting from scattering (indicated by *), contribute to the fluorescence signal from the imaging spot. (Adapted from Svoboda K & Yasuda R [2006] *Neuron* 50:823–839. With permission from Elsevier Inc. See also Denk W, Strickler JH & Webb WW [1990] *Science* 248:73–76.)

Box 13–3: From *in vitro* preparations to awake, behaving animals: a comparison of recording methods

What method should researchers choose for recording neuronal activity? The answer depends on the biological question the experiment intends to address and the preparation used to address the question. In this box, we compare the pros and cons of the recording methods discussed in Sections 13.20–13.22 in the context of different experimental preparations: from neuronal cell cultures and tissue explants *in vitro* to anesthetized animals and awake, behaving animals *in vivo* (Table 13–2).

Electrode-based recording methods directly measure membrane potentials, and hence have superb sensitivity and temporal resolution. They are widely used in reduced preparations such as cultured neurons or *in vitro* explants such as brain slices, providing insight into the properties of individual neurons and helping researchers investigate synaptic transmission and plasticity as assayed from single neurons or a group of neurons in aggregate (measured by local field potentials). The reduced preparations also offer easier access to target neurons, so intracellular recording methods are preferred since they have superb sensitivity for detecting sub-threshold membrane potential changes and support an array of approaches through which researchers can manipulate the recorded neurons by injecting currents or molecules. Intracellular recordings (particularly whole-cell recording) have been the predominant method for studies using slice preparations of the mammalian brain. Optical imaging methods can be employed in reduced preparations if recording the activity of many individual neurons is desirable (for instance to observe retinal waves) or when subcellular resolution of neuronal activity is needed.

Recording from *in vivo* preparations poses more challenges regardless of method because intact systems have greater inherent complexity, the neurons to be recorded are harder to access, and the animals must be kept alive (and, in some cases, awake and behaving) during the recording process. However, obtaining recordings from living animals, including from awake, behaving animals, is essential for addressing many questions in neurobiology related to perception, cognition, and the neural basis of behavior (for example, see Section 4.29). Recording with extracellular electrodes has historically been the predominant method *in vivo*, particularly in awake, behaving animals, due to its overall superior ability to maintain stable recordings (that is, to record from the same cells over long periods of time), to penetrate deep into tissues, and to record many neurons simultaneously (Table 13–2). For example, the discovery of place cells in the hippocampus and grid cells in the entorhinal cortex relied on extracellular recordings in freely moving rodents (see Box 10–2). Intracellular recordings, whether with a sharp electrode or whole-cell patch electrode, are difficult to maintain when animals are awake and moving, because physical movement can shift the electrode. Likewise, optical imaging requires stability and is sensitive to movement. However, intracellular recording offers superior sensitivity, and optical imaging can record many neurons at once and over long periods of time using genetically encoded indicators. Thus, researchers have developed methods to improve stability for *in vivo* recording.

One way to provide the mechanical stability essential for both intracellular recording and optical imaging is to use

Table 13–2: Comparison of electrophysiological and optical imaging methods for recording neuronal activity

Property	Electrophysiology		Optical imaging with Ca^{2+} indicators ¹
	Extracellular recording	Intracellular recording	
Sensitivity to electrical signal	spikes	spikes and sub-threshold activity	generally less sensitive ²
Spatial resolution	cellular to network	cellular to subcellular ³	cellular and subcellular
Temporal resolution	<1 millisecond	<1 millisecond	10s to 100s of milliseconds for a single imaging plane
Number of neurons recorded simultaneously	up to hundreds	at most a few	thousands or more
Stability during movement	good	poor	poor
Depth of recording	any depth	easier superficially	limited ⁴
Duration of recording	days to weeks	10s of minutes	hours with chemical indicators; months with protein indicators
Cell-type-specific recording	poor	good	excellent with protein indicators
Biases	active neurons; dominant cell types	large cells	cells that take up or express the indicators well

¹ Most other indicators share similar properties.

² GCaMP6 can detect single action potentials, but cannot resolve high-frequency spikes (see Figure 13–38D).

³ Whole-cell patch recording can be applied to large dendrites (see Figure 13–35).

⁴ <10 μm with conventional fluorescence microscopy for cellular resolution; ~100 μm with confocal microscopy; ~500 μm with two-photon microscopy.

Box 13–3: From *In vitro* preparations to awake, behaving animals: a comparison of recording methods

head-fixed animals, that is, to restrain head movement with respect to the recording equipment such as a microscope or a micromanipulator that holds an electrode. (The head-fixed preparation was originally developed for extracellular recording of neurons in the visual cortex of awake, behaving monkeys; if the monkey cannot move its head and is trained to fixate, then a stimulus on the screen always falls on the same spot on the retina; see Figure 4–52.) For instance, a head-fixed mouse can be trained to associate an odor with a water reward and to move its tongue to fetch the reward while its motor cortical neurons are being optically imaged during the learning process (Figure 13–40A). A more sophisticated preparation involves virtual reality feedback. For example, a head-fixed mouse navigates on a spherical treadmill made of a ball floating on air; the mouse's movement is used to adjust the visual scene as if the animal was navigating in a real environment (Figure 13–40B; Movie 13–3). The mouse can be trained to run on linear tracks or make turning choices in virtual reality while its neurons are being recorded with a whole-cell patch electrode or a two-photon microscope. An alternative to the head-fixed preparation is the use of a miniature fluorescence microscope. Miniature microscopes have been designed that can be attached to the head of a freely moving mouse, making it possible to record neuronal activity while the animal explores its environment

(Figure 13–40C and Movie 13–4). Further development and refinement of these preparations will expand researchers' ability to record neuronal activity under different experimental settings.

Even optical imaging, which allows simultaneous recording of thousands of neurons, can only provide information about the activities of a very small fraction of the nervous system. A method that can in principle provide information about the activity of neurons throughout the brain at cellular resolution is the expression of immediate early genes (see Section 3.23) after the animal has experienced sensory stimuli or behavioral episodes. Important limitations of this method include the requirement that it be done *post mortem* in fixed brain tissue, the slow temporal resolution (transcription operates on a timescale of minutes or more, whereas neuronal activity operates on a timescale of milliseconds), and its indirectness—it is unclear what kind of activity patterns trigger immediate early gene expression. Nevertheless, with proper design, immediate early gene expression has been used successfully for many applications, from identifying sensory receptors for specific stimuli (see Figure 6–22B) to allowing genetic access to memory traces (see Figure 10–36).

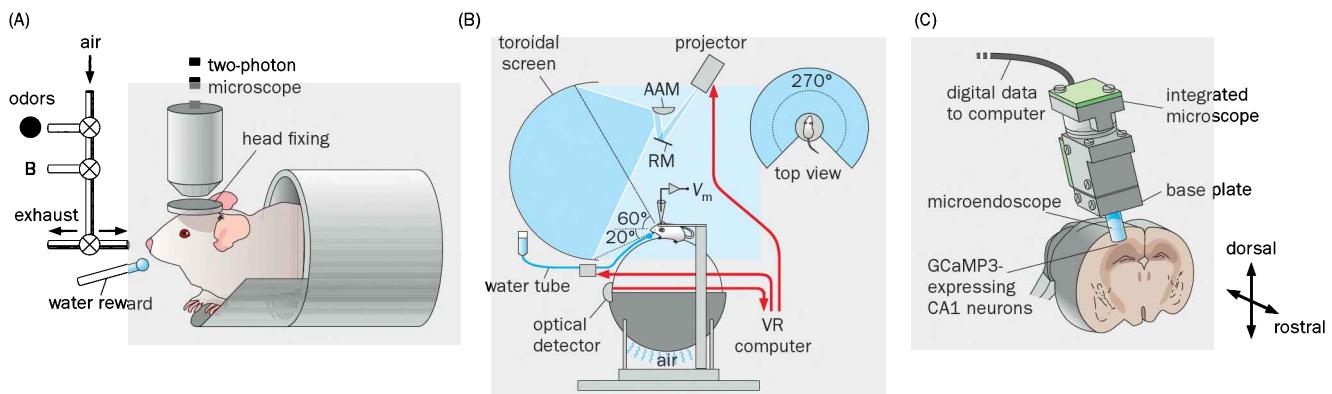


Figure 13–40 Three examples of recording neuronal activity in behaving mice. (A) In this head-fixed preparation, a metal plate is surgically attached to the head of a mouse. During a two-photon imaging experiment, the head plate is mounted onto the microscope to prevent movement of the mouse's head relative to the objective, thus stabilizing the imaging field. A head-fixed thirsty mouse can be trained to extend its tongue only when odor A but not odor B is presented in order to receive a water reward. The motor cortical area that controls tongue extension can be imaged during the learning process. (B) In this virtual reality (VR) preparation, a head-fixed mouse is placed on a ball floating on air while being presented with visual stimuli through a projector. The motion of the mouse causes the ball to move; the movement vector is measured by an optical detector and fed into a VR computer to control the projector such that the mouse's movement changes the scene in the projector as if it were moving in real world (see Movie 13–3). Mice could

be trained to run along linear tracks in this setting while their hippocampal place cells were subjected to whole-cell recordings of membrane potentials (V_m) or two-photon imaging (not shown). AAM, angular amplification mirror; RM, reflecting mirror. (C) A miniature fluorescence microscope that weighs 1.9 g can be attached to the head of a freely moving mouse. With an attached microendoscope, this miniature microscope can image over a period of more than a month the place fields of hundreds of CA1 pyramidal cells expressing the genetically encoded Ca^{2+} indicator GCaMP3 (see also Movie 13–4). (A, adapted from Komiyama T, Sato TR, O'Connor DH et al. [2010] *Nature* 464:1182–1186. With permission from Macmillan Publishers Ltd.; B, adapted from Harvey CD, Collman F, Dombcek DA et al. [2009] *Nature* 461:941–946. With permission from Macmillan Publishers Ltd; C, adapted from Ziv Y, Burns LD, Cocker ED et al. [2013] *Nat Neurosci* 16:264–266. With permission from Macmillan Publishers Ltd.)

13.23 Neuronal inactivation can be used to reveal which neurons are essential for circuit function and behavior

Inactivation of neurons can determine their necessity to the normal function of the nervous system. The crudest method of inactivating neurons is a lesion, which removes or destroys a chunk of nervous tissue, either by accident or by design. For instance, lesions in the aphasia patients of Broca and Wernicke (see Section 1.10) and in H.M. (see Section 10.1) helped pinpoint brain regions important for speech and episodic memory in humans. Lesions in animal models, which can be produced by passing large currents through an electrode or by injecting toxic chemicals at stereotactic positions, offer opportunities to systematically examine the requirement of specific regions for brain function and behavior (for example, see Figure 10-40). Whereas lesions cause permanent damage, researchers can also transiently inactivate specific brain regions by injecting pharmacological agents such as GABA_A receptor agonists such as muscimol (see Box 3-2) to enhance inhibition, glutamate receptor antagonists to reduce excitation, or Na⁺ channel inhibitors to block action potentials (for example, see Figure 10-39B). Experiments using lesions and drugs can assess the functions of specific brain regions but cannot differentiate the roles of specific neuronal types within a region.

In some invertebrate animals, many neurons are individually identifiable and make decisive contributions to circuit function and animal behavior. For example, individual neurons can be identified in *C. elegans* and subsequently ablated using a high-intensity laser; this approach, which takes advantage of *C. elegans*' transparency and the small number of neurons in its nervous system, has been used to identify neurons involved in many specific functions, such as the detection of volatile chemicals (see Section 6.11). In animals with large neurons, injecting hyperpolarizing currents with an intracellular or patch electrode can transiently inactivate a neuron to assess its contribution to circuit function or animal behavior (see Section 8.5).

In animals with more complex nervous systems, such as vertebrates, specific neural functions are often carried out by populations of neurons with similar anatomical and physiological properties. It is therefore more difficult to assess neuronal function by inactivating individual neurons. Genetic approaches can overcome this limitation. Since populations of neurons with similar functions often share similar gene expression patterns, researchers can use genetic methods (see Sections 13.10–13.12) to express in a neuronal population of interest an effector transgene capable of silencing most or all of these neurons at once. Based on our knowledge of neuronal communication (see Chapters 2 and 3), many effective approaches to inactivating neurons have been developed. For example, the gene encoding tetanus toxin (see Box 3-2), which cleaves synaptobrevin, a SNARE protein essential for neurotransmitter release (see Figure 3-8), has been used for blocking synaptic transmission from target neurons (Figure 13-41). Another widely used method of reducing neuronal action potential firing is to overexpress Kir2.1, an inward rectifier K⁺ channel (see Box 2-4); because the K⁺ equilibrium potential is always more hyperpolarized than the resting potential, increasing K⁺ conductance causes hyperpolarization of target neurons and makes it more difficult for them to reach firing threshold.

Killing or long-term silencing of neurons may induce compensatory changes in neural circuits. Therefore, a particularly informative method of inactivating neurons to assess their normal function is to silence them acutely and reversibly. Although effectors such as tetanus toxin and Kir2.1 can be temporally regulated at the transcriptional level when they are expressed as transgenes (see Section 13.10), such regulation is usually slow (hours to days). Expression of a temperature-sensitive mutant of Shibire protein (Shi^{ts}), which reversibly blocks synaptic vesicle recycling only at high temperatures (see Figure 3-14), has been a powerful tool in fruit flies to inactivate target neurons transiently (within minutes of a temperature shift; for example, see Figure 9-7). However, the Shi^{ts} strategy cannot be used in mammals, which have a constant body temperature.

Chemicals can be used to selectively silence neurons that express corresponding receptors; this **chemogenetic** approach has been increasingly used in

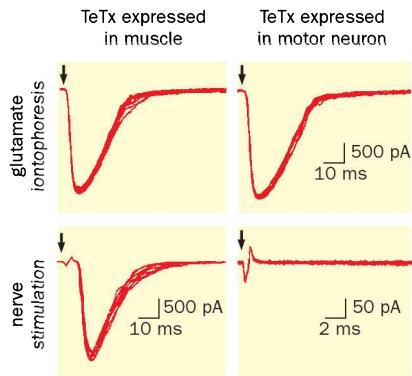


Figure 13-41 Transgenic expression of tetanus toxin (TeTx) blocks synaptic transmission. In each panel, 10 current traces from voltage clamp recordings of *Drosophila* muscles are superimposed. When TeTx was expressed in muscles (left) as a control, both glutamate iontophoresis (see Figure 3-1) and nerve stimulation induced robust postsynaptic currents. When TeTx was expressed in motor neurons, muscles still responded to glutamate iontophoresis, but no longer produced postsynaptic currents in response to nerve stimulation, indicating a blockage of glutamate release from motor axon terminals. Downward arrows: onset of iontophoresis (top panels) or nerve stimulation (bottom panels). (Adapted from Sweeney ST, Broadie K, Keane J et al. [1995] *Neuron* 14:341–351. With permission from Elsevier Inc.)

mammals. We discuss two examples here, both of which utilize chemicals that can cross the blood-brain barrier and have rapid onset and metabolism. The first example, named DREADD (for designer receptors exclusively activated by a designer drug), uses a mutant metabotropic acetylcholine (ACh) receptor called hM₄D, which binds a chemical, CNO (clozapine-N-oxide), but not endogenous ACh. CNO does not have endogenous targets, but binding of CNO and hM₄D leads to hyperpolarization of neurons through opening of an inward rectifier K⁺ channel, which results in effective silencing of hM₄D-expressing neurons (Figure 13–42A). The second approach uses a mutant ligand-binding domain

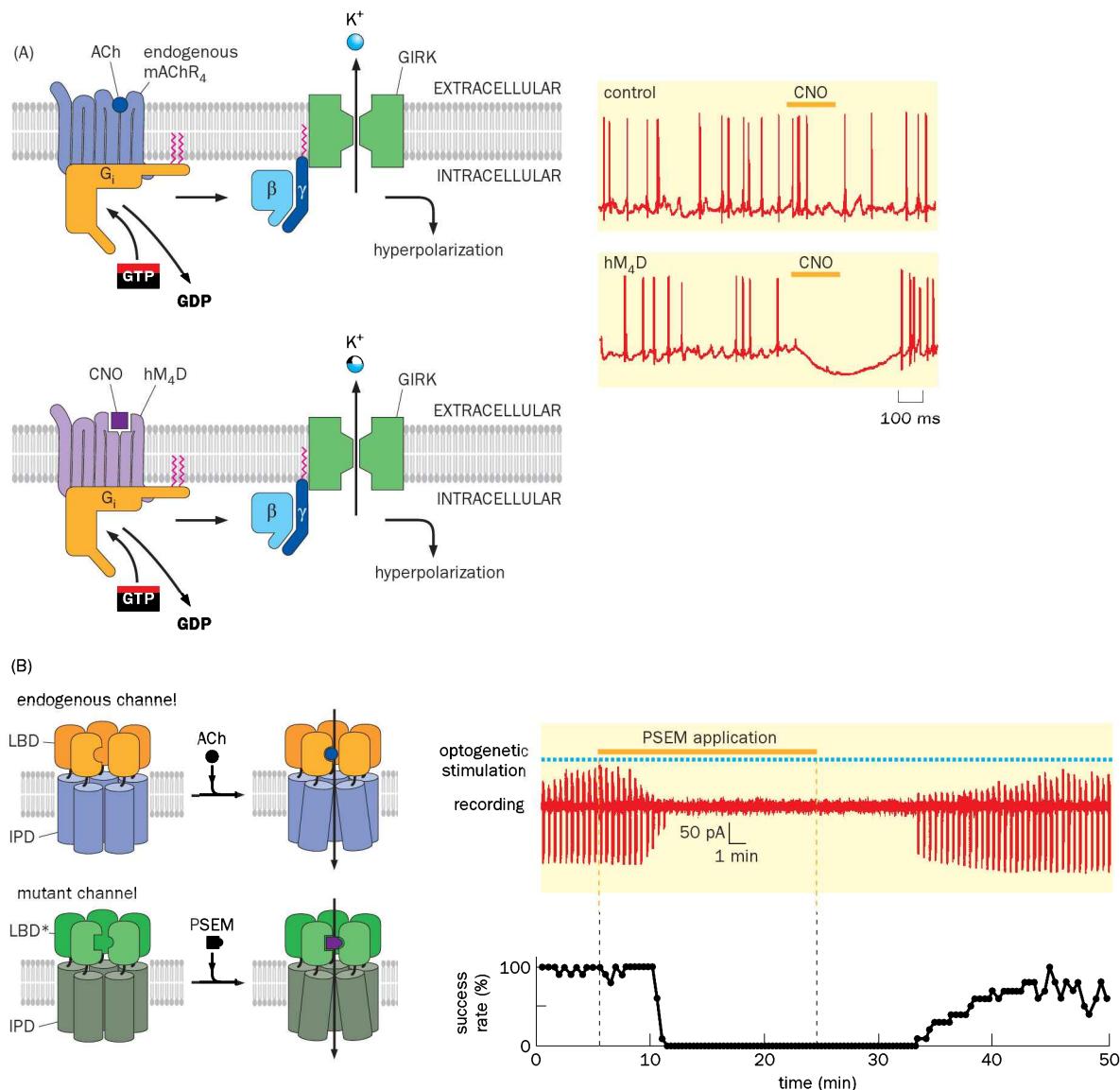


Figure 13–42 Chemogenetic approaches to silence neuronal activity. (A) Top left, binding of acetylcholine (ACh) to the endogenous metabotropic ACh receptor (mAChR₄) is coupled to a G_i protein that activates an inward rectifier K⁺ channel (GIRK), causing hyperpolarization of the neuron. Bottom left, a mutant mAChR₄ (hM₄D) no longer binds ACh but binds clozapine-N-oxide (CNO) with high affinity; CNO binding to hM₄D triggers neuronal hyperpolarization. Right, voltage traces showing that CNO application (horizontal bar) induced hyperpolarization and inhibited spontaneous firing of an hM₄D-expressing cultured hippocampal neuron (lower trace) but had no effect on a normal neuron (upper trace). (B) Left, whereas the normal ligand-binding domain (LBD) of the nicotinic ACh receptor (nAChR) binds to ACh but not pharmacologically selective effector molecule (PSEM), the mutant LBD (LBD*) binds to PSEM but not ACh. When fused to ion pore domains

(IPD) of different channels, LBD* can confer PSEM regulation of different ion conductances. Top right, cell-attached recording of a hypothalamus AgRP neuron (see Section 8.17) expressing channelrhodopsin (ChR2) and an LBD* fused to the IPD of a glycine receptor in a slice preparation, showing that PSEM application inhibits optogenetically induced neuronal firing. Blue, photostimulation period (each rectangle represents bursts of 10 light pulses within 1 s every 30 s); yellow bar, PSEM application period. Bottom right, quantification of the success rate of action potentials induced by optogenetic stimulation. (A, adapted from Armbuster BN, Li X, Pausch MH et al. [2007] Proc Natl Acad Sci USA 104:5163–5168. Copyright The National Academy of Sciences, USA; B, adapted from Magnus CJ, Lee PH, Atasoy D et al. [2011] Science 333:1292–1296.)

(LBD) of the ionotropic ACh receptor that binds to a chemical called PSEM (pharmacologically selective effector molecule) but not endogenous ACh. When this mutant LBD was fused to the ion pore domain of a glycine receptor, the hybrid receptor became a PSEM-gated Cl^- channel, which can cause effective neuronal silencing in response to PSEM application (Figure 13–42B).

While chemogenetic methods such as the PSEM-based approach can induce reversible neuronal silencing within 20 min of drug application (Figure 13–42B), optogenetics offers a method of manipulating neuronal activity with a higher temporal precision that matches the timescale of electrical signaling (see Section 13.25).

13.24 Neuronal activation can establish sufficiency of neuronal activity in circuit function and behavior

Electrical stimulation can be used to mimic the activation of neurons. Indeed, the first discovery that the nervous system communicates using electrical signals was based on the observation that muscles contracted when nerves were electrically stimulated (see Section 1.8). Many fundamental findings in neuronal communication, such as the phenomena and mechanisms of synaptic transmission and long-term potentiation, involved the experimental stimulation of nerve fibers (for example, Figures 3–1 and 10–8). Experiments using electrical stimulation also led to the discovery of the sensory and motor homunculi in the human brain (see Section 1.11), suggested brain regions related to reward processing (see Section 10.24), and helped establish causal relationships between neuronal activity and visual perception (see Section 4.29). Electrical stimulation in humans can be used to treat brain disorders such as Parkinson disease (see Section 11.13).

In most *in vivo* preparations, electrical stimulation has been delivered using an extracellular electrode. In principle, the strength and frequency of electrical stimulation can be controlled to match the endogenous firing patterns of neurons. However, in the complex milieu of the central nervous system, where different types of neurons and passing axons are intermingled, it is difficult to control what types of neurons are being activated by a stimulating electrode. Indeed, electrical stimulation usually activates a mixture of excitatory neurons, inhibitory neurons, and projecting axons that pass near the electrode tip, making it difficult to assign the effects of stimulation to the activities of specific types of neurons. The recent development of genetically encoded effectors that can activate neurons and be targeted to specific types (see Sections 13.10 to 13.12) has begun to overcome this limitation. These effectors depolarize and therefore activate target neurons in response to an experimentally applied trigger, such as heat, a chemical, or light. Ideally, the effector does not act in the absence of the trigger, and the trigger has no impact on the systems being investigated in the absence of the effector. This way, activation can be specifically restricted to those neurons that express the effector molecule at the time of trigger application.

For example, an effective way to activate neurons in the fruit fly is to express in the neurons of interest a transgene that encodes a temperature-gated TrpA1 channel, which causes neuronal depolarization in response to heat (Figure 13–43). By simply changing the temperature of the environment, researchers can investigate the behavioral consequences of selectively activating specific neurons in freely moving flies (for example, see Figure 9–11B). This method is simple and noninvasive, but it can only be employed if the behavior being investigated is insensitive to temperature, and it cannot be used in animals such as mammals that have a constant body temperature. The temporal resolution of neuronal activation is limited by how fast temperature changes can occur (usually over the course of seconds to minutes).

Neurons can also be activated by chemicals. Indeed, both of the chemogenetic approaches discussed in Section 13.23 can also be adapted to activate neurons rather than silence them. For example, by creating CNO-binding mutations in a metabotropic AChR isoform that couples to G_s instead of G_p , CNO application results in an increase of intracellular cAMP, which can depolarize certain neurons

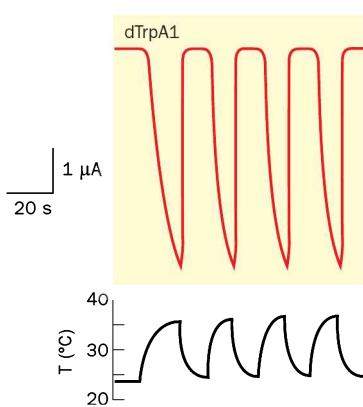


Figure 13–43 Turning heat into depolarization. Expression of a *Drosophila* TrpA1 channel in *Xenopus* oocytes results in heat-induced depolarization. In response to heat pulses (bottom), inward (depolarizing) currents are induced in *Xenopus* oocytes voltage clamped at -60 mV (top). (Adapted from Hamada FN, Rosenzweig M, Kang K et al. [2008] *Nature* 454:217–220. With permission from Macmillan Publishers Ltd.)

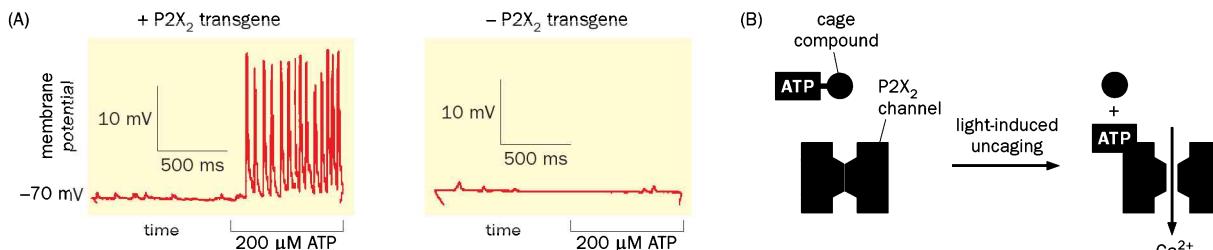


Figure 13–44 Neuronal activation by supplying ATP to neurons that express an ATP-gated channel. **(A)** Intracellular recording of *Drosophila* larva muscle in response to ATP application in a dissected neuromuscular preparation. Left, when motor neurons express the P2X₂ transgene, ATP application depolarizes the motor neuron, evoking neurotransmitter release and endplate potentials in muscles. Right, in the absence of the P2X₂ transgene, ATP application does not induce endplate potentials in muscles. The smaller depolarization events

that occur under both conditions are miniature endplate potentials reflecting spontaneous transmitter release (see Section 3.2). **(B)** Schematic of neuronal activation in behaving flies. Flies were injected with caged ATP, which does not bind to P2X₂ channels. Light triggers the uncaging of ATP, which binds to and activates the P2X₂ channel, causing Ca²⁺ influx and activation of P2X₂-expressing neurons. (Adapted from Lima SQ & Miesenbock G [2005] *Cell* 121:141–152. With permission from Elsevier Inc.)

through cyclic nucleotide-gated channels (see Figure 6–4). By fusing a mutant LBD to the ion pore domain of a cation channel, PSEM application can likewise cause neuronal activation.

Neurons can also be activated by combinations of chemicals and light. For example, a mammalian ATP-gated P2X₂ channel has been used in *Drosophila* for neuronal activation. *Drosophila* does not have its own ATP-gated channels, nor does it have sufficient extracellular ATP to activate a mammalian P2X₂ channel produced from a transgene. Thus, application of exogenous ATP can be a trigger to activate *Drosophila* neurons that express transgenic P2X₂ (Figure 13–44A). The temporal resolution of this approach is limited by the slow time course of ATP application and clearance. To achieve fast activation of neurons in behaving animals, caged ATP (ATP modified chemically so it cannot activate its receptor) can be injected into the central nervous systems of transgenic flies that express the P2X₂ channel. A flash of light triggers uncaging of the ATP (removal of chemical modification and thereby release of free ATP) and activation of the P2X₂-expressing neurons (Figure 13–44B). This strategy has been used to effectively induce various behaviors, from mimicking aversive stimuli (see Figure 10–29B) to singing courtship songs (see Figure 9–13), depending on the type of neurons in which P2X₂ was expressed. Thus, these experiments have helped to establish causal relationships between the activity of specific types of neurons and the behaviors they control.

13.25 Optogenetics allows control of the activity of genetically targeted neurons with millisecond precision

Broadly speaking, **optogenetics** is an approach for altering neuronal activity by using light to activate an effector that is genetically targeted to specific neurons. Thus, it includes the strategy we just discussed—using light to uncage ATP and activate neurons that express an ATP-gated channel. In most cases, optogenetics refers to the use of microbial opsins as the effectors because of their simplicity, effectiveness, and general applicability. Since the term was first introduced in 2006, optogenetics has had a huge impact in many areas of neuroscience, especially on how researchers evaluate the function of neural circuits and their roles in behavior.

The most remarkable and widely used optogenetic effector for neuronal activation is **channelrhodopsin-2 (ChR2)**, a protein first found in the green alga *Chlamydomonas reinhardtii*. As we discussed in Section 12.13, ChR2 is a seven-transmembrane type I rhodopsin; instead of coupling to G proteins, it is itself a cation channel that opens in response to blue-light stimulation (Figure 13–45A; see also Figure 12–21B). Although ChR2 requires all-trans retinal as a cofactor, mammalian neurons have sufficient endogenous all-trans retinal to support

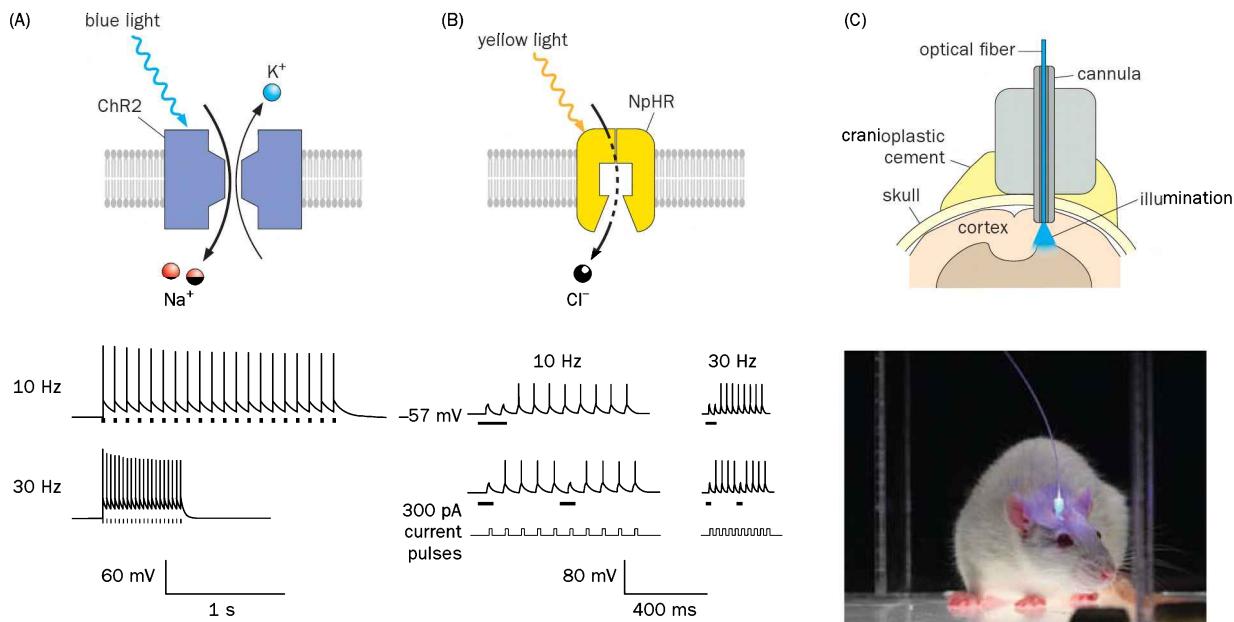


Figure 13–45 Optogenetics for precise temporal control of neuronal activity. (A) Top, channelrhopsin-2 (ChR2) from a green alga is a cation channel that is gated by blue light and causes neuronal depolarization from the resting potential because it allows more Na⁺ influx than K⁺ efflux. Bottom, voltage traces show that in culture the firing of a hippocampal neuron that expresses ChR2 can be precisely controlled by 10-ms blue light pulses (blue dashes at the bottom) up to 30 Hz. (B) Top, halorhodopsin (NpHR) from an archaeal species is a Cl⁻ pump that is activated by yellow light. Bottom, voltage traces show that yellow light pulses (yellow dashes at the bottom) can cancel with millisecond precision the action potentials produced by depolarizing current pulses in cultured neurons. (C) Optogenetics *in vivo*. A cannula is surgically introduced into the brain at a region of interest. Viral

vectors that carry opsin transgenes can be introduced through the cannula at the desired depth, allowing ChR2 or NpHR to be expressed in the neurons of interest prior to the experiment. During the experiment, an optical fiber delivering blue or yellow light is introduced into the cannula, so that light-induced behavior can be observed in a freely moving rodent (shown at the bottom, see Movie 13–5). (A, adapted from Boyden ES, Zhang F, Bamberg E et al. [2005] *Nat Neurosci* 8:1263–1268. With permission from Macmillan Publishers Ltd; B, adapted from Zhang F, Wang LP, Brauner M et al. [2007] *Nature* 446:633–639. With permission from Macmillan Publishers Ltd; C, schematic adapted from Zhang F, Aravanis AM, Adamantidis A et al. [2007] *Nat Rev Neurosci* 8:577–581. With permission from Macmillan Publishers Ltd. Image courtesy of Karl Deisseroth.)

ChR2 function; thus, expression of ChR2 alone is sufficient to allow robust depolarization and firing of mammalian neurons in response to light. Indeed, pulses of blue-light stimulation can precisely direct the firing of ChR2-expressing neurons at rates as high as 30 Hz (Figure 13–45A, bottom), achieving control of target neuron activity with millisecond precision. In nervous systems that lack sufficient all-trans retinal (such as *Drosophila* and *C. elegans*), supplemental retinal provided through food is often sufficient for ChR2 function. Many ChR2 variants have been developed by *in vitro* mutagenesis with enhanced expression, photocurrent, and faster or slower off time constant (how long the current decays after light is off) for different applications.

Optogenetic approaches can also be used to reversibly inactivate neurons with high temporal precision. For this purpose, an archaeal type I rhodopsin called **halorhodopsin**, which is a yellow-light-activated inward Cl⁻ pump (Figure 13–45B), can be expressed in neurons. Yellow light stimulation hyperpolarizes neurons that express halorhodopsin, making it more difficult for these neurons to fire action potentials in response to depolarizing signals (see Figure 4–47). Indeed, in cultured neurons expressing halorhodopsin, short pulses of yellow light were sufficient to block with high temporal precision action potentials induced by depolarizing current pulses (Figure 13–45B, bottom). **Archaeerhodopsin** (Arch), a light-driven outward proton pump from an archaeal species, has also been widely used as an optogenetic effector for light-induced neuronal silencing.

In order to achieve optogenetic manipulation of neurons in behaving animals, fiber optics-based systems have been developed to activate or silence neurons with laser pulses in specific brain regions that have previously been transduced by viruses that allow the expression of optogenetic effectors (Figure 13–45C);

optogenetic effectors can also be expressed by transgenic animals. The optical fibers are usually ~200 μm in diameter, thin enough to minimize damage but still able to effectively excite ChR2-expressing neurons in approximately one cubic millimeter at the fiber's tip. Thus, the behaviors of freely moving animals can be assayed while specific populations of neurons in specific brain regions are activated or silenced (see Movie 13–5).

Because optogenetic methods manipulate neuronal activity with the same timescale as fast neuronal communication (with spikes and in the millisecond range), they are well suited for probing neuronal signaling and computation. Still, a limitation of the optogenetic approach is that for neuronal activation to achieve its biological effect, a large fraction of the neurons of interest must be accessible to light delivered by optical fiber; for neuronal inactivation, access to the full population of relevant neurons may be required. Fiber optic implantation is also associated with physical damage. By comparison, the chemogenetic approaches discussed in Section 13.23, while not as temporally precise, can assess the entire population of neurons expressing the relevant receptor. They are minimally invasive if the chemicals can cross the blood-brain barrier and do not have side effects in the absence of the effector. Thus, these methods complement each other.

In summary, classic methods to activate or silence neurons, including electrical stimulation, lesion, and pharmacology, have now been supplemented by a variety of genetically encoded effectors to activate or inactivate specific neuronal types with light, heat, or chemicals in a number of model organisms. Coupled with the development of genetic tools that provide access to an increasing number of cell types, these approaches are making a significant impact on our understanding of how neural circuits operate and how they control behavior.

13.26 Synaptic connections can be mapped by physiological and optogenetic methods

Having studied methods for recording and manipulating neuronal activity, we now return to the subject of mapping neuronal connections discussed in Section 13.19 to study how physiological methods can be employed to address this important problem.

A widely used method for determining whether two neurons are directly connected is to place an electrode in each of the neurons and record the two neurons simultaneously in response to manipulating the activity of one of them (Figure 13–46). If injecting a depolarizing current into neuron A to produce an action potential causes depolarization of neuron B within the timeframe of a monosynaptic connection (usually a few milliseconds), we can conclude that neuron A forms excitatory synapses directly onto neuron B (Figure 13–46A). If firing of neuron A causes hyperpolarization of neuron B within a few milliseconds, then neuron A forms inhibitory synapses onto neuron B (Figure 13–46B). Likewise, stimulating neuron B and recording from neuron A can test whether these neurons are connected by reciprocal $B \rightarrow A$ chemical synapses. If hyperpolarization of neuron A causes hyperpolarization of neuron B, and vice versa, then we can conclude that these two neurons are connected by an electrical synapse (Figure 13–46C), as chemical synapses do not transmit hyperpolarizing signals (see Box 3–5). Paired recordings have been performed extensively in invertebrates with large neurons using intracellular electrodes (see Figure 8–13) and in mammalian brain slices using whole-cell patch recording (see Figure 4–46). Such tests provide definitive evidence that two neurons are functionally connected, can determine the type (for example, excitatory, inhibitory, electrical) and strength of the connection, but are labor intensive for mapping large-scale neuronal connections and are not applicable to map long-range connectivity.

A higher throughput mapping method developed in mammalian brain slices utilizes laser uncaging of neurotransmitters. For example, a brain slice can be placed in medium that contains caged glutamate (glutamate modified chemically so it cannot activate its receptor). A focal laser stimulation leads to uncaging (removal of the chemical modification) and thus local release of glutamate, which causes neurons near the laser stimulation site to fire action potentials. If

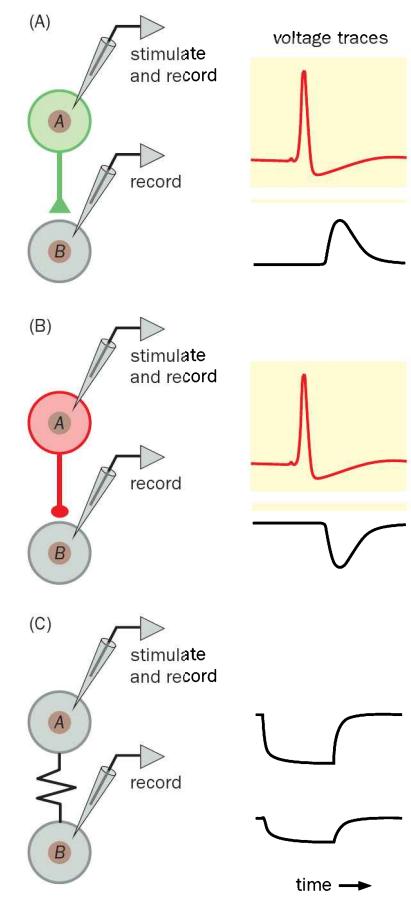


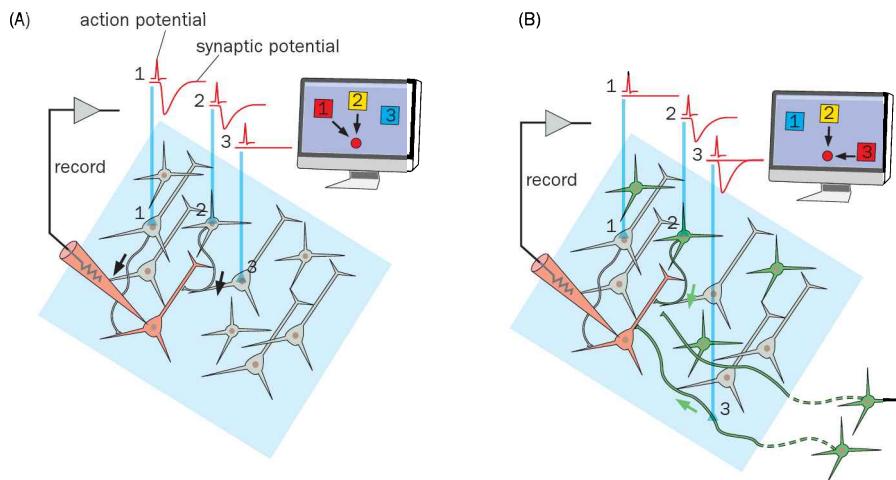
Figure 13–46 Mapping neuronal connections with paired recording.

(A) Depolarizing an excitatory neuron A causes it to fire an action potential that elicits a depolarizing membrane potential in its postsynaptic partner neuron B.

(B) Depolarizing an inhibitory neuron A causes it to fire an action potential that elicits a hyperpolarizing membrane potential in its postsynaptic partner neuron B. **(C)** Hyperpolarizing neuron A causes hyperpolarization of neuron B via an electrical synapse.

Figure 13–47 Mapping neuronal connections with optical and electrophysiological methods.

electrophysiological methods. Arrows indicate the direction of signal flow. For simplicity, axons of neurons that do not form a synapse with the recorded neuron are not drawn. **(A)** Photo uncaging. The brain slice is incubated with caged glutamate. When a scanning laser (blue) reaches a neuron that is presynaptic to the recorded neuron, local release of glutamate causes this neuron to fire action potentials, which produce excitatory postsynaptic potentials in the recorded postsynaptic neuron (the y axes of synaptic potential and action potential are not at the same scale). A two-dimensional input map can be produced after the laser systematically scans through the entire slice. In the illustration, neurons 1 and 2 are presynaptic partners of the recorded neuron, but neuron 3 is not. **(B)** ChR2-assisted circuit mapping. A defined subpopulation of neurons (green) express channelrhodopsin (ChR2), causing them to fire action potentials in response to blue light stimulation. In this scheme, stimulating neuron 1 does not activate the recorded neuron because it does not express ChR2. Stimulating neuron 2 activates the recorded neuron because it expresses ChR2 and synapses onto the recorded neurons. Note that this method can also be used to map connections between ChR2-expressing presynaptic neurons whose cell bodies lie outside the slice (bottom right) and the recorded neuron, because stimulating their axons (3) and terminals (not shown) is often sufficient to elicit synaptic response in postsynaptic neurons. Although drawn in the same schematic, the axon-stimulation experiment is performed separately, to ensure that the only source of ChR2-expressing neurons is defined and located outside the slice. (Adapted from Luo L, Callaway EM & Svoboda K [2008] *Neuron* 57:634–660. With permission from Elsevier Inc. The original methods were described in Callaway EM & Katz LC [1993] *Proc Natl Acad Sci USA* 90:7661–7665 and Petreanu L, Huber D, Sobczyk A et al. [2007] *Nat Neurosci* 10:663–668.)



one or a few neurons near the laser stimulation site form monosynaptic excitatory connections with a postsynaptic neuron of interest that is being recorded using intracellular or whole-cell recording, then laser uncaging will produce excitatory postsynaptic potentials in the recorded neuron. After the laser scans through a defined area of the brain slice, investigators can create a two-dimensional map of all excitatory neurons in the area that connect with the target neuron that is being electrically recorded (**Figure 13–47A**).

A more widely used approach to this type of experiment replaces laser uncaging of a neurotransmitter with photoactivation of ChR2-expressing neurons (Figure 13–47B), a method called CRACM (ChR2-assisted circuit mapping). The advantage of CRACM is that the type of presynaptic neurons can be genetically defined by the promoter that drives ChR2 expression. Moreover, unlike paired recordings and laser uncaging discussed above, which are limited to mapping connections within the brain slice, CRACM can also map long-range connections. If the only source of ChR2 expression is from a defined neuronal population outside the brain slice, photoactivation of ChR2 molecules in their axons and terminals is often sufficient to cause neurotransmitter release at synaptic terminals, which can be detected by recording of postsynaptic neurons located within the slice.

None of these physiological mapping methods can be easily applied *in vivo*, at least in the complex mammalian brain. Thus, these methods complement anatomical methods such as serial EM reconstruction and trans-synaptic tracing (see Section 13.19) in mapping synaptic connections.

BEHAVIORAL ANALYSES

A major goal of neurobiology is to understand how behaviors arise from the molecular and cellular properties of neurons, the wiring specificity of neural circuits established during development and modified by experience, and the spatiotemporal patterns of neuronal activity at the times when behaviors occur. Insightful and quantitative analysis of animal behavior is instrumental for studying many neurobiological problems, from sensory perception and motor control to emotion and cognition. Unlike in human studies, where verbal reports are revealing, animal studies depend on observation and measurement of behavior in order to infer what animals sense, feel, learn, and understand.

In neurobiology research, behavioral analyses serve three broad purposes. First, behavioral analyses aim to explain the behavior itself (for example, see Chapter 9): what is the function of the behavior for animal survival and reproduction, what external factors influence it, what are its constituent motor actions, and what are the underlying neural bases? Second, behavioral analyses are used as quantitative assays for the functions of brain regions, circuits, and neurons in

specific neurobiological processes being investigated, such as sensory perception (see Figure 4–52) or learning and memory (see Figure 10–32). Third, behavioral analyses are used to test the effects of manipulating specific genes (see Figure 10–33) or to assess animal models of human brain disorders (see Figure 11–7). Given the inseparable links between genes, neurons, circuits, and behaviors (see Figure 10–7), these purposes have considerable overlap. In the following sections, we first highlight two general approaches in behavioral analysis that are applicable to all of these purposes and then discuss behavioral assays commonly used to assess the functions of genes, neurons, and circuits and to model human brain disorders.

13.27 Studying animal behavior in natural environments can reveal behavioral repertoires and their adaptive value

From an evolutionary perspective, behaviors are products of natural selection that allow animals to interact with their environments in ways that improve their probability for survival and reproduction. Thus, an influential approach to animal behaviors is to study them in the natural environment. This neuroethological approach can reveal an animal's behavioral repertoire (what behaviors an animal is capable of exhibiting), the relationships between different behaviors (for example, whether one behavior precedes or follows another in a sequence, or whether two behaviors are mutually exclusive in their occurrence), and the adaptive values of specific behaviors.

The principal methods of neuroethology include observation and measurement in carefully designed field studies. We use the study of honeybee dancing as an example to illustrate. Honeybees are social insects that can perform sophisticated behavioral tasks (see Figure 10–23). They are also expert nectar collectors and pollinators. Once forager bees find a good source of nectar, sometimes kilometers away from their hive, they communicate with their fellow bees (forager recruits) to direct these hive-mates to the same place. How do bees achieve this? Researchers have set up observation hives with glass windows so that they can observe the behaviors of foragers in the hive environment. Once foragers locate a good source of nectar, they return to the hive and perform dances to convey information to forager recruits. When the source of the nectar is more than 50 m from the hive, foragers typically perform a tail-wagging dance following a trajectory that resembles the Arabic numeral 8 (Figure 13–48A). By placing scented feeding bowls at different distances and directions from the hives and measuring the dances of bees and the subsequent foraging of forager recruits, researchers reached the following set of conclusions. The richness of the nectar is indicated by the vigor of the dance. The distance to the nectar source is signaled by the duration of tail wagging, which takes place as the forager bee dances in a straight path between the 8s two circular halves (Figure 13–48B). The orientation of the figure-8 dance in the hive signals the direction of the outgoing flight with respect to the sun (Figure 13–48C). Finally, the scent that foragers carry informs forager recruits about the kind of nectar they should look for once they arrive at the vicinity of the nectar source. Experiments using foraging behavior as readout validated these conclusions (Figure 13–48D). The adaptive value for efficiently locating nectar is obvious for the bee colonies, and efficient nectar foraging is also beneficial to the plants that produce the nectar and are pollinated by the bees.

The spirit of the neuroethological approach can be extended to the laboratory, where animal behaviors can be observed, recorded, and quantitatively measured in settings that resemble the natural environment but offer greater technical ease compared with field studies. For instance, the complete recording of *Drosophila* mating behaviors in a laboratory setting (see Figure 9–2 and Movie 9–1) enabled these behaviors to be dissected into discrete components. Quantitative plots of different behaviors exhibited over time can be used to compare individuals receiving different experimental treatments and thereby to study the neural mechanisms underlying these behaviors (see Figure 9–11B). The development of high-speed video recording and automatic video analysis have further enhanced the sensitivity and throughput of behavioral observations and measurements.

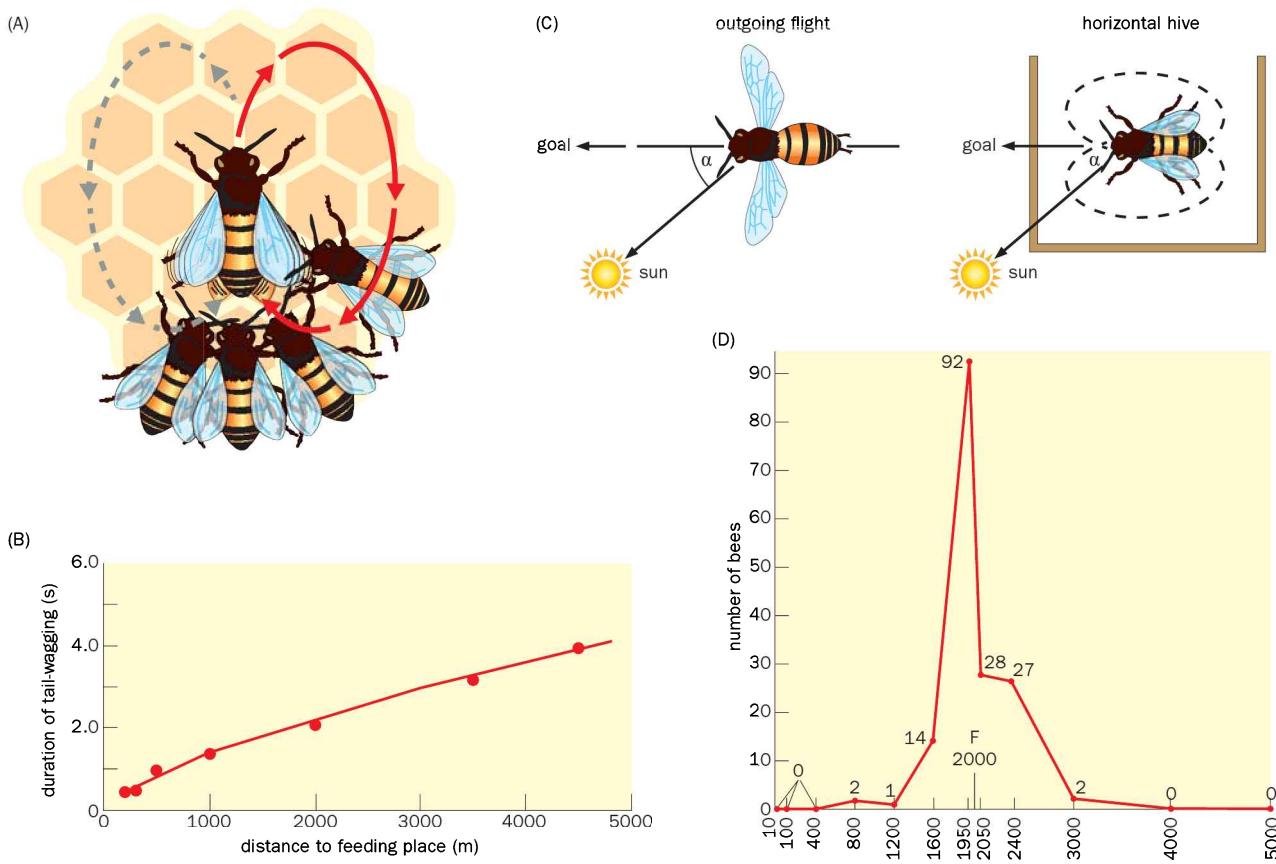


Figure 13–48 Behavioral analysis of honeybee foraging. (A) Illustration of the figure-8 dance of a forager bee in an observation hive. The forager alternates between the right half (solid red arrows and trajectory) and the left half (dashed gray arrows and trajectory) of the figure 8. The four forager recruits take in the information by moving with the forager and maintaining close contact with her, particularly during the straight part of the dance, when the forager exhibits tail wagging. (B) The relationship between the duration of tail wagging, measured from film recordings, and the foraging distance signaled by the dancing bee. (C) The angle (α) of

the outgoing flight with respect to the sun (left) is signaled by the angle at which the figure-8 dance orients with respect to the sun in this horizontal hive (right). (D) In this field experiment, foragers were fed at a scented plate (F) 2000 m away from the hive. Afterwards, similarly scented plates without food were placed at many distances (numbers on the x axis) and the visits of forager recruits were quantified; the y axis values adjacent to the data points reveal that the forager recruits preferred distances around 2000 m. (Adapted from von Frisch K [1974] *Science* 185:663–668.)

13.28 Studying behaviors in highly controlled conditions facilitates investigation of their neural basis

Behavior is influenced by many factors: external stimuli, internal drives and brain states, and the individual animal's genetic make-up and life experience. Thus, another influential approach, which at face value might seem to be the polar opposite of neuroethology, is to study behavior under as much experimental control as possible so that known factors that might influence behavior can be varied one at a time in order to study their contributions to the behavior. To achieve this, researchers carry out behavioral studies in inbred animal strains to decrease genetic variability—all individuals within an inbred strain are essentially genetically identical—and use animals of the same sex and age reared under similar conditions to decrease variability in experience. Behaviors can be performed in a fixed apparatus (for example, see Figure 10–22) to reduce the variability of external factors, and standardized conditions can be implemented to control for internal factors, such as the circadian cycle of the animal and the time when the animal last ate or drank prior to a behavioral experiment.

This approach has yielded great insights. The discovery of classical and operant conditioning and the identification of factors that affect those learning processes were made using this approach (see Section 10.14). The operant conditioning paradigm has been particularly influential in studying internal factors

such as drives that affect a behavior. Behavioral paradigms are often designed to take advantage of internal drives. For instance, in two commonly employed paradigms, thirsty animals are motivated by a potential water reward to choose to perform an action (such as pressing a button) or to do nothing (go/no-go task), or to choose one of the two actions (two-alternative forced choice task, such as a saccade toward one of the two alterative targets). These tasks can be used to study sensory perception, decision-making, motor execution, and memory (see Figures 4–53, 4–54, 6–73, 8–27, and 10–46). The combination of behavioral analysis with methods for recording and manipulating the activity of relevant neurons and circuits (see Box 13–3) has enabled researchers to establish causal links between behavior, neuronal activity, and circuit function.

The two general approaches outlined above are complementary, and many behavioral paradigms incorporate the merits of both. Ideally, the choice of which behaviors to study under highly controlled experimental conditions and the design of the assays used to study these behaviors should be based on an understanding of the behavioral repertoire of animals in their natural environment. For instance, the Morris water maze (see Figure 10–32) takes advantage of the aptitude of rodents for using external landmarks to navigate and their preference not to swim. The **closed-loop** design of behavioral experiments, in which the behavior of an animal changes the environmental stimuli that induce the behavior in the first place (for example, see Figure 10–44), can allow animals to explore naturalistic virtual environments in highly controlled settings. For instance, an influential closed-loop preparation was developed while studying visual control of the flight behavior of houseflies. In a cylinder that delivers panoramic visual stimuli, a test fly is suspended from a torque compensator, which measures the rotation of the fly as it attempts to orient toward visual stimuli, and sends a signal to control the rotation of the panorama cylinder (Figure 13–49). In this way, the fly's intended movement alters the visual scene as if the fly were actually moving through the scene. This design allows researchers to study in an immobilized fly some of the flight behaviors that might be observed in a more naturalistic setting, such as tracking of objects, and at the same time makes possible the precise delivery of visual stimuli and the quantitative measurement of the fly's behavioral output. This setup also enables researchers to investigate the underlying neural bases of behavior by performing electrophysiological recordings of brain neurons.

Conceptually similar approaches to the closed-loop design for studying the flight behavior of a housefly have also been developed for other animals. For example, navigation of head-fixed mice through complex virtual reality environments has facilitated investigation of the neuronal activity associated with spatial navigation using optical imaging and whole-cell recording methods that cannot easily be applied to freely moving mice (see Figure 13–40B). Indeed, feedback signals can be obtained from recordings of motor neurons rather than the motion itself, as in the use of paralyzed zebrafish larvae. In this preparation, a zebrafish larva was visually stimulated by the backward motion of a grating on a computer screen to simulate the flow of water; this virtual water flow induced a compensatory forward swim in the fish. Extracellular recordings of the motor neurons were used as a measure of this fictive motion, and the recorded signals were fed back to control the apparent speed of the grating, forming a closed loop (Figure 13–50A). When researchers adjusted the gain of the feedback signals, fish adjusted their swimming speed correspondingly to match the virtual change of the water flow speed (Figure 13–50B). This preparation enabled researchers to image neurons from the entire brains of paralyzed zebrafish larvae using a genetically encoded Ca^{2+} indicator, and to identify neurons in the cerebellum and the inferior olive (see Section 8.8) whose activities correlate with the adjustment of speed in response to changes in gain.

In summary, by combining the insights derived from neuroethological approaches with the merits of experimental controls, researchers can develop sophisticated behavioral paradigms that enable quantitative analysis of behavior (or fictive behavior) while recording and manipulating neuronal activity. Thanks to the techniques described in previous sections, these approaches are becoming increasingly powerful for dissecting the neural bases of complex behaviors.

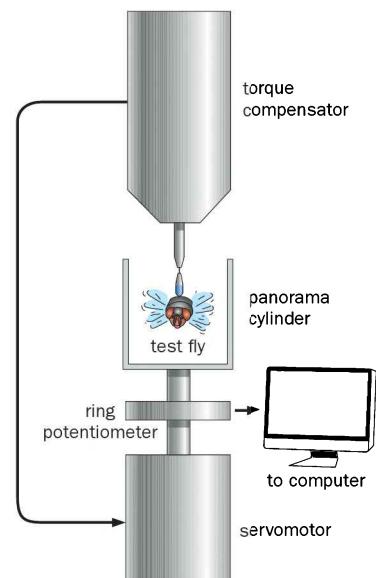


Figure 13–49 A simplified scheme of a closed-loop design to study the flight behavior of flies. A housefly is suspended from a torque compensator. Signals from the torque compensator provide a quantitative measure of the fly's intended behavioral output. These signals are sent to a servomotor, which controls the rotation of the panorama cylinder through a ring potentiometer. Thus, the fly's intended behavioral output controls its visual environment. (Adapted from Reichardt W & Poggio T [1976] *Q Rev Biophys* 3:311–375.)

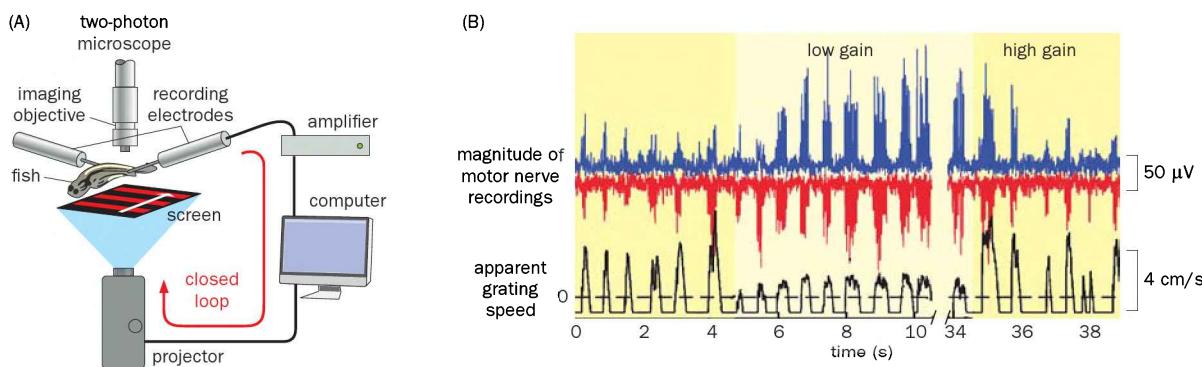


Figure 13–50 Closed-loop control of fictive swimming by a zebrafish larva. (A) Experimental setup. A paralyzed zebrafish larva receives visual stimuli from a computer screen, on which the image of a grating moves to mimic the backward flow of water (white arrow). Forward fictive swims are measured by extracellular recordings of the motor neurons. These signals are fed back in real time to control the apparent grating speed. In this set up, neuronal activities in the brain can be measured using a two-photon microscope and genetically encoded Ca^{2+} indicator expressed in all neurons. (B) Example recordings of a larva's response to experimentally altered feedback

gain. The blue and red traces represent simultaneous recordings of motor nerves from two sides of the larva. For ease of viewing the red trace is flipped vertically relative to the blue trace. When the gain is low (pale yellow background), the fish increases its fictive swimming speed as illustrated by an increase in motor nerve output; when the gain is high (bright yellow background), the fish decreases its fictive swimming speed as illustrated by a decrease in motor nerve output. The bottom trace indicates the apparent grating speed. (Adapted from Ahrens MB, Li JM, Oger MB et al. [2012] *Nature* 485:471–477. With permission from Macmillan Publishers Ltd.)

13.29 Behavioral assays can be used to evaluate the functions of genes and neurons and to model human brain disorders

In the final section, we highlight behavioral assays that are commonly used to assess phenotypes associated with the disruption of specific genes or the activation or inactivation of specific neuronal populations. These assays are also frequently used to study animal models of human brain disorders and the effects of pharmacological intervention. We focus on assays designed for rats and mice, as they are the most widely used mammalian models (see Section 13.4).

Behavioral assays can be used to assess general sensorimotor functions. The simplest assay is to record an animal's behavior in its home cage with a video camera over several days followed by manual or automatic analysis of videos. This recording allows scientists to assess general motor activity, circadian rhythms, and eating, drinking, sleeping, and nest-building patterns in the laboratory housing environment with minimal handling. Another assay is to place the animal in an open field, which is essentially a box with walls but no top, and videotape, analyze, and quantify its trajectory within a given period of time. Motor coordination can also be tested by recording the footprints of an animal with painted feet (see Figure 8–19), or by measuring the length of time an animal can remain on a rotating rod (Figure 13–51), the speed of which can be held constant or can increase over time so that the task becomes progressively more difficult. These assays evaluate the basic functions of the nervous system, including those of the cerebral cortex, cerebellum, basal ganglia, and spinal cord. More specific assays can be used to examine specific sensory functions; for example, the hot plate assay tests temperature and pain perception by measuring the time it takes for an animal to flick a tail or lick a hind paw placed on a plate as the temperature of the plate rises.

Behavioral assays have also been designed to assess animals' cognition, such as learning and memory. The Morris water maze and the contextual and auditory fear conditioning assays (see Figures 10–32 and 10–40) are widely used to test the functions of the hippocampus and amygdala in learning and memory. Another often-used assay is the radial arm maze, which tests spatial and working memory. The maze consists of a number of arms (usually eight), with food pellets located at the ends of the arms (Figure 13–52A). The maze is placed in a room in which numerous visual cues are scattered outside the maze. After becoming habituated to the apparatus and the room, food-restricted rats placed at the center of the maze efficiently visit all of the arms to collect the food, with minimal revisiting of arms from which food had already been collected. Behavioral experiments



Figure 13–51 The rotarod assay for testing motor coordination. At the beginning of the experiment, mice are placed on a stationary rod; the separate compartments shown in the photograph allow simultaneous testing of multiple animals. The rod is connected to a motor with an adjustable speed. Typical experiments include running the motor at a constant speed (for example, 10 revolutions per minute, or rpm) or a speed that increases (for example, from 5 to 30 rpm), and measuring the period of time each mouse remains on the rod. In addition to testing motor coordination, the rotarod can also test motor skill learning (see Figure 8–18D). (Courtesy of Mehrdad Shamloo.)

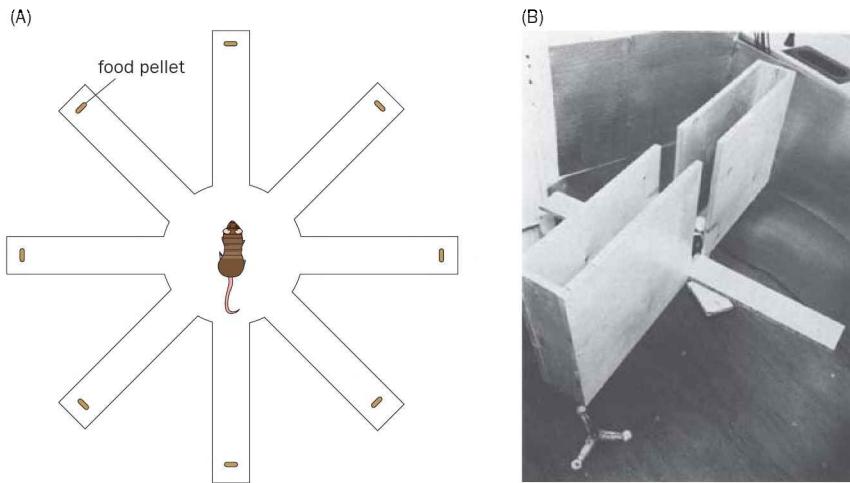


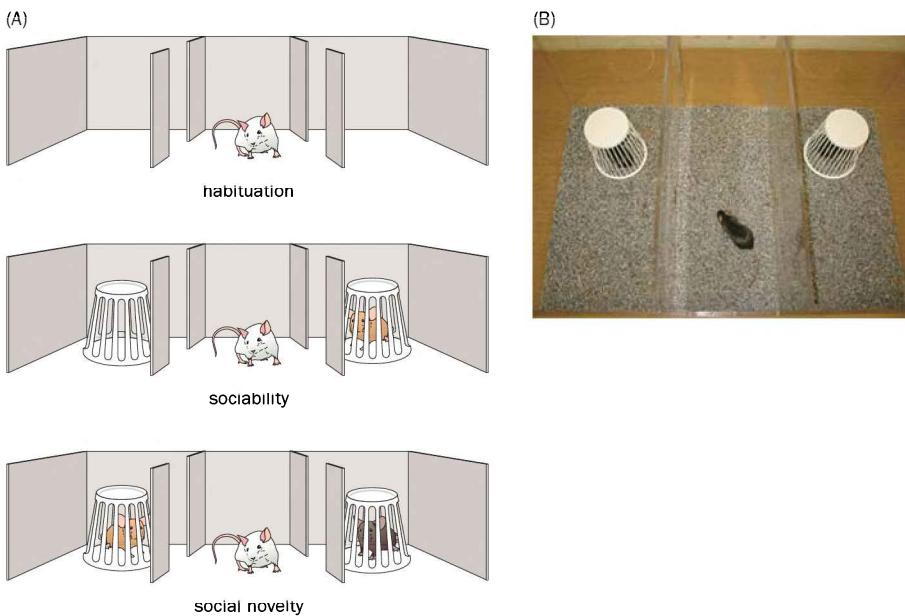
Figure 13–52 Mazes used to test memory and anxiety. **(A)** Top view diagram of a radial arm maze for testing spatial and working memory. Food pellets are located at the ends of the arms. A food-restricted rat placed in the center of the maze will visit each arm to consume the pellets with minimal repetition, utilizing extra-maze cues (not shown) for navigation. **(B)** Photograph of an elevated plus-maze with a rat located at the intersection, between the opened and closed arms. The fractions of time spent in the closed arms and open arms can be used to quantify anxiety in rats or mice and can be modulated by anxiolytic and anxiogenic drugs. (A, adapted from Olton DS & Samuelson RJ [1976] *J Exp Psychol Animal Behav Proc* 2:97–116. With permission from the American Psychological Association; B, from Pellow S & File SE [1986] *Pharmacol Biochem Behav* 24:525–529. With permission from Elsevier Inc.)

indicate that rats rely mostly on extra-maze cues rather than intra-maze cues such as odor trails they leave behind or sequential strategies such as visiting adjacent arms one-by-one. By recording the trajectories of animals visiting different arms in the maze, scientists can assess the functions of the hippocampus, which has an essential role in spatial memory, and the prefrontal cortex, which has been implicated in working memory.

Perhaps the most challenging behavioral assays are those aimed at modeling human psychiatric disorders, because it is difficult to assess whether a mouse or a rat is experiencing a condition that resembles schizophrenia, depression, anxiety, or autism. However, scientists have devised behavioral paradigms to measure phenotypes that could reflect some aspects of these mental states. For instance, one measure of anxiety-like states is based on the open field test mentioned above. When mice are placed in an open field, their natural tendency is to stay near its periphery, presumably because mice are more susceptible to predation when they are exposed than when they are hidden. Normal mice venture into the center occasionally. Mice that visit the center with reduced frequency can be considered anxious. Another commonly used anxiety assay is the elevated plus-maze (Figure 13–52B). Here, a mouse (or a rat) is placed onto a four-arm maze that is elevated from the floor, with two arms covered on the sides with high walls and the other two arms exposed. Although mice prefer the closed arms, normal mice also spend a fraction of time exploring the open arms. Putatively anxious mice spend significantly less time in the open arms. Administering anxiolytic drugs such as benzodiazepines (see Section 11.17) can increase the tendency of animals to enter the center of an open field or the open arms of an elevated plus-maze, whereas anxiogenic drugs do the opposite, suggesting that these behavioral assays test phenomena that may be related to anxiety in humans.

Assays have also been designed to test social behaviors; these assays are often used in studies of mouse models of human autism-spectrum disorders. For example, a three-chamber social interaction assay for mice (Figure 13–53) resembles the partner preference assay for prairie voles discussed in Section 9.24. Here, a mouse is first allowed to habituate in a three-chamber apparatus where it can move freely between chambers. Then, a small wire cage is placed in each of the two side chambers, one containing a live mouse and the other empty. Most strains of mice spend more time in the chamber containing the conspecific than in the chamber with the empty cage. The relative time spent in each of the two chambers can be a measure of sociability; a reduction of time in the side chamber with the mouse corresponds to reduced sociability. An additional test of social novelty is to place one mouse in each of the side chambers, one familiar to the mouse being tested and the other unfamiliar. Mice usually spend more time investigating the unfamiliar mouse, so the relative time spent in the two chambers can be a measure of preference for social novelty (Figure 13–53). To validate that the observed behavior is social in nature, control experiments with non-social objects or odors

Figure 13–53 Three-chamber social interaction assays. (A) Schematic drawings of testing procedures. Prior to social interaction, a test mouse becomes habituated to an empty three-chamber environment in which it can move freely between the chambers (top). Next, additional mice can be added to investigate social interactions. The number of visits and total time spent in the chamber containing a live mouse compared to the chamber with an empty cage can be used to assess sociability (middle). The number of visits and total time spent in the chamber containing a familiar mouse (orange) compared to the chamber with an unfamiliar mouse (brown) can be used to assess social novelty (bottom). (B) Photograph of a social novelty assay being conducted in a three-chamber social interaction apparatus. (From Moy SS, Nadler JJ, Perez A et al. [2004] *Genes Brain Behav* 3:287–302. With permission from John Wiley & Sons.)



must be conducted in an identical experimental setup; these control experiments can exclude possible confounding variables, such as the failure to recognize or discriminate between different odors or objects.

Behavior is sensitive to a large number of factors, so it cannot be overemphasized that in performing each of the assays described above, care must be taken to control the experimental conditions (discussed in Section 13.28) and thereby minimize unintended influences on behavior by factors not being tested. Proper control groups should always accompany the experimental group. Multiple behavioral assays are often used in combination to identify the effects of specific perturbations on the nervous systems, as defects in more complex assays can have multiple interpretations. For instance, if an animal exhibits a defect in the auditory fear conditioning assay, the animal might have a defect in hearing, in sensing electrical shocks, or in associating these two events (that is, learning). Control experiments on hearing and shock sensitivity are necessary in order to determine whether or not the defect is related to learning. The interpretation of behavioral results also depends on the nature of experimental perturbation. For instance, if the experimental manipulation is to knockout a gene of interest or to apply a systemic pharmacological agent, then in principle the entire nervous system could be affected and could contribute to the observed behavioral phenotypes. If the experimental manipulation is a conditional knockout of a gene in specific neuronal types or the activation and silencing of specific brain regions or neuronal populations, then the behavioral phenotypes reflect alterations of the manipulated brain regions or neuronal populations.

SUMMARY AND PERSPECTIVES

Neurobiology research has been carried out in diverse animal models. These animal models have been chosen for the technical ease with which they can be studied (for example, their neurons recorded, genes manipulated, or explants studied *in vitro*), for the special faculties they exhibit, for the sophistication of their behavior, or for their resemblance to humans. Indeed, recent revolutions in genomics and non-invasive imaging, combined with a long history of medicine and experimental psychology, have made humans an increasingly attractive organism to study neurobiology. Whereas many molecular and cellular processes in the operation of nervous systems are well conserved across animals, we do not yet know

the extent to which information processing at the circuitry and systems levels follows general principles that apply across nervous systems of varying complexity. Regardless, studies in diverse animal models enrich our understanding of the diversity of life and the evolution of the nervous system.

All neurobiological processes are ultimately the direct or indirect consequences of gene actions. The two most widely used molecular-genetic manipulations in neurobiological research are the disruption of endogenous genes and the expression of transgenes. Gene disruption can be performed in the context of a forward genetic screen or by utilizing reverse genetic methods such as homologous-recombination-mediated knockout or RNAi-mediated knockdown. Many methods have been employed for expressing transgenes with sophisticated spatiotemporal controls. Both gene disruption and transgene expression can reveal how genes function in specific neurobiological processes. Transgene expression is also instrumental in providing genetic access to specific neuronal populations for the purposes of investigating their anatomical organization, their physiological properties, and the functional consequences of manipulating their activity. Future challenges include not only expanding access to specific cell types with increasing precision in genetic models such as flies and mice, but also broadening genetic tools to other animal models.

Classic anatomical methods such as cell staining, axon tracing, and single-cell labeling have provided foundations for our present understanding of how the nervous system is organized. A deeper appreciation of this organization requires building further connections between molecules, neurons, and the brain. One frontier is the fine-structural analysis of how individual molecules form complexes in different parts of the neuron and the dynamics of these molecular complexes; this will deepen our understanding of the mechanisms by which individual neurons function. Another frontier is the construction of connection diagrams for complex nervous systems, ultimately to the resolution of individual synapses; this will serve as a blueprint to decipher the information processing principles of neural circuits.

Extracellular, intracellular, and patch recordings of electrical activity have contributed fundamentally to our understanding of how information flows within individual neurons, across synapses, in small circuits, and over large networks. These electrophysiological methods have more recently been supplemented by optical imaging, which enables the simultaneous recording of many neurons of specific cell types and over longer periods using genetically encoded Ca^{2+} indicators. Future challenges include combining the sensitivity and temporal resolution of electrophysiological recording with the breadth, cell-type specificity, and duration of optical imaging, to expand the methods available to record neuronal activity in behaving animals, and to develop a conceptual framework to convert rich data into an understanding of the principles of neural circuit operation and the neural basis of behavior.

A crucial approach that links neuronal activity, circuit function, and behavior is the manipulation of neuronal activity with precise spatiotemporal control. Classic lesion, pharmacology, and electrical stimulation methods to inactivate and activate brain regions have been supplemented in recent years with sophisticated control of neuronal activity by light, heat, and chemicals. New tools developed in recent years, in particular the ability to control neuronal activity with light, have made it possible to activate and inactivate genetically defined neuronal populations at spatiotemporal scales that begin to match those of neuronal signaling.

Ultimately, the combination of methods we have studied in this chapter, such as deleting or misexpressing specific genes in defined cell types, measuring, activating, and silencing the activity of specific neuronal populations with high spatiotemporal precision, and quantitative analysis of animal behavior, will help establish causal links between genes, neurons, circuits, and animal behaviors. These links will deepen our understanding of the nervous system in health and disease. With the rapid pace of tool developments and their wide applications, neurobiology research has never seen a more exciting time.

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