Chapter 1

Introduction to Neuroscience and Neuronal Manipulation Tools

The brain is the most complex and highly adaptable organ in the human body. Every thought, sensation, perception, movement, motivation, emotion, mood, and memory we experience is produced as a continuous stream of information. This information exists as an encoded array of complex and simultaneous physical, chemical, and biological events all accomplished in the brain by individual nerve cells and the connections between them.

Neurons are the Excitatory Cells of the Brain

The human brain is composed of approximately 86 billion neurons (nerve cells) and 85 billion nonneuronal (glial) cells organized into distinct anatomical regions[1]. Neurons are the functional unit of the brain. They are electrically excitable and their activity affects the electrical state of adjacent neurons. In contrast, glial cells are not directly involved in electrical signaling. Rather, they are deemed support cells, providing structure, regulation, and protection to the neurons. Glial cells also insulate the nerve cell axons and synaptic connections necessary for the conduction of electrical signals.

At rest, all cells including neurons maintain a separation of positive and negative ions on either side of the plasma membrane. A resting nerve cell has an excess of positive

charge on the outside of the membrane and an excess of negative charge on the inside. This separation of charge creates an electrical potential difference, or voltage, across the membrane called the resting membrane potential. A typical quiescent neuron has a resting membrane potential of -65 mV. As excitable cells, neurons differ from other cells in their ability to rapidly and dramatically change their membrane potential.

Rapid changes in membrane potential are mediated by ion channels. Ion channels are integral membrane proteins found in all cells of the body, however, those present in nerve cells are optimally tuned for rapid information processing. Ion channels of nerve cells open in response to specific electrical, mechanical, or chemical stimuli to conduct charge-specific ionic current at rates up to 10^8 ions/channel/second. Some channels are selective for a particular ion over others with the same charge. The most abundant, permeable ions in biological systems include the positively charged cations potassium (K^+), sodium (Na^+), and calcium (Ca^{2+}), and the negatively charged anion chloride (Cl^-). These ions are not distributed equally across the membrane; the concentration of K^+ ions is higher inside the cell, while the concentrations of Na^+, Cl^-, and Ca^{2+} are higher outside the cell. This unequal distribution generates a concentration gradient. Thus, the direction of passive ion flow is subject to both chemical and electrical driving forces due to concentration and ionic charge differentials. Passive diffusion of ions down their electrochemical gradient will proceed until reaching the point at which the electrical driving force in one direction exactly opposes the chemical driving force in the opposite direction and there is no longer a net flow. The membrane voltage at which this occurs is called the equilibrium potential (or Nernst potential) for that particular ion. The equilibrium potential of an ion is dependent on the valence charge of that ion, z, and the

concentrations of that ion inside, $[X]_i$, and outside, $[X]_o$, of the cell, and can be calculated using the Nernst Equation, defined as

$$E_X = \frac{RT}{zF} \ln \frac{[X]_o}{[X]_i}$$

where R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the temperature (in Kelvin), and F is the Faraday constant (9.65×10^{-4} C mol^{-1}).

At rest, a nerve cell membrane is mostly permeable to K^+ ions, therefore the membrane voltage (resting membrane potential) is close to the potassium equilibrium potential, E_K. A net flow of cations or anions into or out of the cell disturbs the charge separation across the membrane, altering the voltage. A reduction of charge separation, or depolarization, leads to a less negative membrane potential (e.g., from -65 mV to -55 mV). An increase in charge separation, or hyperpolarization, results in a more negative membrane potential (e.g., from -65mV to -75mV).

Neuronal Communication

When depolarization approaches a critical membrane potential, called the threshold voltage, it triggers the opening of voltage-gated Na^+ channels present in the cell membrane. This allows Na^+ ions to flow into the cell (i.e., down their electrochemical gradient), causing further depolarization, which facilitates the opening of even more voltage-gated Na^+ channels, rapidly driving the membrane potential toward E_{Na}. In this

depolarized state, the Na^+ channels begin to inactivate, while voltage-gated K^+ channels, which opened more slowly in response to the initial depolarization, remain open. Slow, outward K^+ current repolarizes the membrane back its resting membrane potential. The entire depolarization-repolarization process occurs within a millisecond. This rapid, transient, all-or-nothing voltage impulse is called an action potential.

The morphology of a typical neuron consists of (1) the cell body (soma), which contains the nucleus including the genes of the cell, (2) dendrites, processes which branch out to receive incoming signals from other neurons, (3) the axon, a single tubular extension which transmits the electrical signal over some distance, and (4) presynaptic terminals, fine branches extending from the axon that communicate the electrical signal at a site called the synapse to the dendrites or soma of receiving (postsynaptic) neurons (Figure 1-1A). The presynaptic terminal and postsynaptic cell are physically separated by a space known as the synaptic cleft (Figure 1-1B). At a synapse, the electrical signal is converted to a chemical signal, in which chemical neurotransmitter molecules are released from the presynaptic cell and diffuse across the synaptic cleft to activate receptors present on the postsynaptic membrane, where the signal is then converted back to an electrical potential. The sign of the signal, inhibitory or excitatory, depends on the type of receptors in the postsynaptic cell, not the identity of the neurotransmitter. All synaptic input of the receiving neuron is integrated at the axon hillock, the initial segment of the axon. This region of the cell membrane contains the highest density of voltage-gated Na^+ channels in the cell, and thus has the lowest threshold for spike initiation. If the summation of input signals reaches the threshold voltage, an action potential will be generated (Figure 1-1C).

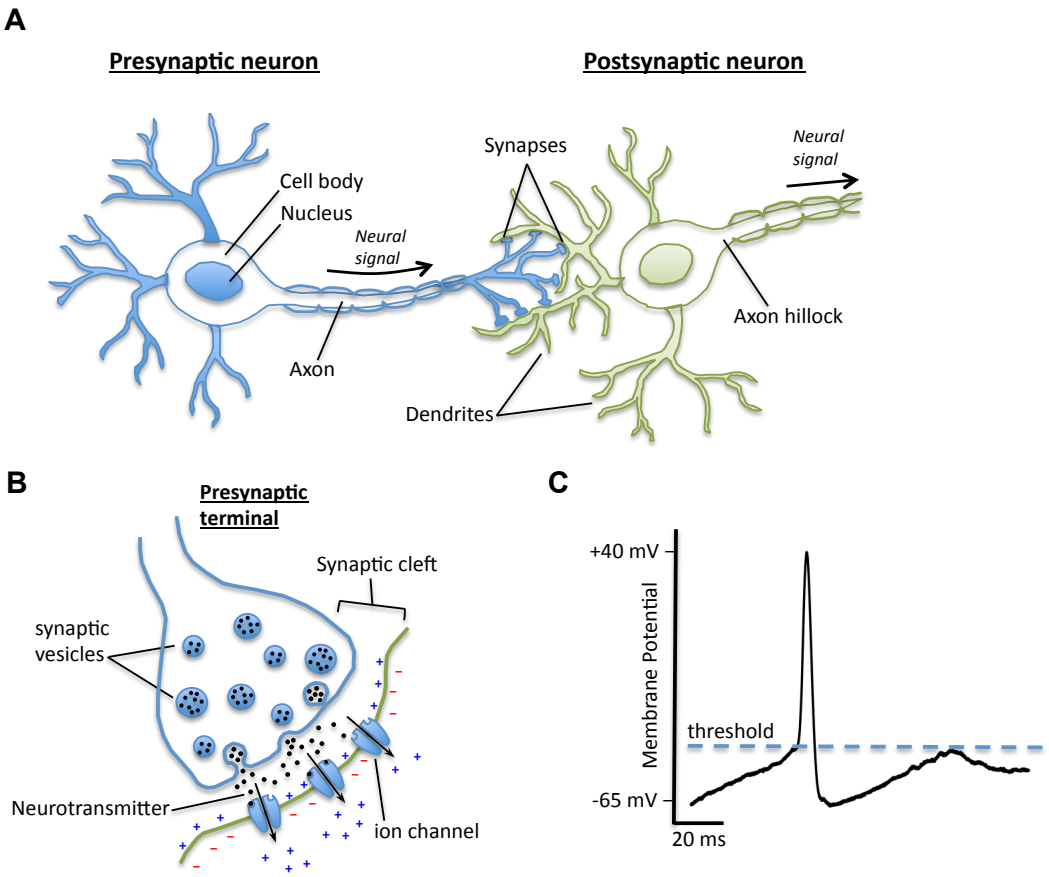

Figure 1-1. Neuronal communication. *A*. Typical neuron morphology consists of the cell body, dendrites, the axon, and presynaptic terminals. The presynaptic neuron communicates the neural signal to the postsynaptic neuron at synapses. *B*. Chemical neurotransmitter molecules packaged in synaptic vesicles are released from the presynaptic cell and diffuse across the synaptic cleft to activate ion channel receptors on the postsynaptic membrane. *C*. Synaptic input of the receiving neuron is integrated at the axon hillock. If the summation of input signals reaches the threshold voltage, an action potential will be generated.

Neural Circuits Convey Information

An action potential initiated at the axon hillock is actively propagated along the axon, regenerating with constant amplitude at regular intervals, until it reaches the presynaptic terminals where the signal is transmitted to other cells. Input signals below threshold voltage will not initiate an action potential, whereas all signals above the threshold will

produce the same all-or-nothing action potentials in succession in a "spike firing" pattern. All spikes fired are the same size and shape, but they differ in frequency (i.e., the number of action potentials and the time intervals between them). Thus, information in the brain is conveyed through neuronal firing patterns and the specific pathways in which they travel.

Nerve cells in the brain are highly organized into signaling pathways and have the same gross anatomical arrangement in every individual. Neurons are clustered into discrete groups that are functionally specialized for processing specific types of information. These regions are projected and interconnected to form extensive neural networks, generating sensory and motor functions, and facilitating learning, memory, and language abilities. The neural pathways for certain higher functions have been precisely mapped in the brain, though exactly how they produce complex cognition and behavior is still poorly understood. The majority of neurological and psychiatric disorders are believed to result from disruption of neural circuits caused by cellular abnormalities and/or molecular imbalance. Therefore, a detailed understanding of neural circuitry will aid in proper diagnoses and treatment strategies for such conditions.

The Study of Neuroscience: A Brief Chronology

The original notion that individual brain regions have distinct functions associated with different behaviors has been around since 1796 with the creation of phrenology by the German physician, Franz Joseph Gall. Phrenologists believed that the brain was the organ of the mind and that one's personality could be determined by the variation of bumps on

their skull. Now considered a pseudoscience, phrenological thinking was an important historical advancement toward the discipline of modern neuroscience. In 1861, the French neurologist Pierre Paul Broca extended the idea of phrenology, arguing that localization of brain function should be based on examining behavior that results from clinical lesion of internal brain regions rather than external inspection of bumps on the head. A short fifteen years later, German neurologist Karl Wernicke proposed that only the most basic mental functions such as perception and movement were localized to single areas of the brain, but more complex cognitive functions resulted from interconnections between several anatomical sites, advancing the idea of 'distributed processing' (i.e., various components of a single behavior are processed in different regions of the brain). At the beginning of the twentieth century, German anatomist Korbinian Brodmann used a staining technique to divide the human cerebral cortex into 52 discrete functional areas based on distinctive structural variation and characteristic organization of the cells. The cytoarchitectonic scheme of Brodmann areas is still widely used and continually updated today.

In the days of Broca and Wernicke, everything known about brain function had come from studying the behavior of brain-damaged patients and determining the site of damage in a postmortem analysis. If a patient had a deficit in some behavior, then execution of that behavior must depend on the lesioned area. In the 1920s, American psychologist Karl Lashley performed intentional lesion studies on laboratory animals by assessing the ability of a rat to complete a maze task after lesioning separate regions of brain cortex. A variety of animal lesion models and behavioral assays have since been created to associate specific brain regions with brain function. When establishing such

correlations, lesion models can be useful for demonstrating the necessity of an anatomical region, but they cannot resolve its particular role within a neural pathway. Disruption of adjacent brain regions during surgery or adaptive rewiring postsurgery may also complicate functional interpretations. Hence, lesion studies often produce confounding results and are not sufficient for investigating neural circuitry.

Need to Manipulate Neuronal Activity

The basic principles of brain organization, and to some extent information processing, have been pieced together using functional data from both brain slices (*in vitro*) and brains of awake, behaving animals (*in vivo*). Functional data can be obtained by various imaging and electrophysiology techniques, while additional pharmacological application and electrical stimulation can be used to directly probe neuronal function and connectivity. However, these methods are also limited in their ability to elucidate neural circuitry. Pharmacology often lacks specificity for particular cell types. Microstimulation excites both excitatory and inhibitory neurons and the precise region or number of stimulated cells is in many cases unknown.

Absolute resolution of intact neural circuits requires the direct manipulation of defined neuronal populations[2,3]. Such manipulation entails the ability to selectively and reversibly turn neuronal activity on and off in a tunable way on a relevant timescale. This can be approached in two different ways: controlling neurotransmitter availability to manipulate signal transmission, or controlling neuronal membrane potential to manipulate signal transduction. Both strategies have been used to induce or inhibit

neuronal activity. Manipulation is achieved via chemical, physical or genetic influences on transcription or protein activation.

Neurotransmitter availability can be restricted by preventing release into the synaptic cleft. For example, cleavage of vesicle-associated membrane protein 2 (VAMP2, also known as synaptobrevin) by inducible transcription of tetanus neurotoxin light chain (TeNT) can be used to inhibit synaptic vesicle fusion and subsequent neurotransmitter release[4,5]. An alternative approach called 'Molecules for Inactivation of Synaptic Transmission' (MISTs), utilizes a small molecule dimerizer to induce cross-linking of genetically modified forms of vesicular proteins including VAMP2 and synaptophysin to interfere with the protein-protein interactions necessary for vesicle fusion[6]. Induced neurotransmitter availability can be achieved with the use of caged neurotransmitters. With this technique, neurotransmitters are rendered biologically inactive, or caged, by chemical modifications with a photocleavable protecting group. A flash of light liberates the active form, imitating neurotransmitter release and permitting photostimulation of synaptic activity. Glutamate uncaging has been used extensively to study circuitry *in vitro*[7], however, most mammalian neurons express glutamate receptors so the technique lacks cellular specificity. The usefulness of TeNT and MISTs methods for *in vivo* studies is also limited due to a slow onset (14 days) of transcriptional induction and issues with delivery of chemical dimerizers. Furthermore, these methods alter the activity of neurotransmitter molecules rather than the neuron itself, so the postsynaptic targets must already be known in order to confirm the manipulated effect by electrophysiology.

Manipulation of neuronal membrane potential to control signal transduction is possible through modification of membrane ion channels or receptors. Rapid current flow

of selective ions into or out of the cell provides the dramatic changes in membrane potential necessary for versatile neuronal signaling. Direct alteration of the membrane potential can enhance the cell's ability to generate an action potential through depolarization, or inhibit the cell's ability to generate an action potential by hyperpolarization or shunting (clamp the $V_m \approx E_K$). Thus, neuronal activity can be induced by cation influx or silenced by K^+ efflux or Cl^- influx. Neurons have successfully been silenced by overexpression of various K^+ channels[8-10]. Since many of these channels are constitutively active, induction and reversal can only be accomplished through transcriptional control. Overexpression of K^+ channels can also yield undesirable effects such as disruption of native potassium channel expression or cell death[11,12]. Another effective silencing strategy uses membrane-tethered toxins to inhibit endogenous sodium channel or nicotinic receptor function[13]. Since toxins are peptides tethered to the membrane by a GPI anchor, they also require regulated gene expression for temporal control. Tethering of the ligand to the receptor with a photoisomerizable moiety addressed the need for controlled initiation and termination of modulating effects. Photoswitchable tethered ligands allow exogenously engineered channels or native channels to become 'light-gated', as light-induced isomerization presents or removes the ligand from its binding site[14,15].

Other strategies have involved chemically induced inhibition of neuronal activity. One study administered the allosteric modulator zolpidem to activate selectively expressed $GABA_A$ chloride channels using a transgenic mouse model in which endogenous $GABA_A$ channels were engineered to abolish sensitivity to zolpidem[16]. A related technique used a serotonin receptor 1A ($5\text{-}HT_{1A}$) knockout mouse and targeted

restoration of 5-HT_{1A} receptor expression with administration of selective serotonergic agonists[17]. Though successful, these methods unfortunately require animals with specialized genetic backgrounds and implement native receptors that can still be activated by endogenous neurotransmitters.

Expression of Foreign Receptor Tools

Many of these methods lack cell specificity, have slow temporal control, limited reversibility, constitutive activity, or interfere with native protein expression. Such issues have clarified the need for more refined control over neuronal activity.

Detailed circuit analysis requires the ability to manipulate and monitor a specific cell type. Cell types may be defined by anatomical characteristics including cell body location, dendritic morphology, axonal projection as well as electrophysiological characteristics and gene expression patterns. Molecular and genetic technology has been used to target gene expression of foreign receptor proteins to specific neuron types that, when activated, can inhibit or enhance neuronal activity within complicated neuronal circuits.

Genetically targeted manipulation must be precisely controlled in space and in time. The expression of an exogenous protein by itself should be innocuous, but when activated should enhance or silence neuronal firing in a selectively inducible and reversible manner. Many successful applications of targeted neuronal manipulation have involved the use of light to activate exogenous ion channels and receptor proteins. These include opsin proteins which are naturally light-sensitive ion channels and pumps

activated by photoisomerization of the chromophore retinal, a native compound of vertebrate nervous systems, to directly photoregulate membrane potential. Light activation of channelrhodopsin, an ion channel from the unicellular green algae *Chlamydomonas reinhardtii*, produces cationic currents to enable action potential firing that is time-locked to pulsed light[18,19]. Conversely, halorhodopsin, a chloride pump from the microorganism *Natronomonas pharaonis,* hyperpolarizes neurons to inhibit the production of action potentials[20]. Such optical control over neuronal activity allows millisecond timescale modulation. However, optical approaches require specialized equipment and are invasive, as light sources must be applied directly to the brain region of interest. Poor light penetration and heat generation also limit its applications to anatomically defined regions and short-termed modulations.

Alternative approaches use small molecule agonists for activation of exogenous receptors and ion channels, extending manipulation capabilities to deep and disperse neuronal populations with virtually limitless opportunities for simultaneous applications. These pharmacologically induced methods come with their own advantages, limitations, and requirements for specificity and are described in the next chapter. One such pharmacological tool, GluCl/IVM, is the subject of experimentation in this thesis.

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