12 Synaptic Electrophysiology of the *Drosophila* Neuromuscular Junction

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ABSTRACT

Chemical synaptic transmission is an important means of neuronal communication in the nervous system. On the arrival of an action potential, the nerve terminal experiences an influx of calcium ions, which in turn trigger the exocytosis of synaptic vesicles (SVs) and the release of neurotransmitters into the synaptic cleft. Transmitters elicit synaptic responses in the postsynaptic cell by binding to and activating specific receptors. This is followed by the recycling of SVs at presynaptic terminals. The *Drosophila* larval neuromuscular junction (NMJ) shares many structural and functional similarities to synapses in other animals, including humans. These include the basic feature of synaptic transmission, as well as the molecular mechanisms regulating the synaptic vesicle cycle. Because of its large size, easy accessibility, and the well-characterized genetics, the fly NMJ remains an excellent model system for dissecting the cellular and molecular mechanisms of synaptic transmission. In this chapter, we describe the theory and practice of electrophysiology as applied to the *Drosophila* larval NMJ preparation. First, the basics of membrane potentials are introduced, with an emphasis on the resting potential and synaptic potential. Second, the equipment and methods required to set up an electrophysiology rig are presented. Third, protocols are provided that explain how to use the rig to record from muscles, determine the passive membrane properties of the muscle (i.e., input resistance and time constant), record synaptic potentials both intracellularly and extracellularly, detect synaptic currents by two-electrode voltage clamp, perform quantal analysis, and study short-term synaptic plasticity.

INTRODUCTION

Neurons use electrical and chemical signals to receive, process, and communicate information. The basic currency of signaling by excitable cells (neurons and muscles) is electrical current. Accordingly, electrophysiology is the study of the electrical properties of cells, and we draw on the theoretical background of electrical phenomenon to help understand the behavior of the nervous system. By analogy, the cell membrane is similar to a simple resistor-capacitor (RC) circuit (see Boxes 1 and 2). In an electronic circuit, a battery (voltage source) is connected via conductors (wires) to a resistor and a capacitor arranged in parallel, with a return wire to the battery that completes the circuit. Relatively simple physical relationships describe the flow of electrons through the wires. The cellular equivalents to an electronic circuit are the resting membrane potential (see below), which acts as the battery; the extracellular and intracellular fluids, which act as the low-resistance conductors; ion channels within cell membranes, which act as the resistors; and the phospholipid bilayer, which acts as a capacitor. An important difference between electronic and cellular circuits is that electrons are the charged particles that flow through an electronic circuit, whereas in a biological circuit a variety of cations and anions (e.g., Na^+, K^+, Ca^{2+}, Cl^-) generate membrane currents.

Drosophila has been used to great advantage as a model system for elucidating the molecular and genetic mechanisms that underlie bioelectric and biochemical signaling. The introduction of the larval NMJ as an experimental preparation established a system that permits relatively easy access to large, electrically excitable cells (Jan and Jan 1976a,b). Other electrical techniques applied to *Drosophila* include electroretinograms (Hotta and Benzer 1969; see Chapter 14), extracellular recording from the

BOX 1. ELECTRICAL TERMINOLOGY

Charge (Q): Electric charge is a fundamental property of subatomic particles. By convention an electron has a charge of –1 and a proton has a charge of +1. In general, particles with the same charge repel each other, and particles with opposite charges attract each other. The unit of charge is known as the coulomb (C) and 1 C is equal to 6.25×10^{18} charges. In biological cells, the charge is carried by ions such as Na^+, K^+, Ca^{2+}, and Cl^-.

Current (I): The directional movement of charged particles is electric current, such as electrons moving through a metal conductor. Current is measured in amperes (A); 1 A is the movement of 1 C of charge per second.

Voltage (V): Also known as electrical potential or potential difference, voltage refers to the difference in electric potential between two points within an electric field, such as a circuit. By definition, potential difference is the amount of work required to move a unit of charge. A 1-V potential difference requires 1 J of work to separate 1 C of charge.

Conductance (g) or Resistance (R or Ω): The flow of current between two points is called conductance; the greater the conductance, the easier it is for current to flow. Resistance is the inverse of conductance; the greater the resistance, the harder it is for current to flow. Conductance is measured in siemens (S) and resistance is measured in ohms (Ω). The biological equivalent of conductance is the permeability of ion channels. The higher the conductance of a membrane, the greater the number of open ion channels.

Capacitance (C): If two conducting materials are separated by an insulating layer, a capacitor is formed. This could be two metal plates separated by a nonconducting material; in the cell, the lipid bilayer of the membrane separates the conducting intracellular and extracellular fluids. If there is an excess of positive charge on one of the conductors and an excess of negative charge on the other, a potential difference develops between the two conductors. Capacitance is measured in farads (F): A 1-F capacitor will have an electrical potential of 1 V when +1 C of charge is on one conductor and –1 C is on the other conductor. An electric current will change the electric potential of a capacitor by moving charge from one conductor to the other. This is called a capacitive current (I_C). Lipid bilayers serve as biological capacitors.

BOX 2. BASIC EQUATIONS AND RC CIRCUITS

Ohm's law: Ohm's law describes the relationship between current, voltage, and conductance (resistance):

$$I = gV.$$

It states that electric current is a product of conductance and the voltage difference applied to the conductor. Ohm's law can also be written as

$$V = IR,$$

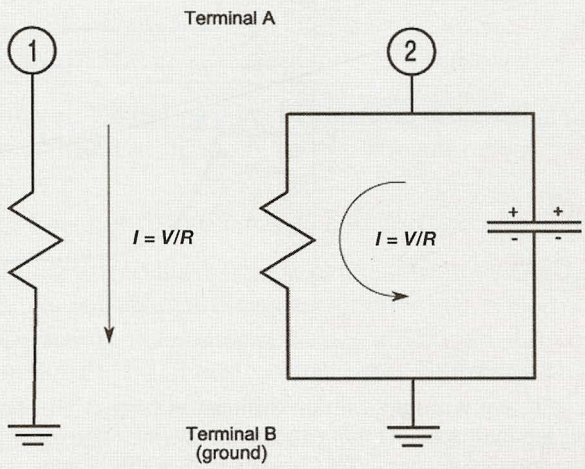
which is the inverse relationship (and the one most often used in our discussions) and states that the voltage between two points is equal to the product of the current and resistance.

Capacitance: The definition of capacitance is given by

$$C = Q/V,$$

which states that capacitance is the ratio of the charges on the conductors to the voltage between the conductors.

An RC circuit:



In circuit (1), there is only a resistor in the conductive path between the two terminals. If an electric potential exists between the two terminals, then current will flow according to Ohm's law. In circuit (2) there is a capacitor in parallel to the resistor. In this case, if there is an electric potential between the two terminals, current will also flow, but the capacitor must be charged or discharged before current will flow through the resistor.

We therefore need to know the *rate* of change of the electric potential of the capacitor,

$$C = \frac{Q}{V}.$$

The rate of change of the voltage for a capacitive current is found from the time derivative,

$$\frac{\partial V}{\partial t} = \frac{I_C}{C},$$

and the capacitive current is therefore

$$I_C = C\frac{\partial V}{\partial t}.$$

(Continued on following page.)

BOX 2. *(CONTINUED)*

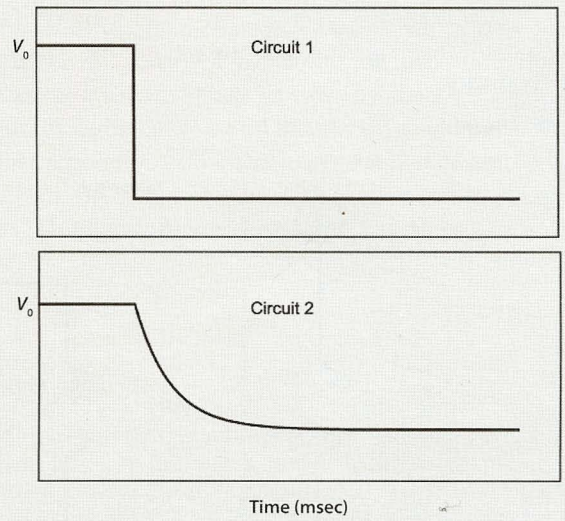
Therefore, to determine the total amount of current flowing in circuit (2), we can add the current through the resistor and the capacitor:

$$I_{\text{total}} = I_C + I_i,$$

$$I_{\text{total}} = C\frac{\partial V}{\partial t} + \frac{V}{R}.$$

From this relationship we can see that if the voltage is constant, then $I_C = 0$; when the voltage changes, capacitance is important.

How does capacitance affect the behavior of a circuit? This can be seen easily by measuring voltage differences across the two terminals in the above circuits when the voltage changes:



Initially, we will start with an arbitrary voltage (V_0) at time 0. If the voltage is instantly changed, we will see for circuit (1) that the voltage we measure also changes instantly, going in a downward direction. However, for circuit (2) we see that the change is not instant but rather takes some period of time to settle down to the new level. This is the property the capacitance adds—it slows down the response of the circuit to changes in voltage.

The relationship between voltage and time in an RC circuit is given by

$$V = V_0\, e^{-t/R},$$

in which t is time, R is the resistance, and C is the capacitance of the circuit. The component of the equation RC is also known as the time constant or tau (τ) and this is an important parameter for neurophysiology. The time constant is equal to $1/e$ or ~36% of V_0.

Functionally, the membrane time constant reflects how fast a neuron or muscle responds to a synaptic signal. For a given cell, membrane capacitance (C) does not change, and therefore membrane resistance determines the time constant. The more channels open (low R), the faster the response becomes.

giant fiber pathway (Tanouye and Wyman 1980; see Chapter 13), and the use of dissociated cell cultures (Wu et al. 1983) to gain access to the relatively small neurons. More recently, in situ electrical recordings from the central nervous system of the embryo, larvae, and adults (Baines and Bate 1998; Rohrbough and Broadie 2002; Wilson et al. 2004; see Chapters 17 and 18), as well as adoption of optophysiology techniques (Karunanithi et al. 1997; Macleod et al. 2002; Marek and Davis 2002; Wang et

al. 2004; Lima and Miesenbock 2005; Rasse et al. 2005; see Chapters 19–21), have expanded the repertoire of techniques available to challenge and measure physiological processes used by neurons.

THE RESTING MEMBRANE POTENTIAL

The physical basis of electrical signaling in cells is the resting membrane potential. Simply stated, there is a charge difference across cell membranes because the inside of a cell is electrically negative when compared with the outside. Understanding the origins of this electrical potential is the foundation for learning how changes in the membrane potential constitute signaling.

How do we know that cells have a resting membrane potential? Julius Bernstein in 1902 was among the first to *predict* that a cell would have a negative resting potential. Direct evidence of cell resting potentials, however, was not available until electrophysiologists measured it in squid giant axons in the 1940s (Hodgkin and Huxley 1945; Hodgkin and Katz 1949). Today, using well-honed techniques, it is relatively straightforward to gain electrical access to the inside of most cells. Glass micropipettes, having a tip diameter on the order of several hundred nanometers (for typical intracellular recording pipettes) are carefully inserted through the plasma membrane and into the cell lumen. Using the appropriate electronic hardware connected to the micropipette, cell resting potentials can be accurately measured and are typically ~50–80 mV less than the outside.

How Is the Membrane Potential Generated?

Two basic factors determine a cell's resting potential: unequal distribution of ions between the inside and outside of a cell and the selective permeability of cell membranes.

Unequal Distribution of Ions between the Inside and Outside of a Cell

The ionic composition of the extracellular fluid is different from that in the intracellular fluid. The extracellular fluid usually contains Na^+ as the major cation, which is electrically balanced by a combination of Cl^- and HCO_3^- ions to maintain electrical neutrality. Smaller contributions to the extracellular charge come from K^+, Ca^{2+}, and Mg^{++}, which again are balanced by Cl^- and HCO_3^- ions.

The major cation of the intracellular fluid is K^+, with a smaller contribution from Na^+. Intracellular free $[Ca^{2+}]$ is usually near 0, because Ca^{2+} are bound to buffers, pumped into intracellular stores, or pumped out of the cell. Intracellular fluid has a greater abundance (compared with the outside of the cell) of large impermeable anions, chiefly proteins, DNA, and RNA. These negative charges are balanced by intracellular K^+, and it is for this reason that there is an imbalance of K^+ across the cell membrane.

Selective Membrane Permeability

The cell membrane is a phospholipid bilayer with associated peripheral and transmembrane proteins. The lipid bilayer portion is impermeable to most things, noteworthy exceptions being O_2, CO_2, and steroid compounds. Charged ions such as K^+ and Na^+ do not readily cross the bilayer. They move in and out of the cell with the aid of transmembrane protein channels and transporters. Among these proteins is a family of ion channels, known as leakage channels, that primarily transport K^+ (Goldstein et al. 1996), making the cell membrane relatively permeable to potassium. The membrane, however, remains relatively impermeable to other ions when it is in the resting state.

The Physical Basis for Membrane Potential

We see now that the resting membrane potential is a function of the ionic composition of the intracellular and extracellular milieus, the charge difference that results, and the selective movement of ions across the membrane with the assistance of ion-selective protein channels and transporters. To

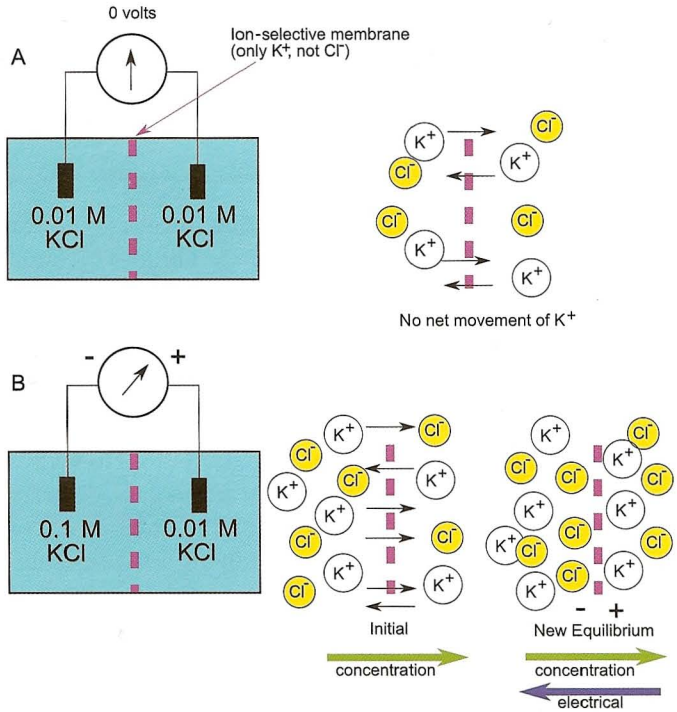


FIGURE 1. Physical basis of the membrane potential. (*A*) In a tank two KCl solutions are separated by a membrane that is only permeable to K^+. A voltmeter measures the electrical difference between the two solutions. If the solutions on each side of the membrane are equal, there is no net movement of ions, so the voltmeter reads 0 V. (*B*) If the solution on the left side is increased to 0.1 M KCl, then there is an initial concentration gradient driving K^+ and Cl^- from left to right. Because the membrane is permeable only to K^+, some K^+ ions will be separated from counterbalancing Cl^- ions at the membrane. This charge separation instantly creates an electrical force, which is equal to but in the opposite direction from the concentration gradient, establishing a new equilibrium. The voltmeter now indicates there is an electrical potential difference between the two solutions.

help us visualize how these factors generate a potential, consider a tank divided into two compartments by a membrane that is only permeable to K^+ (Fig. 1). Each compartment is filled with 0.01 M KCl, and a voltmeter is used to measure the electrical difference across the membrane (Fig. 1A). The meter indicates that there is no potential difference—that is, there is zero voltage across the membrane. This illustrates that selective permeability without a chemical gradient is insufficient to generate a voltage difference across the membrane.

Next replace the solution in one of the compartments with a 0.1 M KCl solution. Now there is an unequal distribution of ions across the membrane and a concentration gradient exists that could drive K^+ and Cl^- from high concentration to low. If the membrane was equally permeable to K^+ and Cl^- (not shown in Fig. 1), then all of the ions in the two compartments would redistribute and eventually reach equal concentrations on both sides of the membrane. At this point, the voltage difference across the membrane would again be 0. Thus, having a concentration difference and unrestricted permeability will not generate a potential difference.

Let us now examine the situation when *the membrane is only permeable to* K^+ and when there is a concentration gradient for KCl across the membrane (Fig. 1B). Following the second law of thermodynamics, K^+ will start to diffuse across the membrane, but the counterbalancing negatively charged Cl^- ions cannot follow these positive charges. A *separation of charge* therefore occurs at the membrane interface. There are now two forces at play: the K^+ concentration gradient moving ions in one direction and a newly developed electrical gradient, which opposes the concentration gradient, because the negatively charged ions attract the positively charged ions. A new equilibrium condition is quickly established in which the force of the concentration gradient is exactly balanced by an opposing electrical force. This electrical force is the membrane potential of this artificial cell. Therefore, both unequal distribution of ions and selective permeability are required to generate a membrane potential.

Biological systems develop a similar separation of charge across the plasma membrane and this is what we measure as the membrane potential. The K^+ leakage channels are the major contributor to the resting permeability of the plasma membrane, but the ionic and cellular environment within and around a cell is obviously more complicated than the above illustration. Many more species of ions are involved, and, importantly, the plasma membrane is permeable to K^+ and at smaller permeability values to other ions as well.

Equilibrium Potentials of Individual Ions

The simplified experiment in the previous section illustrates that having unequal concentrations of ions across a semipermeable membrane gives rise to an electrical force. If the concentrations of ions are known, then the electrical force that is required to balance the force of the concentration gradient can be predicted using the Nernst equation:

$$E_{\text{ion}} = \frac{RT}{zF} \ln \frac{[\text{ion outside cell}]}{[\text{ion inside cell}]},$$

in which E_{ion} is the equilibrium potential for the ion, R is the universal gas constant, T is absolute temperature, z is the valence of the ion, and F is Faraday's constant. This equation is often simplified for monovalent cations when the cell is at 20°C:

$$E_{\text{ion}}\ (\text{mV}) = 58 \log \frac{[\text{ion outside cell}]}{[\text{ion inside cell}]}.$$

Note the change from natural logarithm to logarithm base-10. In our example above, if the 0.1 M KCl solution is considered to be inside the cell and the 0.01 M KCl solution to be outside the cell, then $E_{K^+} = -58$ mV. This relationship can be used to calculate the equilibrium potential for any ion. The reader will see that this value is independent of permeability. Cations that are at a higher concentration inside the cell will have a negative equilibrium potential, whereas cations that are at a higher concentration outside the cell will have a positive equilibrium potential. For example, typically $[Na^+]_{\text{outside}} = 100$ mM, whereas $[Na^+]_{\text{inside}} = 5$ mM, so the equilibrium potential for sodium is $E_{Na^+} = +75$ mV.

The Resting Potential for an Entire Cell

Cells are in the presence of many different ions, both inside and outside the cell. To understand the resting potential for an entire cell, we need to consider the concentrations of those ions and their relative permeability. The major contributing cations are Na^+ and K^+, and Cl^- is the major anion. The Goldman–Hodgkin–Katz (GHK) equation describes the relationship between the voltage of the cell membrane (V_m), the permeability (P), and the concentrations of ions as

$$V_m = \frac{RT}{zF} \ln \frac{P_K[K^+]_{out} + P_{Na}[Na^+]_{out} + P_{Cl}[Cl^-]_{in}}{P_K[K^+]_{in} + P_{Na}[Na^+]_{in} + P_{Cl}[Cl^-]_{out}}.$$

Note the reversal of terms for Cl^-, which reflects the negative valence of the chloride ion. Once again converting to logarithm base-10 and assuming a temperature of 20°C, the relationship becomes

$$V_m = 58 \log \frac{P_K[K^+]_{out} + P_{Na}[Na^+]_{out} + P_{Cl}[Cl^-]_{in}}{P_K[K^+]_{in} + P_{Na}[Na^+]_{in} + P_{Cl}[Cl^-]_{out}}.$$

Much of the work to derive this relationship was performed on the squid axon, in which it was found that at rest the permeabilities of K^+:Na^+:Cl^- are 1:0.03:0.1. That is, in the resting squid axon, the permeability of K^+ is 10 times greater than that of Cl^- and 33.3 times greater than that of Na^+. If $[K^+]_{out} = 10$ mM, $[K^+]_{in} = 400$ mM, $[Na^+]_{out} = 460$ mM, $[Na^+]_{in} = 50$ mM, $[Cl^-]_{out} = 540$ mM, $[Cl^-]_{in} = 40$ mM, then

$$V_m = 58 \log \frac{1(10) + 0.03(460) + 0.1(40)}{1(400) + 0.03(50) + 0.1(540)}$$

$$= -70 \text{ mV}.$$

Comparing the simplified version of the GHK equation with that of the Nernst equation, one sees that the resting membrane potential of the cell will always be close to the equilibrium potential of the most permeable ion. In this example, because K^+ is the most permeable ion, V_m approaches E_{K^+} with smaller contributions from the other ions.

The Na^+/K^+ ATPase

When the cell membrane is selectively permeable to a single ion (e.g., K^+), passive diffusion of K^+ down its chemical gradient first generates a negative potential inside. This electrical gradient works in favor of keeping K^+ inside and will eventually become strong enough to completely stop the net efflux of K^+. To reach this equilibrium state, very few ions are required near the membrane to maintain a separation of charges compared with the number of ions that are present in solution, such that the $[K^+]$ on both sides of the membrane remains unaltered. At equilibrium, the net flow of K^+ is 0 and no energy is required to maintain equilibrium. If, on the other hand, the cell is permeable only to Na^+, then the equilibrium potential will be completely different. Unlike K^+, which sets the membrane potential to E_{K^+} (~ –90 mV), Na^+ will bring the membrane potential to its own equilibrium potential, E_{Na^+}, which is typically more depolarized (~+50 to +75 mV).

A more complex and dynamic situation arises, however, when a cell membrane is permeable to more than one ion. In a cell whose membrane is predominantly permeable to K^+, a slight influx of Na^+ will disturb the equilibrium state of K^+. This creates a severe problem for maintaining Na^+ and K^+ distributions across the membrane. One can imagine that each Na^+ influx will lead to a K^+ efflux, and this vicious tug of war is impossible to stop without introducing a "peacemaker." In biological cells, this peacemaker is the Na^+/K^+ pump, which pumps Na^+ out and K^+ in, such that $[Na^+]$ and $[K^+]$ do not change over time. There is a price, however, for maintaining the ion concentrations because the Na^+/K^+ pump has to accomplish its task by going against each ion's electrochemical gradient. A significant amount of cellular energy, in the form of adenosine triphosphate (ATP), is consumed in this process. The Na^+/K^+ pump is also an ATPase. In most cells, it pumps in two K^+ ions in exchange for three Na^+ ions out, thereby also making a direct contribution to the generation of the resting potential.

Membrane Permeability and Ion Channels

If you understand the fundamental principle that the membrane potential of the cell is primarily a function of ion permeability (because ion concentrations are relatively stable), then understanding neural signaling is made much easier. What controls ion permeability? The K^+ leakage channel can be regarded as an unregulated K^+ pore that allows K^+ to flow in or out of the cell. However, there are also a host of ion channels that alter their permeabilities to allow ions to cross the cell membrane. There are two broad categories of such "regulated" ion channels, defined by what regulates their opening and closing: voltage-gated channels (see Chapter 17) and ligand-gated channels.

As the name implies, it is the membrane voltage that controls the opening and closing of voltage-gated channels. Most of these channels are closed at the resting membrane voltage (V_m), but they open up when V_m is depolarized (becomes more positive). These channels usually allow only one specific ion to cross through the channel. As such, there are voltage-gated Na^+ channels, voltage-gated K^+ channels, and voltage-gated Ca^{2+} channels. Within each category there are a variety of channel types, which confer specific properties on the cells that express them.

Ligand-gated channels open when a ligand binds to a receptor site on the channel protein. At the fly NMJ, these are neurotransmitter (glutamate) receptors (indeed the phrases ligand-gated channel and neurotransmitter receptors can be used interchangeably). Unlike voltage-gated channels, ligand-gated channels are often permeable to more than one ion. For example, Na^+, K^+, and Ca^{2+} can pass through the larval NMJ glutamate receptor once it is opened by the transmitter glutamate (Jan and Jan 1976a).

Action Potentials, Synaptic Potentials, and Driving Force

Knowing that there are a variety of regulated ion channels on the plasma membrane of a cell, let us return to our general relationship between membrane permeability and membrane potential. At rest, we know that because K^+ is the most permeable ion, V_m is set slightly more positive than E_{K^+}. What would happen if suddenly the membrane permeability of Na^+ became substantially greater than that of K^+ (i.e., $P_{Na^+} >> P_{K^+}$)? Because our rule of thumb is that the membrane potential is close to the equilibrium potential of the most permeable ion, then we would predict that $V_m \approx E_{Na^+}$. Using the values from the example above, $[Na^+]_{out}$ = 460 mM, $[Na^+]_{in}$ = 50 mM, and using the Nernst equation, E_{Na^+} = +56 mV. If under the new condition of high Na^+ permeability, it is assumed that $P_{K^+}:P_{Na^+}:P_{Cl^-}$ is 1:15:0.1, then, using the GHK equation, the cell's membrane potential would be +44 mV.

This is exactly what happens in a neuron during the rising phase of an action potential. An external stimulus depolarizes the neural membrane, and if the stimulus is strong enough, voltage-gated Na^+ channels open to greatly increase P_{Na^+}, which shifts the membrane potential to a positive value. The voltage-gated Na^+ channels do not stay open long (a few milliseconds), as P_{Na^+} returns to the prestimulus level and the membrane potential also returns to the resting level. With a brief delay, the stimulus also activates voltage-gated K^+ channels. This results in an efflux of K^+ ions and a rapid repolarization of the membrane. Therefore, during the falling phase of an action potential, as the Na^+ channels inactivate and the K^+ channels open, the membrane is once again most permeable to K^+ and the membrane potential once again returns to a voltage near E_{K^+}.

A similar change in ion permeability and membrane potential occurs at the synapse, except that the initiating event is the binding of neurotransmitter molecules to the neurotransmitter receptor. At the *Drosophila* larval NMJ, the excitatory neurotransmitter, glutamate, is released into the synaptic cleft and binds to glutamate receptors (GluRs) in the postsynaptic membrane of the muscle, causing an ion channel to open. As these ligand-gated channels are not specific to one ion, it is more difficult to predict what V_m the membrane approaches on channel opening. By analogy to the voltage-gated channels, the V_m will change according to the *mixture* of equilibrium potentials of the permeable ions.

In synaptic physiology this equilibrium potential is given the name "reversal potential" (V_{rev}), which refers to the membrane potential at which the net receptor/channel current is 0. At this potential, Na^+ influx and K^+ efflux are equal but in opposite directions. The current will flow either inward or outward if the membrane potential deviates away from the reversal potential. For glutamate receptors at the fly NMJ, the reversal potential is ~–10 mV. Practically this means that during synaptic transmission the V_m of the muscle cell will move from its resting value toward V_{rev}. Thus, Na^+ influx dominates K^+ efflux when the membrane potential is hyperpolarized below –10 mV. Under voltage-clamp conditions, it is possible to reveal that K^+ efflux overpowers Na^+ influx when the membrane is depolarized beyond –10 mV. One important practical application of the concept of the reversal potential in synaptic studies is that the peak amplitude of the excitatory junction potential (EJP; the evoked postsynaptic potential in the fly muscle) will always be below but never reach its reversal potential, no matter how much transmitter is released. This phenomenon is known as a "ceiling" effect. A detailed explanation of V_{rev} can be found in specialized neurophysiology textbooks.

Finally, we introduce the concept of an ionic electromotive force (EMF_{ion}) (also called driving force). Essentially, this is the difference between the equilibrium potential for an ion (E_{ion}) and the membrane potential (V_m) at any given point in time:

$$EMF_{ion} = V_m - E_{ion}.$$

According to Ohm's law, an ion current (I_{ion}) with a conductance G_{ion} can be expressed as

$$I_{ion} = G_{ion}(V_m - E_{ion}).$$

The importance of this relationship can be seen by considering the situation in which $V_m = E_{ion}$; that is, $EMF_{ion} = 0$. Under such conditions even if an ion channel for that ion is open there will be no net movement of the ion (I_{ion} =0), because there is no driving force.

On the other hand, if V_m is far from E_{ion}, then there will be a large driving force for that ion. This is the common situation for Na^+ at the resting membrane potential of most cells. V_{rest} for most cells

is a negative value (e.g., –70 mV), whereas E_{Na^+} is usually a positive value (e.g., +50 mV). Therefore, in this example the driving force for Na^+ is 120 mV. If Na^+ channels are fully open under these conditions, then there is a large force that will push Na^+ through the channel. In contrast, Na^+ influx at the peak of an action potential (e.g., +48 mV) will be minimal even if all of the Na^+ channels are open. This is because the driving force (2 mV) is very small when the peak of the action potentials closely approaches E_{Na^+}. Hence, ion permeability, as well as the driving force, determines the impact of an ion on the membrane potential.

The concepts of relative permeability and driving force and their dynamics can be illustrated by a "spring" model, as shown in Figure 2. At rest, the thick and thin springs represent a high permeability for K^+ and a low permeability for Na^+, respectively. Because the resting potential is close to E_{K^+}, the driving force (the length of the spring) for K^+ is much smaller (shorter) than the driving force for Na^+. Although the equilibrium potentials for Na^+ and K^+ do not usually change for a given cell, both the permeability and driving force can be highly dynamic. For example, the Na^+ spring becomes much stronger relative to the K^+ spring at the peak of an action potential. At this point, the driving force for Na^+ reaches a minimal level, whereas it is at its largest for K^+. Readers should keep in mind that after a brief delay the K^+ spring strengthens dramatically, whereas the Na^+ spring weakens during repolarization of the action potential (not shown). This spring model also applies to synaptic potentials. The example shown here is an excitatory postsynaptic potential (EPSP) mediated by nonselective cation channels, such as the nicotinic acetylcholine receptor at mammalian NMJs and the glutamate receptor at fly NMJs. At the reversal potential, when the peak of the EPSP approaches –10 mV, the springs for Na^+ and K^+ are represented as equally strong. Hence, it is the relative strength of the springs—that is, the permeability of permeable ions—that determines the membrane potential.

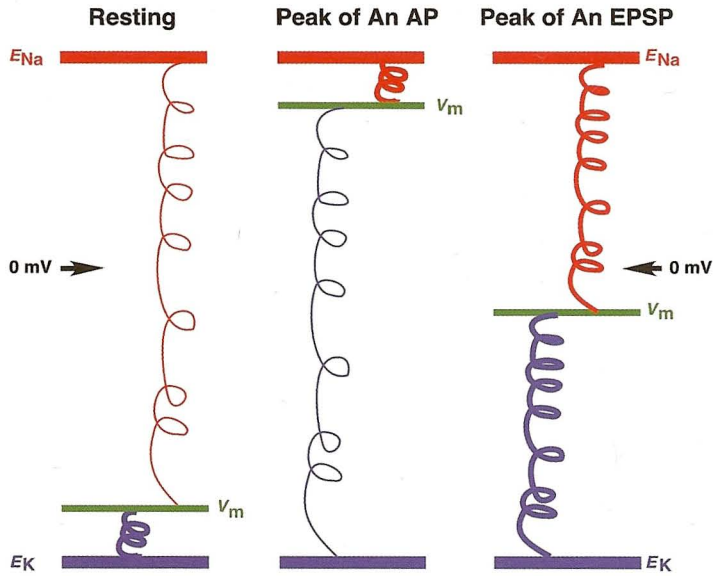


FIGURE 2. A spring model of relative permeability, driving force, and their dynamics. This cartoon models the relative permeability, the driving force, and membrane potentials of a typical cell at three different states. The thickness of the spring correlates positively with the permeability of an ion channel, whereas the length of the spring represents the driving force. At rest, the dominant strength of the K^+ spring (P_{K^+}) brings the membrane potential (V_m) toward E_{K^+}. But the driving force for Na^+ is much higher than that for K^+. The equilibrium potentials for Na^+ (E_{Na^+}) and K^+ (E_{K^+}) rarely change for a given cell. However, both the permeability and driving force can be highly dynamic. During the peak of an action potential, the spring strength and length reverse for K^+ and Na^+ such that P_{Na^+} overpowers P_{K^+}, thereby bringing the membrane potential near E_{Na^+}. In a synaptic potential mediated by nonselective cation channels such as the glutamate receptor at the fly NMJ, the spring is usually equally strong at the reversal potential. Hence, the peak of the EPSP approaches –10 mV. The fundamental take-home message is that it is the strength of the spring (i.e., relative permeability) of permeable ions that determines the membrane potential.

SYNAPTIC TRANSMISSION

Synaptic transmission is the process by which neurons secrete neurotransmitter molecules from the nerve terminal onto target cells. Synaptic physiology has been studied for a long time and remains a favorite topic among neuroscientists. Although a complete review of synaptic transmission is beyond the scope of this chapter, highlighting some of the achievements and contemporary advancements is worthwhile. Synaptic physiology began when Bernard Katz and his colleagues recorded the first synaptic response at the frog NMJ in the 1950s using an intracellular microelectrode. Their important studies, summarized in an elegant book (Katz 1966), provided the essential elements for our understanding of synaptic transmission. Their major findings were as follows.

1. There appeared to be a spontaneous release of neurotransmitter.
2. Action potentials arriving at the nerve terminal caused the release of neurotransmitter.
3. Transmitter release required that calcium be in the extracellular fluid at the time the action potential arrived.
4. Neurotransmitter is released in defined units, which Katz called quantal units.
5. The units appeared to be the same as the spontaneously released units (Fig. 3).

Indeed in subsequent years much of the effort in synaptic physiology has been to test, retest, extend, and expand on these early ideas. A recent study appears to challenge the basic tenet of Katz's quantal theory (Fredj and Burrone 2009).

Nearly simultaneous with the development of Katz's data on synaptic physiology, the first glimpses of the nerve terminal ultrastructure were obtained with an electron microscope. These initial views of the synapse revealed the presence of small clear vesicular structures in the nerve terminal. This observation naturally gave rise to the notion that Katz's quantal units were in fact the synaptic vesicles. This remained a controversial notion, however, until the important work of Heuser, Reese, and others, who showed a correspondence between quantal release as measured by electrophysiology and vesicular release as observed with the electron microscope (Heuser et al. 1979).

In the late 1980s studies began to reveal the molecular nature of synaptic transmission. Synaptophysin, VAMP (vesicle-associated membrane protein)/synaptobrevin, and SNAP-25 (synaptosomal-associated protein 25) were among the first synaptic proteins to be identified and characterized (Trimble et al. 1988; Oyler et al. 1989; Sudhof et al. 1989). This was followed by the identification of synaptotagmin (Perin et al. 1990), syntaxin (Bennett et al. 1992), and Rab3 (Fischer et al. 1990) in the early 1990s (also see reviews by Jahn and Sudhof 1994; Hay and Scheller 1997; Chen and Scheller 2001; Jahn et al. 2003). Research into the molecular nature of cellular processes was (of course) not limited to neuroscience, and studies on neural proteins soon became greatly influenced by molecular studies in related vesicle trafficking disciplines within the broader framework of cell biology. Particularly noteworthy was the identification of endocytic protein dynamin (Chen et al. 1991; van der Bliek and Meyerowitz 1991) following earlier discoveries of clathrin (Pearse 1976) and AP2 (Keen 1987), as well as seminal studies emerging from the vesicle trafficking field (see reviews by Cremona and De Camilli 1997; Brodsky et al. 2001; Conner and Schmid 2003). Most noteworthy are the studies that first identified *N*-ethylmaleimide-sensitive factor (NSF) and the other SNARE (soluble NSF attachment receptor) proteins and the realization that VAMP, syntaxin, and SNAP-25 were members of large protein families involved in a host of vesicular transport processes throughout the cell (Sollner and Rothman 1994). It had long been known that tetanus toxin and the botulinum toxins have profound effects on synaptic transmission. The discovery that these toxins were proteases that specifically cleaved VAMP, syntaxin, and SNAP-25 led to studies using them as probes that helped explain synaptic processes at the molecular level (Jahn and Niemann 1994). In particular, identifying the target molecules of these toxins quickly assigned a function to these recently discovered synaptic molecules.

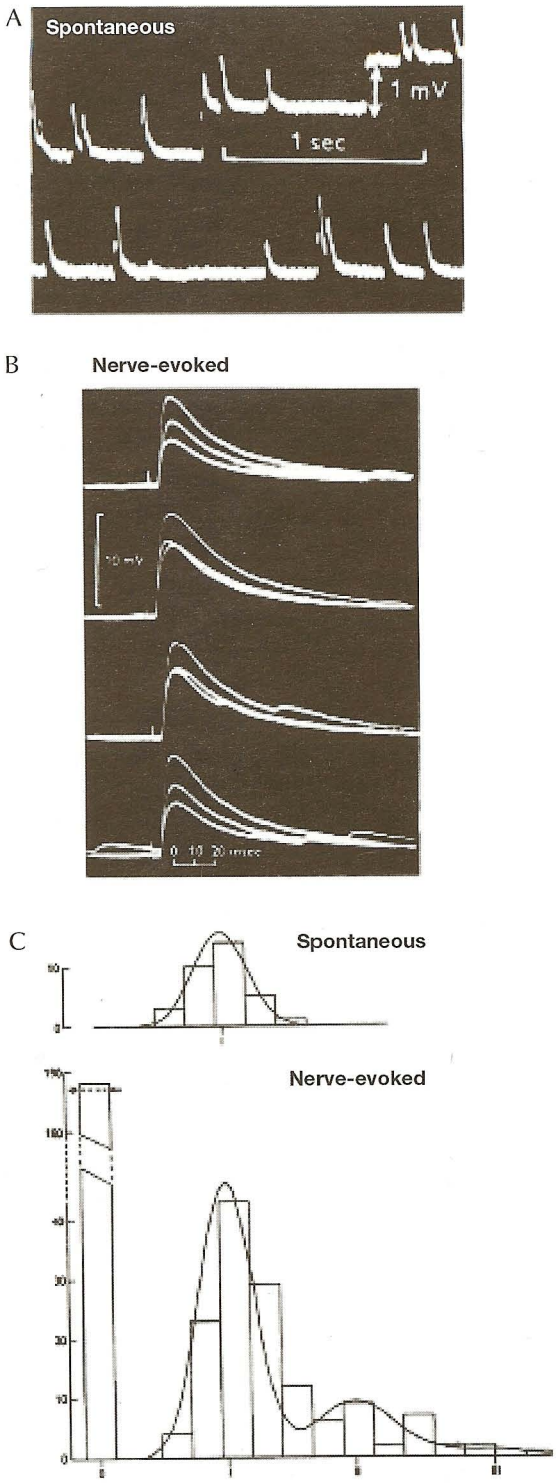


FIGURE 3. Quantal units of transmitter release. (*A*) A constant feature of synaptic transmission is the spontaneous (i.e., in the absence of action potentials) release of neurotransmitter. These miniature synaptic events appear similar to those caused by action potentials at low extracellular [Ca^{2+}]. (*B*) Nerve-evoked EJPs at medium extracellular [Ca^{2+}]. (*C*) If one collects EJP amplitude data of spontaneous events and nerve-stimulated events (under low-release conditions), two observations can be made. First, the amplitude distribution of the spontaneous events is the same as for the smallest nerve-stimulated events; second, the larger nerve-stimulated EJPs fluctuate in a manner that suggests the larger EJPs are multiples of the smallest EJP. These observations gave rise to the quantal hypothesis, which is that neurotransmitter is released from the nerve terminal in small packets (or quanta). (Reprinted, with permission, from del Castillo and Katz 1954.)

A Brief History of Neurogenetic and Electrophysiological Studies of Synaptic Transmission

Neurogenetic studies in *Drosophila* began with the premise that mutations of single molecules would alter the function of the nervous system, which would cause detectable changes in the behaviors of flies (Benzer 1973). With advances in molecular cloning techniques, it became possible to identify the genes whose mutations affected neural function. Touchstone publications include the report of temperature-sensitive alleles of *shibire*, which cause paralysis by blocking endocytosis of synaptic vesicles. It was discovered that *shibire* encodes the dynamin protein (Poodry and Edgar 1979; Koenig and Ikeda 1989; van der Bliek and Meyerowitz 1991). A temperature-sensitive allele of another *Drosophila* gene, *comatose*, also causes temperature-dependent paralysis. *comatose* encodes NSF (Ordway et al. 1994; Pallanck et al. 1995). Following these early studies, a number of other mutations affecting synaptic transmission have been discovered using forward genetics (e.g., *stoned*, Stimson et al. 1998; Fergestad et al. 1999; *awd*, Krishnan et al. 2001).

Using reverse genetics, researchers within the *Drosophila* community began looking for fly homologs of the proteins being characterized in other systems. The general strategy was to perform searches, such as screening cDNA libraries, to identify the genetic regions that encode known synaptic proteins, and then to make new mutants of these genes to study their effects on synaptic function. In this way synaptotagmin, syntaxin, SNAP-25, VAMP/synaptobrevin, and complexin, among others, have been analyzed in *Drosophila* (DiAntonio et al. 1993; Littleton et al. 1993; Broadie et al. 1994; Schulze et al. 1995; Deitcher et al. 1998; Rao et al. 2001; Huntwork and Littleton 2007). Using reverse genetics, a number of endocytotic mutants (such as AP2, AP180, and endophilin) have also been generated and studied to understand their roles in synaptic vesicle recycling (Gonzalez-Gaitan and Jackle 1997; Zhang et al. 1998; Verstreken et al. 2002).

The creation of transgenics has also been a valuable method in understanding synaptic transmission in *Drosophila* (Spradling and Rubin 1982; Brand and Perrimon 1993). These studies include expression of genes bearing engineered site-directed mutants, expression of exogenous tetanus toxin, gene replacement strategies, acute inactivation of synaptic proteins, and tissue-specific and temporally controlled gene expression (e.g., Sweeney et al. 1995; Osterwalder et al. 2001; Roman et al. 2001; Marek and Davis 2002; Venken and Bellen 2007; also see Chapters 22 and 23). A noteworthy example is research into the Ca^{2+}-binding properties of synaptotagmin I (Syt I) (Mackler et al. 2002; Robinson et al. 2002). Syt I was found to have two Ca^{2+}-binding C2 domains that interact with phospholipid and SNAREs, suggesting that it triggers transmitter release (Perin et al. 1990). Direct testing of Syt I's ability to perform this function was only achieved by constructing site-directed mutants that alter the Ca^{2+}-coordinating amino acids and introducing the transgene into *syt I*-null flies (Mackler et al. 2002; Robinson et al. 2002). These studies underscore the importance of combining molecular genetics with electrophysiology in understanding synaptic transmission.

USING *DROSOPHILA* LARVAL NMJ TO STUDY SYNAPTIC TRANSMISSION

The fly larval body wall muscles are ideal for studying synaptic transmission. They do not express voltage-gated Na^+ channels (Hong and Ganetzky 1994) and thus do not produce rapid action potentials. Neither do they exhibit Ca^{2+}-dependent regenerate potentials unless the tracheal system remains intact and is perfused with air (Yamaoka and Ikeda 1988). Hence, all recordings from the body wall muscle are passive membrane properties or pure synaptic responses. Furthermore, the muscle is isopotential, making it possible to record synaptic potentials anywhere in the muscle (in contrast to the frog NMJ in which minis [spontaneous miniature synaptic potentials] can only be detected when the electrode is inserted near the synapse). For these reasons the fly larval NMJ preparation has become the gold standard in the *Drosophila* neurobiology field ever since it was first developed for electrophysiological studies (Jan and Jan 1976a,b).

The key to understanding the electrophysiology of synaptic transmission lies in understanding the concepts of equilibrium potentials, driving force, selective permeability, and the dynamic inter-

play of different channels open at a specific time. The intracellular recording technique detailed in the following protocols is relatively simple, yet powerful, allowing one to address all of the basic questions concerning passive membrane properties and synaptic transmission. The technique also lays the foundation for more advanced techniques, such as voltage clamp and patch clamp. All of these experimental methods are based on similar biophysical principles and have methodological and operational features in common. Hence, we highly recommend that the reader first master the skills of intracellular recording (Protocols 1 and 2) before moving on to the focal loose patch and the two-electrode voltage-clamp (TEVC) techniques (Protocols 3 and 4).

In the CSHL course, students were provided with a number of "mystery" mutant strains so that they could use their newly acquired skills to characterize the mutant phenotypes and compare them with the wild-type larvae. These mutants included *Syx*$^{3\text{-}69}$, *snap-25*^{ts}, *lap*, K_{ir} mutant, and *eag, Sh* double mutants. *Syx*$^{3\text{-}69}$ and *snap-25*^{ts} mutants were chosen because they have higher rates of minis and produce larger EJPs compared with the wild type (Rao et al. 2001; Lagow et al. 2007). Once a student was more experienced, mutant flies were provided that had reduced synaptic transmission (such as *syt*, DiAntonio et al. 1993; Littleton et al. 1993; Mackler et al. 2002; *lap*, Zhang et al. 1998; Bao et al. 2005). The *eag, Sh* double mutant is unique because the motor axons are hyperexcitable, making it possible to observe spontaneous EJPs without nerve stimulation (Feng et al. 2004). The K_{ir} mutant is ideal for studying the effect of leak potassium channels on resting potential and on passive membrane properties (Paradis et al. 2001). A bonus of the K_{ir} mutant is that it gives students the opportunity to appreciate the power of retrograde compensation in maintaining synaptic homeostasis (Paradis et al. 2001).

Electrophysiology Equipment

The electrophysiology setup is commonly referred to as a rig. The rig used in the following protocols is based on equipment and software used in the Cold Spring Harbor Laboratory (CSHL) course, Neurobiology of *Drosophila*. Manufacturers regularly update their products and periodically replace old products with new models. Equipment from reputable commercial suppliers other than those mentioned here should work equally well. Hence, we strongly recommend that you contact the vendors and work with them closely before purchasing a piece of equipment for a rig. Contact information for the major suppliers of electrophysiology equipment may be found at the end of the chapter under the section WWW Resources. Here we describe briefly the components of an electrophysiology rig. Figure 4 shows examples of the major rig components using either an upright compound microscope (Fig. 4A–C) or a stereomicroscope (Fig. 4D).

Vibration Isolation Table: The table is intended to reduce vibration and minimize mechanical damage to cells. Suggested suppliers are Newport Corporation and Technical Manufacturing Corporation.

Microscope: A microscope is used to visualize the preparation and electrodes. Any stereomicroscope with a long working distance is sufficient for most of the intracellular recording and TEVC experiments routinely conducted with fly larval NMJ preparations. Examples of stereomicroscopes include the Leica M50, Nikon SMZ645, and Olympus SZX7. In fact, any dissection microscope will do the job, provided you can clearly visualize the body wall muscle. Some investigators prefer an upright compound microscope equipped with 5x and 10x air objectives and a 60x water-immersion objective with long working distance (e.g., Olympus BX51WI with LUMPFL 60xW WD3.3 mm objective). A compound microscope is more costly, but experiments can be conducted that are not possible with a stereomicroscope. For example, with a compound microscope, single-bouton recordings can be made within the NMJ, the morphology of a single synaptic bouton can be explored, and optical imaging of the NMJ (using green fluorescent protein [GFP], red fluorescent protein [RFP], or calcium indicators) can be performed at the level of a single synaptic bouton.

Amplifier: It amplifies small synaptic or membrane electrical signals above noise levels so those physiological signals can be visualized and recorded. In the CSHL course, an AxoClamp2B or AxoPatch 200B (Molecular Devices) amplifier was used. However, any good amplifier from a reputable supplier will work well. The amplifier should come with holders for microelectrode and head

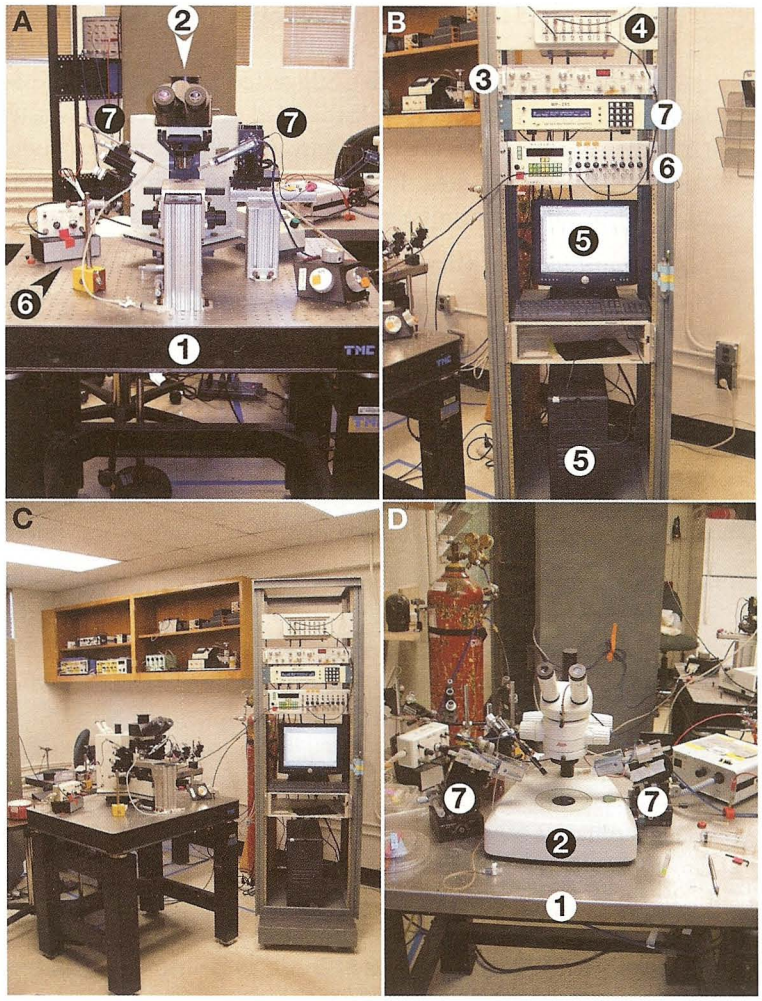


FIGURE 4. Examples of electrophysiology rigs. These pictures illustrate the major components of an electrophysiology setup commonly used in fly neurophysiology laboratories. (1) Vibration isolation (reduction) table; (2) microscopes (compound and stereomicroscope); (3) amplifier; (4) analog-to-digital (AD) and digital-to-analog (DA) interface board; (5) computer, monitor, and acquisition and analysis software (latter two not shown); (6) stimulator and stimulus isolator; (7) micromanipulators (motorized and manual). (*A–C*) A rig using an upright compound microscope. This configuration can be used for a variety of both basic and sophisticated experiments, including patch clamping of neurons or muscles (in embryos, larvae, and adults), two-electrode voltage clamping (TEVC), intracellular recording, single-bouton focal recording, and optical imaging. (*D*) A rig using a stereomicroscope. This simple setup is highly functional for intracellular recordings and TEVC in larvae or adult muscles. It is, however, not suitable for recordings from embryos or single boutons.

stages. Make sure that the outer diameter of the capillary glass to be used for manufacturing microelectrodes matches the size of the electrode holder. Suggested suppliers are Molecular Devices, HEKA, and Warner Instruments.

AD/DA Interface Board: This device changes analog (A) signals to digital (D) form so that a computer can record electrical traces. It also converts digital signals into analog ones, such as when your computer sends command signals to stimulate the nerve. A suggested product is Digidata 1440A (Molecular Devices).

Computer and Software: The software used in the course is called pClamp 10.0, which is compatible with the Digidata 1440A AD/DA interface board. The pClamp package has two separate programs: The Clampex program makes it possible to observe and acquire data and the Clampfit program is used for data analysis. In addition, the Mini Analysis Program (Synaptosoft Inc.) is used to analyze spontaneous and miniature synaptic potentials.

Stimulator: To excite an axon to fire action potentials, a brief electrical shock must be delivered to it. A stimulator and isolator are used to program the stimulation paradigms (such as stimulation frequency, duration, and strength). Suggested products are the S48 square pulse stimulator and SIU5 isolator (Grass Technologies/Astro-Med, Inc.) or the Master-8 and ISO-Flex stimulus isolator (A.M.P.I., Israel). If you are only delivering simple pulses (such as a single stimulus or two pulses), a stimulator is not required. Instead, use the built-in software with the Clampex program as a "stimulator" (for safety reasons a stimulus isolator must be used).

Micromanipulators: Micromanipulators help position the electrode(s) and assist in impaling the cell membrane with minimal physical damage. Larval NMJ recordings require one "coarse" manual manipulator (e.g., Narishige MM-3; Siskiyou MX110), which is usually placed on the left side and is used to position the stimulating ("suction") electrode. Two "fine" manipulators are needed to help position the recording electrode and impale muscle fibers. A manipulator with fine movement can be manual (e.g., Narishige NMN-25), hydraulic (e.g., Narishige MHW-3), or motorized (e.g., Sutter Instruments MP-225 or MP-285; Burleigh PCS-5000). The fine manipulator is a critically important piece of equipment because it is used to direct the electrode to impale muscles and to hold the electrode in a stable position. It is prudent to obtain the best micromanipulator possible.

Microelectrode puller: This device is used to pull the high-quality microelectrodes needed for impaling cell membranes. As one of the most important (but often overlooked) pieces of electrophysiology equipment, an electrode puller will determine how reliable your electrodes will be over time. Suggested products are the P-97 or P-1000 Flaming/Brown Micropipette Puller (Sutter Instruments) or the PC-10 Puller (Narishige).

Other Items:
BNC cables
Glass capillaries for manufacturing microelectrodes
Grounding wires and silver wires
Light source
MicroFil (World Precision Instruments)
Syringe (1-mL), filled with 3 M KCl and to be used with MicroFil to backfill a microelectrode

Video Monitor System: This is optional, but for teaching purposes it is extremely useful, because it allows up to five students to simultaneously visualize NMJ preparations and to watch their own actions (such as electrode position/movement) on a large monitor.

Oscilloscope: This is optional, although an oscilloscope has more functions than does a computer.

No two electrophysiology rigs are identical, because each laboratory configures theirs to suit their needs. In addition, different experiments require minor modifications to the rig, such as varying the number of micromanipulators needed depending on the number of electrodes being used. Historically, the system for signal observation and data storage has gone through the most dramatic change (and improvement) within the last 20 years. Instead of the traditional oscilloscope, chart recorder, and magnetic tapes, electrophysiological data are now visualized, acquired, and stored in digital formats in a computer using specially designed software.

Using Axoclamp 2B and the Digidata data acquisition systems as examples, a diagram of wiring connections for an electrophysiology rig that can be used for both intracellular recording and TEVC is provided in Figure 5.

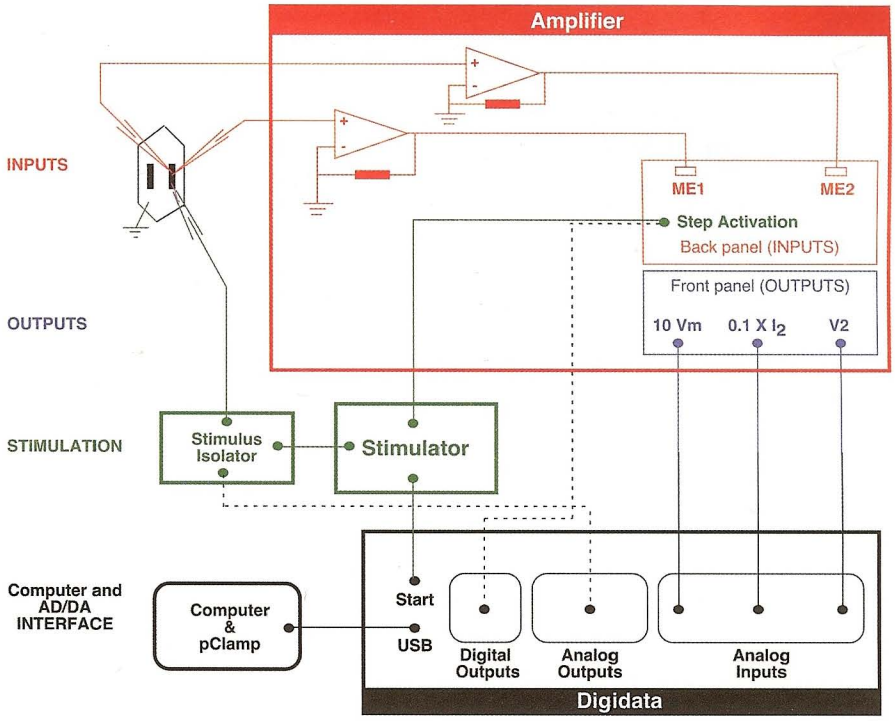


FIGURE 5. A wiring diagram for an electrophysiology rig. A rig can be divided into four major components based on the flow and processing of electrical signals: inputs, outputs, stimulation, and interface and data acquisition. Once connected through a BNC cable as illustrated here, these components will become a functional electrophysiology rig. The wiring diagram shown here is based on the AxoClamp 2B amplifier, Digidata, and pClamp software. However, this diagram can be used as a reference for any other similar products. Membrane potentials recorded by microelectrodes are detected by specific probes and then sent into an amplifier through corresponding channels (such as ME1 and ME2). Once amplified by the amplifier, the electrical signals travel to the AD interface board to be converted into digital forms and then acquired by the computer using specially designed software. The interface board is also capable of producing signals to command either the stimulator or the amplifier. A voltage or current delivered through the stimulus isolator and a suction electrode is used to stimulate the motor axons into firing action potentials. A stimulator is used to program different stimulus parameters so that one can control the stimulus duration and frequency. The software and DA interface board can also be used to control the stimulus pulse, but they do not have great flexibility for users if one wants to have more complex stimulus paradigms.