THE UPS AND DOWNS OF ELECTRICAL ACTIVITY IN THE BRAIN:
STUDYING HOW COMPONENTS OF THE ACTION POTENTIAL WAVEFORM
MODULATE NEUROTRANSMISSION

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ABSTRACT OF THE THESIS

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STUDYING HOW COMPONENTS OF THE ACTION POTENTIAL WAVEFORM

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Action potentials (APs) are travelling waves of electrical activity in neurons composed of a rapid increase in membrane potential called a depolarization, followed by a repolarizing phase returning the membrane to a resting potential. Although APs are often thought of as all-or-nothing events, this is not necessarily the case. The AP waveform is generated by voltage-gated sodium and potassium channels whose composition, density and activity vary between and within neurons based on the function and output of that neuron. In addition to signal propagation, the purpose of an AP is to initiate the cascade of neurotransmitter release, beginning with the opening of voltage-gated calcium channels activated by the AP. Here, we set out to better determine how AP waveforms affect calcium influx and subsequent neurotransmitter release. The patch clamp technique has emerged as the best method to measure and study macroscopic electrical activity in neurons. Presynaptic APs at most synapses in the brain are difficult to study due to the small size of most presynaptic terminals. However, the calyx of Held synapse in the mammalian auditory brainstem is large enough to allow patch clamp recordings. In mouse brain slices, perfused with sodium and potassium channel blockers, various
voltage protocols were tested to determine how modulation of AP kinetics alter calcium activity.

First, various depolarization and repolarization rates were studied with test pulses of equivalent stimulus duration at 1 ms, showing that a repolarization/depolarization ratio between 1.5 and 2.3 is optimal for eliciting calcium influx. Additionally, depolarizations that follow the AP were studied and found to have no appreciable effect on calcium activity within the physiological range for AP durations in this neuron. However, if the repolarization rate is sufficiently fast, these currents were found to significantly alter the timing and magnitude of calcium influx. Finally, the AP was shown to minimize calcium channel inactivation to promote consistent and reliable neurotransmitter release. These findings will serve as the starting point for future work performing simultaneous pre- and postsynaptic patch clamp recordings to study transmission. This work promotes a better understanding of how AP kinetics affect calcium channel activity and thus neurotransmission.
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Introduction

The brain is the source of some of the greatest and most perplexing mysteries persisting in the world today. It is comprised of an array of approximately 100 billion interconnected neurons communicating with one another to form complex systems that make up the very essence of our being. All of this culminates into phenomena such as sensation, emotion, individuality, problem solving, simple, complex, and abstract thought, perception, and consciousness- each one a miracle in its own right, taken for granted as commonplace causalities of existence. The ability to convert the physical stimuli of the world into interpretable signals is one element of what makes this possible, allowing organisms to interact with the world around them. There are numerous ways in which this is carried out, but once these signals are generated, they must be transmitted to regions of the brain where they can processed. An immense amount of work has been done to better understand the means through which messages are sent throughout the brain, and it seems we are still only scratching at the surface of this grand riddle.

Over the past two centuries, numerous theories as to how the brain performs neurotransmission have been posed, but it is only in the past half century or so that researchers have truly begun to comprehend the manner in which the network of neurons in the brain is organized and the electrical and chemical mechanisms through which signals propagate. Techniques have emerged allowing researchers to study the electrical properties across the cell membrane, and these have revealed fast, voltage dependent pulses known as action potentials. When a cell membrane is excited beyond a certain threshold value, voltage-gated ion channel proteins, which span the membrane, open and allow ions specific to the type of channel to flow into the cell in accordance with their
electrochemical gradient. Voltage-gated sodium and calcium channel generally respond to depolarization activity which, if sufficient in magnitude, results in a rapid further depolarization of the cell membrane. This is followed by a rapid repolarization as voltage-gated potassium channels open and allow potassium ions within the cell to flow outward, thus leaving behind a net negative charge as the positive ions leave the neuron. This activity constitutes the waveform of an action potential, which initiates a cascade of rapid depolarization and repolarization that propagates down the axonal portion of the neuron.

Action potentials have been shown to be the mechanism by which the majority of nerve cells in the peripheral and central nervous systems (PNS and CNS, respectively) transmit signals throughout their axonal processes. The communication interface between two neurons is called a synapse, which consists of a presynaptic neuron and a postsynaptic region of the target neuron. Action potentials arriving at terminal of a chemical synapse (described in greater detail later) are responsible for initiating the process through which neurotransmitters, small molecules packaged within membranous vesicles, are passed between cells. Released neurotransmitter molecules diffuse across the small extracellular space between neurons to adjacent regions of target cells where they bind to receptors that produce an electrical response.

Action potentials are vital to maintain proper function throughout the body. Though often portrayed as all-or-nothing events, action potentials actually come in many different shapes and sizes, or waveforms, dependent upon the neuron they generated within, and can even vary in different regions of the same neuron. By studying the effect that different action potential waveforms have on synaptic transmission, we can cultivate a
better understanding of how ion channels and synapses function, and how changes in the waveform can enhance or reduce neurotransmission.

*Synaptic Transmission- How the Brain Communicates*

In the latter portion of the 19th century, there were two main schools of thought on how the nervous system was constructed- there was the reticular theory, which believed that the nervous system was comprised of a continuous, fibrous network that transmits signals throughout the brain, and the neuron theory, which proposed the nervous system to be a compilation of individual cells, specifically neurons, in conjunction with cell theory\(^1\). In 1873, Camillo Golgi devised a method for imaging the structures in the brain by hardening the tissue with potassium bichromate and loading the nerve cells with silver nitrate; this technique was dubbed the black reaction, also known as the Golgi method\(^1\). With this technique, Golgi was able to view the nervous system with a specificity and clarity not previously achieved. Golgi considered what he saw to support the reticular theory because pathways appeared to be continuous.

However, others researchers, most notably Santiago Ramon y Cajal, believed neurons are individual cells with very small spaces between neighboring neurons that provided a region for nerve cells to communicate and transmit messages. In 1909, Cajal published a compendium of images that he painstakingly sketched during staining experiments using the black reaction, several of which were locations that he considered likely for the existence of these spaces\(^2\). There is some debate as to whom should be credited for coining the term "synapse", with most attributing the name to Arthur Sherrington in 1897, but there was no single discovery that proved their existence; it was a compendium
that began with recognizing the importance of the nervous system, dating back to the time of Plato and the ancient Greeks, when the earliest theories on the importance of the nervous system were formulated\(^3\), and continuing with synaptic research being conducted to this day.

Synapses are the region in which signals are transmitted from neuron to neuron. There are two types of synapses: electrical synapses and chemical synapses\(^4\). Electrical synapses, which are the less common of the two, function by transferring voltage shifts between neurons through specialized channels known as gap junctions. These gap junctions form a continuous connection that allows ions and even small molecules to flow between cells, but they account for a relatively low number of synapses. Chemical synapses are much more common and relatively more complex. They are generally comprised of extensions of the pre- and postsynaptic neurons (axons and dendrites, respectively, also referred to as processes) in close proximity to one another, with a thin space (~20 nm) separating them referred to as the synaptic cleft. The postsynaptic region contains ligand gated receptor proteins that open in response to binding the neurotransmitters that is released by the presynaptic neuron from vesicles inside of the terminal. Given that there are continuous electrical synapses and discrete chemical synapses throughout the brain, the reticular and neuron theories were in a sense both correct in describing the overall organization. Electrical synapses form a connection between neurons, while chemical synapses contain a small interstitial space, both of which allow for the exchange of electrical and chemical signals. However, the latter is much more common and has led to what is now referred to as the neuron doctrine becoming the accepted dogma.
Chemical Synapse Function

Neurotransmission across a chemical synapse, depicted in Figure 1, can be best described beginning with the arrival of an action potential at the terminal of the presynaptic neuron, resulting in a depolarization of the cell membrane at the terminal. This depolarization causes a shift in voltage sensing regions of voltage gated calcium channels (VGCCs) to produce a conformational change that causes opening of the channels. External calcium is then able to flow freely into the neuron along its electrochemical gradient, and continues to flow throughout the repolarization phase until the membrane potential is repolarized to a voltage level that causes closing of the channels. Calcium levels inside the terminal are in the range of 40 nM, so the influx of calcium causes a large and abrupt change in free calcium in the terminal that is thought to reach levels up to 400 nM in response to a single action potential\(^5\). Calcium concentrations in areas adjacent to an open calcium channel experience higher local concentrations\(^6\). Calcium binds to proteins that are responsible for fusing neurotransmitter filled vesicles with the cell membrane as described below.

Neurotransmitter filled vesicles are present in several populations, referred to as pools, including the reserve pool, recycling pool, and readily releasable pool. The reserve pool represents 80-90% of the overall vesicle population and is scattered throughout the presynaptic terminal, while the recycling pool contributes 10-20% of the population and is located somewhat closer to synaptic release sites\(^7\). The readily releasable pool is the smallest population, only 1-2% of the total pool size, but is bound to the membrane and can be released very quickly\(^7\). Neurotransmitter vesicles contain tethering proteins called synaptobrevin, which function to bind the vesicles near the release face to protein
complexes of SNAP-25 and syntaxin on the membrane of the presynaptic neuron. Calcium that enters the cell during action potential activity binds to another protein on the surface of the vesicles called synaptotagmin, catalyzing the fusion of the neurotransmitter vesicle with membrane of the presynaptic terminal in a process known as exocytosis. The resulting fusion with the membrane releases the neurotransmitter formerly contained within the vesicle into the synaptic cleft.
Figure 1: Neurotransmission cascade at a chemical synapse.
Neurotransmitters are stored in submembranous vesicles in the presynaptic terminal. Once an action potential invades the presynaptic terminal, it causes a depolarization in the cell membrane that triggers the activation of voltage-gated calcium channels. The channels open, allowing calcium to flow into the cell and bind to protein clusters binding docked vesicles to the membrane, catalyzing exocytosis of the vesicles. The neurotransmitter diffuses throughout the synaptic cleft, binding with receptor proteins on the postsynaptic cell membrane, which function to open ion channels in order to depolarize or hyperpolarize the postsynaptic cell. If a sufficient depolarization of the postsynaptic membrane occurs, a postsynaptic action potential will be generated. Meanwhile, vesicles in the presynaptic neuron are retrieved from the cell membrane and loaded with neurotransmitter to refresh the vesicle pool. Obtained from open source NCBI bookshelf; Purves' Neuroscience.

An action potential invades the presynaptic terminal.

Depolarization of presynaptic terminal causes opening of voltage-gated Ca$^{2+}$ channels.

Influx of Ca$^{2+}$ through channels.

Ca$^{2+}$ causes vesicles to fuse with presynaptic membrane.

Transmitter is released into synaptic cleft via exocytosis.

Retrieval of vesicular membrane from plasma membrane.

Postsynaptic current causes excitatory or inhibitory postsynaptic potential that changes the excitability of the postsynaptic cell.

Opening or closing of postsynaptic channels.

Transmitter binds to receptor molecules in postsynaptic membrane.
Once released, the neurotransmitter diffuses across the synaptic cleft where it can bind to receptor proteins located on the postsynaptic cell membrane. There are two main types of receptors involved in neurotransmitter binding: ionotrophic and metabotropic. Ionotrophic receptors, also known as directly ligand-gated ion channels, span the thickness of the postsynaptic cell membrane, forming a pore that connects the cytoplasmic region of the neuron to the synaptic cleft. When an appropriately structured ligand, be it neurotransmitter or other small molecules with the appropriate structure, binds to the receptor, it undergoes a conformational change and allows ions to flow across the membrane. Ionotrophic receptors are fast acting, depolarizing or hyperpolarizing the postsynaptic neuron within one to two milliseconds following the arrival of the action potential in the presynaptic terminal. Metabotropic receptors, often referred to as G-protein linked receptors, are decidedly more complicated in their function. These receptors consist of an extracellular binding site where neurotransmitter can bind, and an intracellular protein called a G-protein. Upon binding the appropriate neurotransmitter, the G-protein is activated, and promotes the opening or closing of ion channels at a nearby location, typically by triggering a cascade of enzymatic reactions. The resulting shift in the membrane potential occurs much slower, but generally remains active over much longer time scales.

Synapses are also classified by the type of activity that is generated in the postsynaptic neuron as a result of the type of neurotransmitter released from the presynaptic neuron, referred to as either excitatory or inhibitory, and the type of receptors the neurotransmitter binds to. In the case of an excitatory synapse, the open channels are specifically structured to allow sodium, and in some cases calcium, to flow into the
neuron, generating a depolarization in the membrane potential of the postsynaptic cell. Glutamate is the most common excitatory neurotransmitter, binding to ionotrophic receptors, such as N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors, or a family of metabotrophic glutamate receptors (mGluRs). For inhibitory synapses, however, the receptors either activate channels that allow potassium ions to flow out of the neuron or, more commonly, permit chloride ions to flow in, causing a hyperpolarization of the membrane potential. Common inhibitory neurotransmitters include glycine, which binds to ionotrophic receptors, and γ-aminobutyric acid (GABA), binding to both ionotrophic (GABA_A) and metabotrophic (GABA_B) receptors. Activity across these many receptor types will culminate to produce a shift in the membrane potential, causing it to depolarize or hyperpolarize. If the net change of the cell membrane in the region of the axon hillock results in a depolarizing potential that surpasses the threshold for voltage gated sodium channel activation (i.e., if the response is sufficiently excitatory), then action potentials are triggered. Thus, the process of converting the electrical signal of the action potential to a chemical signal in the form of neurotransmitters, then back to an electrical signal continues throughout the brain, transmitting signals from neuron to neuron.

**Electrophysiology and the Study of Excitable Membranes**

Electrophysiology consists of the study of electrical activity in biological organisms. It can vary in terms of scale from individual cells, such as neurons or muscle cells, all the way up to entire organs, such as the heart or brain. When it was realized that this electrical activity was linked to the flow of ions across cell membranes, researchers needed a means of reliably assessing this behavior in order to understand how it
functioned to drive various elements of the body. While many of these techniques are still being honed to this day, the study of individual cells was a particularly difficult hurdle to overcome, and equivalently intriguing, as these are the very building blocks of our being; understanding their function is the key to unlocking many of the biological mysteries that continue to perplex researchers across the globe. Among the earliest techniques was intracellular recording using sharp electrodes, one inserted into the cell being studied (called the intracellular electrode) and another kept extracellular to act as a comparison (the reference electrode). With this approach, researchers were able to observe changes across the membrane of cells by comparing the potential difference (i.e. voltage) between these electrodes to study how the cells functioned at a resting state and in response to stimuli.

One issue with the early intracellular recordings was the inability to influence the electrical activity of the cells without altering the composition of the salt solutions or performing bath application of agonists aimed at shifting the membrane potential by interacting with ion channels. In these experiments, maintaining a constant resting potential between stimuli and leading up to stimulation was difficult, and typically made data comparisons complicated or not possible. With this in mind, researchers began searching for ways and devising methods reliably controlling and exciting the membrane. In the early 1940's, Kenneth Cole devised a method for "clamping" the membranes of cells being studied using a negative feedback control loop (see Figure 2). This was carried out in a preparation involving the squid giant axon, which served as one of the popular modes of experimentation with excitable membranes in the early days of electrophysiology due to its unique size; this axon is capable of reaching 0.5-1 mm in
Similar to the original method of intracellular recording, sharp electrodes were inserted into the interior of the cell and in the external solution. The difference measured by these electrodes, yielding the membrane potential of the cell, was determined by an initial comparative amplifier unit and transmitted to a feedback amplifier. This feedback amplifier was also linked with a control unit where the researcher sets the desired membrane potential of the cell. Utilizing the exceptional size of the squid axon, an additional electrode consisting of a thin silver wire was thread up through the axon in order to control the cell's membrane potential along the length of the electrode. The feedback amplifier determined the amount of current needed to maintain the desired membrane potential by comparing the control signal to the output of the comparative amplifier. This information was used to output a feedback signal, which was passed through a monitor unit and into the threaded electrode, functioning to clamp the membrane of the cell at a specified voltage. The control unit was manipulated based on information obtained from the monitor unit to achieve this potential, giving the researcher control over the membrane potential and allowing for the manipulation of ion channel activity using electrical currents. Information output by the feedback amplifier could be utilized to determine how the cell responded to manipulation of the currents that were delivered to it, allowing researchers to study the function of these excitable membranes with a whole new level of control and interaction.
Figure 2: Schematic of voltage clamp configuration.
The electrode used to clamp the potential of the cell is threaded into the axon. A sharp intracellular electrode is inserted into the cell and compared with an extracellular reference electrode via an amplifier unit to determine the membrane potential of the cell. This information is compared with a command signal from a control unit using a feedback amplifier. This amplifier outputs a current based on this information to adjust the membrane potential of the cell to the desired level, passes it through a monitor unit, and on to the clamp electrode where it is delivered to the cell to effectively voltage clamp the cell membrane. Adapted from information provided in The Axon Guide\textsuperscript{11}.

This technique gave way to two potential ways to study electrical activity in cells, by either controlling the voltage and recording corresponding shifts in current or simply performing passive readings of the voltage, referred to as voltage clamp and current clamp, respectively. In the case of the somewhat poorly named current clamp, the feedback component was not utilized, meaning that the membrane potential was not controlled, though it could still be modulated through current injection. This produces a passive recording of any activity that was elicited in the form of a voltage readout. This mode continues to be an excellent way to visualize the waveform of an action potential, but is limited in its ability to study many channel properties due to the shortcomings of its
passive nature. Voltage clamp mode, on the other hand, made full use of the feedback amplifier and is capable of controlling the membrane potential by injecting current to compensate for any departures from the set voltage level, referred to as the holding potential. This gave researchers complete control over the voltage of the cell, allowing them to inject various electrical stimuli and study how the cell responded. Around this time, Alan Hodgkin, Andrew Huxley, and Bernard Katz were investigating the membrane permeability of the squid giant synapse, focusing largely on the effects of sodium ions\textsuperscript{12,13}. Hodgkin and Huxley were particularly interested in the voltage clamp technique, and published a series of papers using it to study the permeability of various ions\textsuperscript{13}. This lead to the first quantification of the action potential and was widely regarded landmark discovery in the field of neuroscience, earning Hodgkin and Huxley the Noble Prize in Physiology or Medicine in 1963.

\textit{Patch Clamp Recording}

In the field of electrophysiology, the patch clamp technique is among the most effective ways to study the electrical activity of individual cells and the corresponding ion channels that generate it. Erwin Neher and Bert Sakmann were the first to obtain patch clamp recordings in frog muscle fibers, using the technique to study single ion channel currents\textsuperscript{14}. Rather than impaling the cell with an electrode, the patch clamp technique functions in a less invasive manner. A single electrode consisting of a fine-tipped, glass micropipette, generally around one micron in diameter, allows researchers to both deliver current stimuli and record the electrical response of the cell in voltage or current clamp configurations. The electrode is typically situated within a specialized holder that is attached to a probe, called a headstage, which is directly connected to the amplifier unit.
(see Figure 12 in Experimental Setup and Methods for an example). This system is grounded by a bath electrode that is placed in the extracellular environment. This electrode in tandem with the pipette electrode functions to measure electrical activity and inject current to offset any physiological currents that occur, thus maintaining the membrane potential set by the experimenter.

The pipette is filled with a solution designed to mimic either the intracellular or extracellular composition of the cell based on the type of patch clamping technique that is performed. The electrode pipette is carefully manipulated into a position next to the cell and an access point on the electrode holder allows gentle positive pressure to be applied to prevent occlusion of the tip opening. Once in position, the electrode is maneuvered until it is pressing up against the cell, as depicted in Figure 3, and the positive pressure is released to allow for the formation of a seal. Slight suction is generally required to coax the cell to adhere to the pipette properly, but is not always necessary. The formation of this seal is a crucial first step of all patch clamping techniques, determining the stability of the recording and thus dictating how efficiently electrical signals can be observed and quantified. Current is typically injected during the formation of the seal to help match the environment inside of the pipette with that of the interior of the cell to facilitate a stable seal. The resistance of this seal must be sufficiently high to verify that the pipette tip is in fact tightly fastened against the cell membrane, allowing a stable recording without leaky regions where solution in the extracellular environment could enter the intracellular/pipette environment and bias recorded activity. Due to the small piccoamp range of currents from single ion channels, a giga-ohm seal (or gigaseal; a seal with a minimum of 1 GΩ resistance) is necessary to maintain a sufficiently low signal-to-noise
Once this seal is achieved, the recording is in a state known as cell attached, or on cell recording. By filling the pipette with a solution designed to mimic the extracellular environment of the cell, single-channel activity can be studied in this orientation, with any ion channels on the membrane sealed within the diameter of the pipette responding to stimuli generated through the pipette electrode. Since this approach does not damage the integrity of the cell membrane, recording success rates are typically rather high and show little decline in function over time. However, manipulation of intracellular environment is difficult in a cell attached mode because there is no direct access to the interior of the cell without the use of membrane permeable molecules. In cell attached mode, it is also difficult to determine the membrane potential of the cell.

**Figure 3:** Formation of seal in patch clamp technique to obtain cell attached configuration. The patch pipette is slowly lowered into position with positive pressure being applied to prevent debris from entering the tip. Positive pressure is maintained while approaching the target cell, then released upon pressing up against the cell, forming a seal onto the cell. Typically slight suction is required to facilitate the formation of a stable seal. Once a seal is made, the pipette-cell interface is in what is known as the cell attached configuration.
This cell attached configuration is also an intermediate phase for all other patch clamp configurations, functioning as a first step. Other single-channel assays that can be performed at this point fall under the category of excised patch recordings, including inside-out and outside-out patch clamp techniques.

Inside-out patch clamping is performed by slowly pulling the pipette away from the cell after obtaining a gigaseal, with the pipette containing extracellular solution. If the seal is adequately strong, the membrane will tear around the outside of the pipette, leaving the portion at the opening of the pipette intact. This technique is employed to expose the membrane to different conditions, generally by applying various bath solutions containing drugs, enzymes, peptides, or other compounds that act on the cytoplasmic side of the membrane. It should be noted that pipette and bath solutions need to be reversed when performing inside-out patches to achieve the appropriate directionality of ion flow.

There are several complications involved with the formation of inside-out patches, most notably being the resealing of any membrane segment that exists outside of the pipette when it is withdrawn from the cell. This often forms a vesicle that must be ruptured to obtain any recordings. This is typically done by carefully removing the pipette from solution briefly, exposing the membrane to air in an attempt to lyse the resealed face, then quickly inserting it back into solution to prevent damage to the inside-out patch. This procedure is extremely delicate and often results in a complete deterioration of the membrane, leading to a relatively low success rate.

Outside-out patch clamping allows very efficient control over the patched region with minimized delay of responses due to the small surface area of membrane. This allows one to record single-channel activity or multichannel activity depending on ion channel
density. Since the outside of the cell membrane faces the outside of the pipette, agonists for ligand gated channels, or modulators that act at the extracellular side of the membrane can be applied. The outside-out patch uses intracellular solution loaded in the pipette, and the procedure to produce this type of recording involves rupturing the cell attached gigaseal before pulling the pipette away from the cell. A small electrical pulse can also be administered to help facilitate the resealing of the remaining membrane at the end of the pipette. Any channels in the membrane can be studied by applying stimuli via the pipette electrode, or by applying agonists to the outside of the patch since this technique is amenable to submillisecond application of compounds using fast perfusion systems. However, if any damage is incurred to the seal during this process, the resulting recording will exhibit a high level of noise, which is a common issue with this technique.

For studying the macroscopic electrical activity in a cell, whole cell patch clamping has become the gold standard configuration, depicted in Figure 4A. This is the patch clamp configuration that was used to acquire all of the data contained in this work. As discussed during the formation of an outside-out patch, it is possible to rupture the membrane area sealed within the pipette, resulting in a low-resistance connection with the cell while maintaining a high resistance seal to the membrane at the tip of the pipette referred to as the whole cell configuration. Breaking through the membrane within the seal to establish access to the cell is a vital step in forming a stable whole cell recording, and there are several ways for this to be carried out. These options include sharp, brief bursts of suction intended to break the membrane and release before damaging the rest of the cell, delivery of quick "zap" currents intended to dialyze the sealed membrane until it ruptures, or some mixture of the two approaches. The suction approach is the more widely used of the two
methods due to the fact that it is generally less damaging to the cell when executed properly, though excessive suction runs the risk of destabilizing a portion of the seal or lysing the cell.

Once a whole cell configuration is obtained, several parameters must be monitored and/or manipulated in order to maintain a stable recording. First, the holding potential (in voltage clamp mode) should be set to a voltage that is typical for the resting potential of the cell type that is being studied. This is typically done during the formation of the seal, but should be maintained throughout the course of experiments. Additionally, there are several resistive components to the electrical activity; these include the pipette resistance, access resistance, and membrane resistance. The pipette resistance refers to the resistance exhibited by the pipette through the opening at the tip, with some small component also being attributed to the glass itself. Access resistance is the resistance of the opening to the cell that is formed when a whole cell orientation is achieved. The access resistance will increase with any debris or residual membrane in the pipette after the rupture is generated. Membrane resistance represents the resistance across the membrane of the cell, which accounts for leak currents through ion channels, as well as leak at the seal during recording. There are also capacitive effects that must be taken into account, which consist of the pipette capacitance and the cell capacitance, also referred to as membrane capacitance. The pipette capacitance is a function of the wall thickness, tip diameter and the pipette recording solution and it can be measured and offset during the cell attached phase. The cell capacitance is due to the separation of charge across the cell membrane. It can be measured after a whole cell configuration and is proportional to the cell membrane.
area, including any additional processes, such as axons and dendrites, or blebs, which are membrane deformations that form if the membrane surface is ruptured and reseals.

Figure 4: Whole cell patch clamp configuration and circuit schematic.

A, Schematic of the resistances and capacitances associated with the whole cell configuration, including the pipette resistance and capacitance ($R_p$ and $C_p$), access resistance ($R_a$), membrane resistance and capacitance ($R_m$ and $C_m$), and series resistance ($R_s$). B, Circuit formed during a whole cell patch clamp recording in voltage clamp mode. Intracellular and extracellular conditions are compared at the probe to determine the membrane potential, which is then compared to the command input using the feedback amplifier. This output is fed back to the pipette in order to control the electrical activity of the neuron as dictated by the command protocol. In current clamp, the circuitry is similar, but there is no feedback due to the recording being a passive measurement. Adapted from information provided in Patch Clamping by Areles Molleman.

All of these factors function to determine the current that needs to be injected in order to hold the cell at a desired potential in voltage clamp. The values of these parameters also serve to indicate the quality of the seal. Recordings with a minimal series resistance demonstrate that currents will be delivered to the cell more quickly and responses will be detected at a faster rate, while capacitance values indicate the amount of cell membrane that is being clamped, informing the experimenter as to how accurate the recording will
be. A portrayal of these parameters can be found in Figure 4A, with an accompanying electronic circuit schematic in Figure 4B.

**The Calyx of Held**

The calyx of Held is a mammalian auditory synapse that is involved in the localization of sound. The first documented discovery of the synapse was in 1893 by Hans Held using the newly developed Golgi method\textsuperscript{16}. Ramon y Cajal was able to sketch the first light microscopy images of the calyx of Held in several mammalian species using Golgi methods, which he published in a 1909 work\textsuperscript{2}. Nearly a century later, it became popular in the field of electrophysiology with researchers studying synaptic transmission and the associated ion channels. Due to the unique size and morphology of the calyx, it is amenable to patch clamp techniques, and became one of the earliest presynaptic terminals where direct recordings could be obtained in the mammalian CNS. In the 1994, Ian Forsythe obtained the first patch clamp recordings\textsuperscript{17} from the calyx of Held in rat brain slices, followed closely by Gerard Borst in 1995, who was able to perform simultaneous patch recordings on the both the pre- and postsynaptic neurons to investigate the resulting excitatory postsynaptic potential (EPSP) following an action potential stimulus in the presynaptic terminal\textsuperscript{18}. This work was the spark that ignited a specialized area of research dedicated to studying ion channel activity and synaptic transmission using the calyx of Held preparation.

**Function in the Auditory Pathway**

In order to accurately interpret activity at a synapse, it is important to understand the role of that synapse in the overall pathway in which it resides. As an auditory synapse, the
calyx of Held plays a role in sound localization through contralateral inhibition. A sound signal travels up the ear canal until it reaches the cochlea, and it tends to reach one ear slightly before the other. When the other ear receives the sound there is a slight delay, as well as a reduction in intensity of the signal because of the anatomical nature of the head functioning as a sound barrier. This delay, which typically amounts to less than a millisecond, is responsible for the localization of low frequency sounds in space, whereas the intensity of the signal leads to the pinpointing of high frequency sounds\textsuperscript{19}.

The resulting vibration of the cochlear hair cells stimulates the mechanosensing stereocilia, which convert this physical signal of the sound into neuronal signals. These signals, in the form of action potentials, are then propagated into the superior olivary complex (SOC), where the computation of sound localization, among other functions, is carried out using the binaural inputs previously described. Action potentials from the cochlea arrive at the ipsilateral anterior ventral cochlear nucleus (aVCN) and form excitatory synapses onto the spherical and globular bushy cells (SBCs and GBCs, respectively). The SBCs form excitatory synapses at the ipsilateral lateral superior olive (LSO) and medial superior olive (MSO), where first comparison of binaural signals takes place. The GBCs extend across the midline of the brainstem and into the contralateral medial nucleus of the trapezoid body (MNTB), where the calyx of Held terminal is formed from the axon of the GBC synapsing onto a principal cell of the MNTB. This glutamatergic synapse is the final excitatory point in this arm of the pathway, with the MNTB converting these signals into inhibitory glycinergic outputs when they synapse on their target cells in the ipsilateral LSO (contralateral to the original cochlear signal). Thus, the balance of excitatory input from ipsilateral signals and inhibitory input from
contralateral signals are processed in the LSO and used to determine the location of the sound\textsuperscript{20}. A diagram of this pathway is shown in Figure 5\textsuperscript{20}.

\textbf{Figure 5: Schematic of the Superior Olivary Complex.}
Cochlear hair cells in the inner ear project axonal processes onto bushy cell in the anterior ventral cochlear nucleus. Spherical bushy cells (SBCs) propagate to the ipsilateral lateral superior olive (LSO), while globular bushy cells (GBCs) extend across the midline of the brain and synapse onto principal cells in the medial nucleus of the trapezoid body (MNTB), forming the calyx of Held presynaptic terminal. The MNTB principal cell forms outputs in the contralateral medial superior olive (MSO) and LSO (relative to the original cochlea neuron). Used with permission from Dr. Henrique von Gersdorff\textsuperscript{20}.

The timing of these inputs is crucial for the LSO's ability to adequately respond to the signaling provided from each ear, with the calyx of Held functioning to process and relay the signals from the GBCs of the contralateral aVCN to produce an inhibitory response on neurons in the LSO while the SBCs of the ipsilateral aVCN provide excitatory currents. This means that in order for either stimulus to win out, it must reach the LSO in an expedient manner and have a proportionately larger signal output. Therefore, contralateral inputs must be able to transmit the signal over significant distances and exhibit minimal delay if they are to properly represent the initial stimulus- two things that the calyx of Held synapse is built for. The axons extending from the GBCs that form the
presynaptic component of the calyx of Held are relatively large in diameter, allowing for faster-than-usual conduction velocity of contralateral signals. Studies in several mammals have shown extremely miniscule delays in inhibitory signals at the LSO, with some being so fast that they precede the excitatory inputs\textsuperscript{20}. The calyx of Held terminal also offers a uniquely large and fenestrated morphology, allowing for increased quanta of neurotransmitter release that functions to ensure reliable, high-throughput neuronal firing\textsuperscript{20}. Additionally, the MNTB is tonotopically organized such that high frequency inputs arrive in medial areas of the region, while low frequency stimuli are sent to more lateral regions\textsuperscript{21}. This arrangement is optimal for preserving the various types of inputs based on their nature as currents.

*Role as a Model Synapse*

There are trillions of synapses present in the brain, but the presynaptic element of the vast majority of these cannot be studied by patch clamp techniques due to the small size of most presynaptic terminals. Presynaptic activity can be inferred from postsynaptic observations, but this can be inconsistent and unreliable. Ideally, both elements of the synapse would be amenable to electrical recording, allowing for neurotransmission to be studied directly. The calyx of Held is one of only a few synapses in the brain that can be studied by pre- and postsynaptic patch clamp recordings. Additionally, for a synapse to be usable to study synaptic plasticity, it must be capable of undergoing activity dependent modulation of neurotransmission\textsuperscript{20}. Finally, it is desirable for a synapse to have a range of firing frequencies and be capable of releasing a sufficient amount of neurotransmitter for the postsynaptic cell to follow the presynaptic action potential input\textsuperscript{20}. The calyx of Held synapse meets these all of requirements, making it an ideal choice as a model synapse for
studying short term plasticity. It should be noted that long term plasticity, which may be correlated to things like memory formation, has not been demonstrated in this preparation, perhaps due to the function of the calyx as a synapse where such long term effects could interfere with its function in sound localization.

The calyx of Held is not alone, however, and there are also other synapses that meet many of these requirements that are worth mentioning. The endbulb of Held, for instance, is a presynaptic terminal projecting onto the SBCs and GBCs in the aVCN that shares similar properties to the calyx of Held. The endbulb has a very comparable morphology to that of the calyx, which rises from the fact that it lies within the same auditory pathway and thus must meet many of the same specifications. Activity in the endbulb has been shown to be significantly less than in the calyx of Held, but to compensate, there are generally around four endbulb of Held synapses per bushy cell. This creates as sort of redundancy in the physiological system, promoting neurotransmission based on inputs from multiple sources. However, this is less ideal for the purposes of studying synaptic activity due to the fact that, in individual recordings, ion channel responses are more muted, neurotransmitter release will be lower, and signal-to-noise ratios will be lower. Additionally, the endbulb is smaller in size, causing it to be more difficult to obtain presynaptic recordings from. This has led to less publications dealing in the endbulb of Held, with the calyx of Held being a superior preparation for most purposes.

In addition to the two auditory synapses, the mossy fiber terminal in the CA3 region of the hippocampus is another presynaptic terminal that meets many of the criteria of a model synapse, and gives researchers an opportunity to investigate a glutamatergic synapse in a cortical network, at a synapse that has been shown to undergo long-term
plasticity changes\textsuperscript{23}. Additionally, the mossy fiber terminal forms synapses with CA3 cells in a more classical sense as a synaptic bouton, as opposed to the calyx of Held, which wraps around the soma of the principal MNTB cells. The CA3 region is also largely unmyelinated, allowing for improved visibility at older ages and, thus, older recording time points. However, research in the mossy fiber terminal has been limited due to technical issues, including a smaller size (2-5 μm diameter, as opposed to the calyx of Held's 10-15 μm surface length) and technical difficulties in the precision machinery required for the production of adequate brain slices for this preparation.

Another large presynaptic terminal is the cerebellar basket terminal, which forms a synapse with Purkinje cells in the cerebellum. Contrary to the mossy fiber terminal and the calyx of Held, the cerebellar basket terminal forms an inhibitory synapse, allowing researchers that ability to study release from a GABAergic nerve terminal. Much like the mossy fiber terminal, there is also a low amount of myelination and a more traditional morphology, allowing for older aged animals (3-5 weeks old in rats) to be studied\textsuperscript{24}. However, also like the mossy fiber terminal, it is not without its shortcomings. In addition to difficulties with slice preparation that have caused research in the terminal to be limited, the smaller size of the basket terminals forces significantly smaller electrode pipette diameters to be used, restricting access to and electrical control of the cells.

There are several important morphological features of the calyx of Held that allow it to meet the criteria of a model synapse. First, the myelinated axon extending from the GBCs down to the MNTB that terminates at the synapse is relatively large, with some having been shown to be up to 12 μm in diameter\textsuperscript{25}. As the axon approaches the trapezoid body during development, a growth cone begins to form at the terminal. This cone spreads into
several finger-like extensions that envelope a significant portion of the principal MNTB cell that they synapse onto, as shown in Figure 6. It has been reported that about 20% of these projections divide to form two separate calyces on two different principal MTNB cells. Additionally, though a single MTNB cell typically only has one calyceal input, there are rare cases (~5% of the time) where two calyces synapse onto the same principal cell. The resulting encapsulation leads to a drastically larger amount of area where the pre- and postsynaptic cells interface at the synaptic cleft, and therefore a correspondingly elevated active zone region. Thus, an immensely increased exchange of neurotransmitter is obtained relative to that which is generated between smaller, traditional synapses, amplifying the ability of the calyx of Held to reliably and expediently pass signals to the MNTB principal neurons and their target neurons in the contralateral LSO.
As the calyx of Held develops, there are several morphological changes that occur that influence its function. The ear canal of mice and rats generally opens once they reach about 11-12 days postnatal and is thought to guide many of these changes through an increased activity in the auditory system. Beginning around two weeks postnatal, the finger-like extensions of the calyx begin to fenestrate further, becoming smaller in width but more numerous and intricate. This leads to a larger number of active zones present throughout the release face of the terminal. An increased clustering of neurotransmitter vesicles is also observed at active zones, effectively increasing the size of the recycling
and readily releasable vesicle pools. At later stages of development, mitochondria have been found to congregate at these active zones, forming clusters that have been shown to regulate internal calcium concentrations through an expedited replenishment of ATP supplies and functioning to mitigate the effects of synaptic depression caused by sustained activity\textsuperscript{28}.

\textit{The Action Potential Waveform and Calcium Channels}

As noted earlier, though the action potential is perceived as an “all or nothing” event, this is not the case, with components of an action potential waveform differing between different neurons, and even at various places within the same neuron. The waveform of an action potential is determined by the activity of voltage-gated sodium and potassium channels and the corresponding shifts in the membrane potential that result from this activity. Different subtypes of these channels in conjunction with varying densities and localization give rise to different action potential waveforms, which are thought to be optimized for different types of neurons\textsuperscript{43}. For example, a neuron that fires action potentials at a high frequency will likely require voltage-gated sodium channels and potassium channels that activate quickly and are resistant to a process called inactivation\textsuperscript{32}, which is a use dependent decrease in ion channel response, discussed in more detail later in this section. The activity of the voltage gated channels can also be modulated by interactions with auxiliary subunits\textsuperscript{29} or direct modification of the protein structure through processes such as phosphorylation\textsuperscript{30}. It should also be noted that the action potential waveform can also be affected by activity from other voltage gated and ligand gated ion channels. In addition, pumps and transporters can cause brief or prolonged changes in the resting potential that can also affect the action potential
waveform. While there are many varieties of action potentials, this section will focus on the action potential properties observed in the calyx of Held and the associated ion channels. An example of the presynaptic action potential waveform in the calyx of Held and its components are depicted in Figure 7.

Figure 7: Presynaptic action potential waveform in the calyx of Held.
The waveform shown here was elicited through afferent fiber stimulation. The action potential initiates with a rapid depolarization of the membrane potential of the neuron until reaching the peak amplitude, then rapidly descends in a repolarization phase towards the resting potential. The width of the action potential is generally measured at half of the peak amplitude and is referred to as the half width. Following the repolarization phase, there is a fast afterhyperpolarization phase where the repolarization reaches its minimal value, followed by a prolonged afterdepolarization raising the membrane potential above the resting potential and slowly returning to baseline.

Hodgkin and Katz first appreciated the dependence of the sodium ion concentration in action potentials of the squid giant axon\textsuperscript{12}. This study laid the groundwork for many subsequent studies on sodium permeability in cell membranes. In the calyx, there is a low density of voltage gated sodium channels (Na\textsubscript{v}) in the terminal itself, but there are large quantities at the axonal heminode, the region where the axon transitions to forming the
calyx. Labeling studies have shown an extensive presence of Na$_{\text{v}}$1.2 type channels, which are quite common in the CNS, present in the axon leading to the calyx$^{26}$. However sodium channels undergo a shift in expression from Na$_{\text{v}}$1.2 to Na$_{\text{v}}$1.6 over the course of development$^{31}$. Thus, propagation of action potentials into the calyx of Held terminal is a relatively passive process that is possible due to this high heminodal density being sufficient to depolarize the terminal consistently.

Voltage gated sodium channels function by undergoing an activation and inactivation process. When in an activatable state, depolarization of the membrane reaching a voltage of around -45 to -55 mV will cause a population of sodium channels to open, triggering a rapid influx of sodium ions from the extracellular environment. This represents the initiation of an action potential, propelling a further depolarization of the membrane to voltage levels as high as +30 mV or greater depending on the developmental stage of the animal. During this depolarization, sodium channels then begin to inactivate, halting additional flow across the membrane and effectively decelerating the depolarization. Inactivated channels will remain in this state until the membrane is sufficiently repolarized or hyperpolarized, at which point they will return to an activatable until an appropriate stimulus causes them to be opened once more. The entirety of this activation and inactivation process has been shown to occur as quickly as three milliseconds in the calyx of Held$^{32}$.

Potassium channels, on the other hand, represent a much more diverse pool of channel types with varying functions, all generally working to maintain the cell at resting potential, including repolarizing the cell membrane during action potentials. These types include low and high voltage gated potassium channels (K$_{\text{L,V}}$/K$_{\text{V,1}}$ and K$_{\text{H,V}}$/K$_{\text{V,3}}$,
respectively) and calcium activated potassium channels ($K_{Ca}$). In the case of $K_{L,V}$ channels, assays blocking various subunits have shown that $K_v1.2$ channels are present in the calyx of Held$^{33}$, with immunohistochemistry revealing the channels to be located in close proximity to $Na_v1.6$ channels$^{32}$. These channels activate at a threshold lower than sodium channels to maintain resting potentials during sub-threshold stimuli and reduce overall excitability of the regions where they are located. As for $K_{H,V}$ channels, studies have shown these to be primarily expressed in the form of $K_v3.1b$ type channels located on the side of the presynaptic terminal opposite the release face, as well as spread throughout the postsynaptic cell body$^{34}$. These high voltage channels function to repolarize the cell during an action potential current. In addition, strong evidence has been shown to indicate the presence of $K_{Ca}$ channels in the calyx of Held, but their function in regulating neurotransmission is still poorly understood$^{26}$.

Once activated, potassium channels allow for an efflux of potassium ions, which, in conjunction with the inactivation of sodium channels, functions to repolarize the cell back to resting levels. In many excitable cells, including the calyx of Held, this results in a hyperpolarization that can go below the original resting potential. In the case of the calyx, this is referred to as a fast afterhyperpolarization (fAHP) to distinguish it from the hyperpolarization following prolonged activity. In younger animals, such as the postnatal day 8 mouse that the waveform shown in Figure 7 was obtained from, this fAHP generally reaches a potential of $-65$ to $-70$ mV, while in later developmental stages it is capable of reaching as low as -$85$ mV$^{35}$. The fAHP has been shown to be independent of the membrane potential of the cell leading up to the action potential, repolarizing the cell to a level determined by the correlation between sodium channel inactivation and
potassium channel activation\textsuperscript{38}. Chloride channels are also present in the calyx of Held, and may assist in maintaining the resting membrane potential, as they do in many other cell types. Additionally, these channels have also been shown to participate in the regulation of the internal pH of many cell types\textsuperscript{36}. Calcium activated chloride channels have been shown to influence the action potential waveform in some neurons, but this has not been shown in presynaptic terminals\textsuperscript{37}.

After the repolarization and fAHP, some cells, including the calyx of Held, exhibit a depolarization following the action potential which is, referred to as an afterdepolarization (ADP). In the calyx of Held, and several other types of neurons, this arises from a resurgent sodium current through voltage-gated sodium channels. This occurs through the actions of an auxiliary subunit of the sodium channels acting as a voltage sensitive blocker, lodging a portion of itself in the pore to lock the channel in an open configuration while blocking sodium conductance into the cell. The subunit dislodges from the pore as the membrane repolarizes and allows for a brief influx of sodium before the channel closes. This results in a delayed and prolonged influx of sodium that produces the afterdepolarization. From the minimum voltage of the fAHP, there is generally about a 5 ms latency until the peak of the ADP. The resulting depolarization increases the potential by a value of 5-20 mV depending on the age of the animal, reaching a level of around -60 to -65 mV\textsuperscript{38}. Though the peak is reached relatively quickly, the entirety of the ADP lasts for a relatively long time scale (on the order of 20-100 ms). The ADP functions to increase the probability of subsequent action potential firing by maintaining the membrane potential at a depolarized level below the activation threshold for voltage-gated sodium channels\textsuperscript{38}. This increases the overall fidelity of the
synapse, making it less susceptible to failures in action potential firing and improving the latency between spikes to increase firing frequencies by a small amount.

Aside from propagating signals from the soma of a cell to its presynaptic terminal(s), the purpose of an action potential is to trigger VGCCs, initiating the process of neurotransmitter release in chemical synapses. As the action potential invades the terminal and causes a depolarization, these calcium channels are activated. As previously discussed, these channels allow calcium to rush into the nerve terminal and promote exocytosis of neurotransmitter filled vesicles. Calcium channel types in the calyx of Held that contribute to the observed calcium response during stimulation have been shown to include N-, P/Q-, and R-type channels\textsuperscript{26}. In mice, it has been shown that once the animal reaches a developmental stage of about postnatal day 10, however, only the activity of the P/Q-type calcium channels (Ca\textsubscript{v}2.1) have an effect on exocytosis\textsuperscript{39}, as is the case for many mammalian CNS terminals.

Ca\textsubscript{v}2.1 type channels are interesting in that they are the only presynaptic VGCC to exhibit calcium dependent facilitation (CDF)\textsuperscript{40}. This facilitation manifests during the first several milliseconds of a train of action potentials, increasing the probability of calcium channel opening and, by extension, the amount of calcium entering the cell per pulse. Calcium dependent inactivation (CDI) is more common across VGCC types, and it is caused by the excess of calcium that enters the cell during a prolonged stimulus, which can be characterized over various time scales depending on the duration of the provided stimulus. This inactivation is thought to contribute to synaptic depression that is observed during maintained bursts of activity\textsuperscript{41}. Through the use of flash photolysis to unbind caged intracellular calcium, each of these effects has been shown to be mediated by the
intracellular calcium concentration with relatively high sensitivity\textsuperscript{40}. Furthermore, when extracellular calcium is replaced by barium, the barium can traverse the calcium channels but is not capable of activating calcium dependent processes. Under these conditions, short term CDF and CDI effects deteriorate, while long term inactivation remains consistent\textsuperscript{40}. This indicates that short term modulation of inactivation and facilitation effects are specific to calcium ions, while long term effects are voltage dependent and independent of the charge carrier. Multiple calcium channels in the vicinity of active zones are typically required to elicit the release of neurotransmitter, but a single action potential has been shown to be sufficient to initiate a large amount of release.

In order to isolate the calcium current ($I_{Ca}$), sodium and potassium channels are generally blocked with various pharmacological agents. The voltage clamp configuration is generally used in the case of assessment of activity across the whole terminal in order to adequately control the recordings. Box waves, which consist of instantaneous voltage steps, are the most common form of stimulus used to study $I_{Ca}$, which consist of a rapid depolarization that is maintained at an elevated potential, then rapidly repolarized. By modulating the duration of the pulse and the depolarized potential, calcium channel properties such as inactivation/facilitation profiles and channel activation quantities can be studied. An example of such a test can be found in Figure 8. Once the cell is depolarized, calcium channels are triggered, shifting them into the open state and allowing calcium to flow into the cell in accordance with its electrochemical gradient. This phase of the response appears as a negative, inward current, where a steady state value is reached that represents the overall calcium influx to the cell. If the pulse duration is sufficiently long, an inactivation profile can be observed through a reduction in the
steady state level of the inward current. This continues until the repolarization phase of the box wave, which produces what is referred to as a tail current. This occurs due to a shift in the driving force as the cell returns to its resting potential while VGCCs remain active until the membrane potential reaches roughly -30 to -40 mV, causing them to deactivate and return to a closed state. This change in state does not occur instantly, though, and as the voltage of the stimulus becomes increasingly negative, the electrical force that drives calcium to enter the cell increases producing a tail current as the channels are transitioned to this closed state, marked by the rapid return of the \( I_{Ca} \) to the baseline.

![Graph showing calcium current elicited by box wave stimulus.](image)

**Figure 8: Example of calcium current elicited by box wave stimulus.**
A 200 ms depolarization from a holding potential of -80 mV to +30 mV was used to elicit a calcium response. A clear inward current can be appreciated throughout the duration of the pulse, followed by a rapid tail current resulting from the increase in driving force exerted during the repolarization of the test pulse. Inactivation of the calcium channels can also be observed as the inward current gradually decreases in amplitude.

In CNS synapses, the assessment of calcium channel activity using action potential-like (AP-like) stimuli is not generally tested. The \( I_{Ca} \) elicited by an action potential is notably different than that observed from prolonged pulses due to the restricted time scale and the shape of the waveform. An action potential functions to quickly open calcium channels during its depolarization phase, then undergoes a repolarization phase that acts to
increase the calcium driving force, as seen with the box wave stimulus (Figure 8) until the deactivation voltage is reached and channels begin to close. This causes the resulting calcium response to take the form of a tail current, with no steady state inward current exhibited due to the fast time scale. The effect of repolarization and depolarization durations and rates on the $I_{Ca}$ has been studied using pseudo-action potentials (pAPs)$^{44}$, which consist of short duration triangular waveforms intended to mimic the dynamics of an action potential more appropriately than the instantaneous shifts exhibited by box waves. Short box waves on the order of 1 ms in duration are often referred to as "AP-like", but these elicit significantly different activity, as demonstrated in later sections of this work. Similar pAP waveforms have been used to compare the amount of neurotransmitter release generated by a box wave versus a train of AP-like stimuli via capacitance measurements$^{42}$.

Inactivation properties of individual calcium channels subtypes have also been assessed in HEK cells transfected with N-, P/Q-, R-, and L-type channels in a comprehensive approach by comparing inactivation exhibited by a square pulse stimulus to that of stimulation from a train of previously recorded action potentials$^{41}$. The action potential train used in this study was constructed by duplicating a single previously recorded action potential twenty times to make a 50 Hz train, 400 ms in duration. Inactivation for that action potential train was found by comparing the $I_{Ca}$ elicited by the first action potential stimulus to that of the last in the train, while inactivation of the long pulse was found by the difference in the inward current from the peak of the activity to the end of the stimulus. It was found that the N-, P/Q-, and R-type channels all inactivated more from an action potential train of 20 pulses with a 1 ms half width than from a square pulse with
a duration equivalent to the sum of the action potential half widths (20 ms), while L-type channels inactivated less than in the square pulse.

Why Studying Modulation of the Action Potential Waveform is Important

Small changes in the action potential waveform have been shown to produce proportionately larger effects on the calcium influx into the cell. Furthermore, due to the non-linearity between calcium influx and synaptic release, even minute changes in the calcium influx can produce large changes in the postsynaptic response. Since the timing and kinetics in these systems are influential on a sub-millisecond scale, any changes in synaptic transmission can have significant impacts on the neuronal response. Given the requirement that numerous inputs must summate in amplitude and time to initiate action potential firing, any change in amount or timing of neurotransmitter release can significantly impact this process. Thus, it is vital to understand how changes in the action potential can be generated, and also how these changes can alter neurotransmission.

One of the most common ways that the waveform is modulated occurs during trains of action potential activity. In many CNS synapses, the action potentials exhibit a reduction in amplitude accompanied by a significant broadening\(^{43}\). While this generates a proportional change in the observed calcium response, the postsynaptic response elicited by the corresponding neurotransmitter release is less predictable. In the calyx of Held, recorded action potentials from the end of a train that are injected back into the synapse as a stimulus have been shown to produce larger EPSPs than action potentials in the beginning of the train\(^{20}\). Therefore the observed change in the kinetics of the action
potential may act to increase the calcium current to possibly compensate and limit the decrease in neurotransmitter release that occurs with repetitive stimulation at a synapse.

It has been shown that action potentials recorded from brain slices of the same animal species and cell type at different stages of development elicit significantly different synaptic responses when played back into brain slices of similarly aged animals and compared. This is due to the fact that the action potential half width reduces during development as a result of changes in ion channel densities and subtypes, causing a proportionate reduction in the calcium response. However, the EPSP elicited remains relatively consistent, while stimulating a young animal's nerve terminal with an action potential from an older animal elicits almost no EPSP. Conversely, the nerve terminal of an older animal stimulated with an action potential from a younger animal generates a significantly larger EPSP than is typical. This is thought to be a consequence of an improved calcium binding efficiency, indicating that this narrowing of the action potential over development is important for controlling the amount of neurotransmitter released during signaling.

Temperature has also been shown to have effects on the waveform of action potentials. Experiments performed near physiological temperature of 37°C showed little modulation of the peak amplitude of action potentials, but a significantly shorter half width when compared to activity observed at room temperature (~22°C), corresponding to faster sodium and potassium channel kinetics. Likewise, calcium activity exhibits a proportional reduction in the half width. However, a drastically increased EPSP rise time and response integral are observed at the near physiological temperature, indicating a
large thermal dependence in calcium binding efficiency, neurotransmitter release, and/or neurotransmitter uptake kinetics\textsuperscript{44}.

In this work, we have sought to investigate the effect that modulations in the depolarization, repolarization, and ADP have on calcium currents and subsequent neurotransmission. It has been shown that even very small changes in the action potential can produce significant changes in the calcium channel response, and result in large changes in the postsynaptic response\textsuperscript{45}. Effects of action potential depolarization and repolarization duration on calcium currents has been studied previously, but only by increasing the duration of either phase individually, thus increasing the duration and broadening the response\textsuperscript{44}. This makes comparisons of the different calcium channel responses to different depolarization and repolarization rates difficult because the overall amount of current being delivered to the cell is changing as the action potential waveform is broadened. We reasoned that conserving the amount of stimulus being provided during different test pulses would allow us to make more meaningful comparisons of the effects that depolarization and repolarization rates have on calcium channel currents. This approach should demonstrate how the depolarization and repolarization of an action potential at physiologically relevant durations interact to elicit calcium channel activity.

We also hypothesize that the ADP following the action potential in the calyx of Held may affect calcium channel activity by altering the driving force for calcium and thus the calcium channel response. This was tested by measuring changes in the calcium channel response to action potential waveforms as the ADP kinetics and amplitude were modulated. We also suspect that calcium channel inactivation or facilitation during repetitive stimulations could be affected by the prolonged depolarization caused by the
ADP. This can be tested with repetitive stimulation using action potential waveforms with and without the ADP. Finally, it has been shown that low, subthreshold levels of depolarization prior to stimulation are capable of altering the postsynaptic response\textsuperscript{46}. Thus, we hypothesize that the ADP would be capable of producing such modulation by affecting the calcium channel activity during a stimulation train.

Previous studies using HEK cells have shown that action potential trains produce more calcium channel inactivation than prolonged depolarization pulses\textsuperscript{41}. However, the calyx of Held synapse functions to promote reliable and consistent neurotransmission even at high firing frequencies, which suggests that it may be more resistant to calcium channel inactivation. We hypothesize that, contrary to previous studies, by maintaining a consistent overall amount of stimulus across several test pulses, we will see a higher amount of calcium channel inactivation following prolonged depolarizations compared to the inactivation generated by trains of action potential-like stimulations. In addition, the prolonged depolarization created by the ADP during the stimulus train could also affect calcium channel inactivation. This was investigated using action potential stimulus trains in the presence and absence of an ADP following each action potential waveform.
Experimental Setup and Methods

Brain Slice Preparation

Postnatal day 8 (P8) to P12 CD1 albino mice obtained from Charles River Laboratories in Wilmington, Massachusetts were used for all experiments conducted in this work. The animals were housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International and protocols used for handling and care were reviewed by the Rutgers University Animal Care and Facilities Committee in accordance with the Animal Welfare Act. Mice were decapitated and the brain stem was dissected out and mounted on the stage of a Leica VT 1200 Vibrating Blade Microtome in a parasagittal orientation using cyanoacrylate glue, demonstrated in Figure 9A. Throughout the process of dissection and mounting, the brain was maintained at a low temperature by immersing it in artificial cerebrospinal fluid (aCSF) solution at 1-2 °C, and it is crucial that the dissection is performed as rapidly as possible to minimize the time that the brain is not kept in this chilled solution in order to keep cell death at minimal levels. The solution was comprised of the following components (in mM): 125 NaCl, 25 NaHCO$_3$, 25 glucose, 3 Myo-Inositol, 2.5 KCl, 2 Na-pyruvate, 1.25 NaH$_2$PO$_4$, 0.4 ascorbic acid, 3 MgCl$_2$, and 0.1 CaCl$_2$ at a pH of 7.3 when oxygenated with carbogen gas (95% oxygen, 5% carbon dioxide); this is a low calcium variation of traditional aCSF used to quiet spontaneous synaptic transmission during slicing to prevent excitotoxicity.
Figure 9: Brain slice preparation.
A. Dissected brain stem mounted on the slicing stage in a parasagittal orientation. B, Vibratome slicing through mounted brain to produce a brain slice of 130 µm thickness. C. Vibratome setup, including carbogen delivery line and surrounding ice water slurry for maintaining oxygenated slices in a low temperature aCSF solution.

Once mounted, the brain stem was loaded into the microtome and partitioned into thin slices (~130 µm thick) while maintained in low-calcium aCSF solution, as shown in Figure 9B. To maintain a sufficiently low temperature, the slicing chamber was surrounded in a slurry of ice and water as seen in Figure 9C. Slices were collected in a timely manner and transferred to a chamber suspended in a water bath maintained at 37 °C using a Julabo Heating Circulator for 30-45 minutes. This chamber consisted of a 150 mL beaker outfitted with a mesh gauze grid stretched across a 35mm petri dish with the bottom removed and two columns intended to house tubing that delivers carbogen in order to oxygenate the chamber (see Figure 10C). The chamber contained aCSF solution
that reflected a more physiological calcium level (1 mM MgCl₂ and 2 mM CaCl₂; henceforth referred to as recording solution) and oxygenated as before; this combined with the elevated temperature allows the slices to recover from damage incurred during slicing and return to a relatively normal resting state. The chamber was then removed from the heating bath and kept at room temperature while recording experiments were carried out for 4-6 hours following the incubation.

**Microscopy and Brain Slice Handling**

Brain slices were inspected using an Olympus BX51 Upright Microscope (shown in Figure 10A) with a 40x LUMPlanFLN objective; it features a numerical aperture of 0.8 for a relatively high-resolution image and a 3.3 mm working distance to allow enough space for electrodes to be navigated in the interstitial area between the brain slice and the objective. A 4x UPlanFLN objective was also used for positioning the slice and initial scanning of the slices to locate the MNTB. The microscope was situated on a Sutter Instruments MP-285 Motorized X-Y Translator operated by a rotary optical encoder unit to allow for sub-micron movements in the viewing field without disturbing the stage, where the brain slices were located. This assembly rested atop a TMC Vibration Control High Performance Air-Table (using Gimbal Piston system and Breadboard tabletop) to eliminate residual vibration from the building and stabilize experiments. Visual light was used for initial scanning of brain slices through the eyepiece at low magnification to locate the MNTB region. To scan the MNTB for presynaptic terminals, a high magnification 40x objective was used along with an IR filter to produce near infrared light (nIR). This was visualized using a Dage-MTI IR-1000 Infrared CCD Monochrome Camera connected to a monitor using an analog signal. The nIR light was combined with
a differential interference contrast (DIC) system to induce three-dimensionality to the image. This is vital when attempting to land an electrode on the optimal location of a target nerve terminal and obtain a stable patch.

The microscope was outfitted with a multichannel reflected light illuminator intended for the BX51 model with a Collimated LED light source (Thor Labs). The LED output blue light with a peak wavelength of 460 nm and a range of roughly 60 nm. This light is passed through an excitation filter with a range of 445-490 nm and transmitter onto the cell being studied; lucifer yellow dye was present in the pipette solution and filled the nerve terminal and the associated axon once a whole cell configuration was obtained. The dye was at a sufficient concentration such that excitation within this range would trigger appreciable fluorescent emissions. These emissions were reflected back up through an emission filter with a range of 500-550 nm in the illuminator and propagated up to the camera, where the image is captured. Pictures of fluorescent and bright-field images were gather post-hoc with an Allied Visual Technologies Manta G-046 digital camera using GigEViewer software.
Figure 10: Components of the experimental setup.
A. The Olympus BX51 Upright Microscope used for inspecting brain slices. B. Microscope stage, including platinum weight, solution lines, and ground electrode. C. Carbogenated slice chamber used for slice storage during incubation following slicing and during experiments.

Slices were transferred from the oxygenated storage chamber onto the stage of the microscope using a dropper and weighted down with a U-shaped piece of platinum with surgical wire strung across it (see Figure 10B) to ensure that there was no movement during experiments. The stage was perfused using an Ismatec Reglo Analog Pump with oxygenated recording solution to maintain slice quality, supplemented with tetraethylammonium (TEA; 20 mM) and tetrodotoxin (TTX; 1 µm). These additions were added to block potassium channel and sodium channel activity, respectively, thus preventing the cells from generating action potentials. The benefits of this technique are two-fold: it both limits the response that is elicited from voltage injections strictly to that
of the VGCCs as well as allowing for the injection various stimuli to assess the manner in which they alter synaptic transmission through the measurement of the calcium activity.

**Electrophysiology**

Recordings were conducted using a HEKA EPC10 USB double patch clamp amplifier being controlled using PatchMaster software in voltage clamp configuration. Fresh electrode pipettes were fabricated daily from thick walled glass (2.0 mm outer diameter, 1.16 mm inner diameter) obtained from Sutter Instruments. The glass was loaded into a Sutter Instruments P-1000 Micropipette Puller and the heat and pull velocity were adjusted to produce sharp tip electrodes with an opening of about one micron, within a resistance range of 2.5-5 MΩ, for use during presynaptic recordings. An example of this fabrication process can be seen in Figure 11.
Figure 11: Fabrication process of electrode pipettes.
A. A thick walled glass cylinder loaded into the Sutter Instruments micropipette puller. Values such as the heat and pulling velocity are set to values that will produce pipettes with a taper and tip diameter desirable for the experiments being conducted. B. Pipettes pulled from the original glass cylinder following a cycle of heating and pulling phases in accordance with the puller protocol. C. Platinum box filament through which the glass cylinder is threaded, which is subjected to pulses of heat throughout the procedure to prime the cylinder for each pulling phase until it fully separates.

Intracellular pipette solution for measuring calcium currents included the following (in mM): 90 cesium methansulfonate, 20 CsCl, 10 TEA, 40 HEPES, 1 MgCl$_2$, 5 EGTA, 5 phosphocreatin, 2 ATP, and 0.2 GTP, and was buffered to pH 7.3 using CsOH. As mentioned above, fluorescent dye (0.25 mg/mL lucifer yellow) was added to the pipette
solution in order to visualize characteristics of the patched cell after recordings, such as axon length and patch location relative to the axon heminode, and to verify that a presynaptic recording was in fact achieved (see Figure 15 for examples). The energy components of the solution (ATP, GTP, and phosphocreatin) were made separate from the main salt solution and added later. This was done because the energy components are more volatile, and this approach allowed for smaller batches to be made more frequently in order to prevent these compounds from degrading.

Pipettes were loaded with the intracellular solution and mounted onto the headstage of a probe which sends signals to the amplifier. The headstage is situated atop a Siskiyou MX7600 Micromanipulator, a 4-axis controller that allows precise manipulation of the pipette with submicron resolution and high stability to prevent drift during a recording. This system is depicted in Figure 12 (A- headstage and manipulator stand; B- controller unit). A silver chloride pellet served as the reference electrode and was connected to a ground input on the headstage. There is also a port included in the pipette holder that allows for the delivery of positive or negative pressure to aid in the formation of a seal. A glass pipette is filled with intracellular solution and loaded onto the pipette holder, threading a silver wire into the pipette. A holding screw in the pipette holder is tightened, compressing o-rings around the pipette to form an air tight seal. It is essential to ensure that the silver wire is submerged in the pipette solution to complete the circuit between the cell and the amplifier. This component is shown in Figure 12C. The pipette is then rotated to face the microscope stage and, using the micromanipulator mentioned earlier, it is guided into the space between the objective and the weighted brain slice (see Figure 12D) until it was visualized on the monitor. Once located, the pipette was descended
down to the brain slice and maneuvered to position adjacent to a previously located presynaptic terminal, then advanced onto the terminal in an attempt to obtain a seal. Once a presynaptic terminal was patch clamped, electrical recordings were obtained by injecting various electrical stimulation protocols through the amplifier using the PatchMaster software.

**Figure 12: Electrode pipette equipment and procedures.**
A, Siskiyou manipulator stand, amplifier probe, and headstage assembly used to house and guide the electrode to its desired location. B, 4-axis controller unit used to guide the manipulator. C, Components of the headstage, including the port through which positive pressure and suction can be applied to keep the pipette clear of debris and aid in the sealing process and the glass micropipette surrounding the silver electrode wire, along with the reference electrode input on the amplifier probe functioning to ground the bath. D, The assembly after being moved into position and manipulated into the space between the objective and the brain slice, approaching the target cell to attempt the formation of a seal.

During recording, cells were held at a resting potential of -75 to -80 mV, which is the typical resting potential at the calyx of Held. Presynaptic voltage clamp recordings used
in this study exhibited a series resistance (see Figure 4) within the range of 5-20 MΩ, which represents the overall resistance of the both the pipette used to make the recording and the opening to the cell within the seal once a whole cell recording is established. It is important that this value is relatively low (typically desired to be less than 25 MΩ) since it is indicative of the access obtained to the cell, which determines how efficiently the cell can be controlled by voltage clamp protocols and how quickly responses to stimuli are elicited and recorded.

Concerning the control of the membrane potential using voltage clamp, it is necessary to understand that a voltage error exists due to the series resistance. For instance, in accordance with Ohm’s law, a 10 MΩ series resistance with a cellular current of 1nA will exhibit a 10mV voltage error. To compensate for this, series resistance compensation of 70% was also employed to correct for the series resistance of the recording. This is a built in function in the PatchMaster software, and functions by over-injecting current during changes in the membrane potential in order to accelerate the response of the cell and compensate for the effect that the resistances are having. It should be noted that, due to the nature in which this feature functions, destabilizing oscillations can be produced when slight instabilities in the recording. As a result, it is often recommended that compensation values of 90% not be exceeded to prevent the oscillatory tendencies\textsuperscript{15}. In this work, a compensation of 70% was determined to be viable to minimize oscillatory tendencies while sufficiently correcting for the relatively low series resistance values maintained in these experiments.

The values of these parameters are discerned by injecting a small test current (5-10 mV for about 10 ms), producing voltage transients associated with the capacitance of the cell
coupled (in series) with the access resistance of the patch. To compensate for this, the amplifier digitally subtracts this capacitance and resistance based on setting the parameter values to an amount that acts to cancel these transient currents. This also provides values for the access resistance and cell capacitance of the recording as demonstrated in Figure 13.

Figure 13: Cancellation of voltage transients.
In order to cancel out the voltage transients that are present once whole cell mode is established, a short 10 mV pulse is injected into the cell to visualize these transients (above). The series resistance and cell capacitance are adjusted until the transients have leveled out (below). These changes correlate to a shift in the current injected to compensate for the transients so they do not appear in data traces. Upon setting a series resistance compensation value, these often require an additional slight adjustment.

A sampling interval of 5 µs was used to obtain the highest possible resolution of recorded activity, while 10 µs was used in some instances where recording durations were exceptionally long, to restrict the data collected within a reasonable sample size when optimal resolution was not required. Currents were filtered by a 4-pole Bessel filter at 3
kHz to remove any residual high-frequency noise present in the recordings. Passive effects such as capacitive and leak currents were adjust for by utilizing the on-line P/4 leak subtraction protocol for all recordings except those involving the injection of real, previously recorded action potentials, which were adjusted for manually with the same P/4 protocols. Leak subtraction protocols involve recording an initial trace at the desired voltage, then four additional pulses with their voltages scaled down by a factor of 0.25 (in the case of P/4 protocols). These traces, which will be below any voltage threshold capable of eliciting activity, are then summated and subtracted from the initial raw data trace, removing any artifacts resulting from stimulus and leak associated with the cell and yielding data that represents real activity demonstrated by the cell. This process is demonstrated in Figure 14.

Figure 14: Leak subtraction procedure.
The raw data trace is shown in red. In the case of a P/4 leak subtraction protocol, four traces are generated at 25% of the voltage of the original pulse. These traces are then summed (shown in blue) and subtracted off from the raw data trace to give the final data trace adjusted for any leak present in the recording (black).

Real action potentials were obtained from previous recordings conducted in P8 CD1 albino mice. This was performed by stimulating axon fibers at the midline of slices using
a bipolar electrode receiving impulses from an A-M Systems Model 2100 Isolated Pulse Stimulator synchronized with the patch clamp amplifier and scanning the MNTB fields for cells that exhibited voltage dependent activity by touching the surface of the cells with an electrode pipette. Once connected cells were located, they were patched onto and protocols for recording action potentials that propagate down the axon from the fiber stimulation were executed in current clamp mode, along with protocols to gather action potentials from current injection for comparative purposes. These experiments were done in the absence of blockers in order to obtain action potentials of the proper waveform; extracellular solution consisted of normal recording solution, and intracellular pipette solution consisted of the following (in mM): 125 potassium gluconate, 20 potassium chloride, 10 HEPES, 5 EGTA, 10 phosphocreatin, 4 ATP, and 0.3 GTP and was buffered to pH 7.3 using CsOH. Reagents used during all experiments were obtained from Sigma Aldrich, with the exception of TTX, which was obtained from Tocris Bioscience, a subsidiary of R&D Systems.

*Patch Optimization and Rejection Criteria*

The stability and quality of the membrane-pipette seal, as well as the access resistance (as described above) are extremely important and therefore they must meet certain criteria tailored based on the types of experiments being performed. This dictates what the rejection criteria must be in order to determine whether a given set of recordings can be included in the assessment of an effect being studied or if the quality of the recording is insufficient to warrant its inclusion. For studying activity on the order of a single action potential, a minimal delay and optimal control of the cell are essential to accurately elicit and examine activity. To this end, a series resistance cutoff of 5-12 MΩ for individual
pulse studies was used, while up to 20 MΩ was determined to be permissible for long pulse and action potential train testing of inactivation properties. A parasagittal orientation was utilized in all studies to limit axonal length, restricting the axon to a few hundred microns long as opposed to the coronal orientation, which often contains axons as long as several millimeters. By limiting this length, the space clamp error of the recording is effectively reduced, allowing for faster and more efficient control over the cell during recordings.

Previous work has demonstrated through two electrode voltage clamp recordings formed on the distal ends of fenestrations of the presynaptic terminal that currents injected through one electrode and recorded in the other exhibit a delay of about 100-200 µs\textsuperscript{44}. To ensure that stimuli and recorded currents were conducted optimally, presynaptic terminals were patched as close to the axonal heminode as possible (see Figure 15A and Figure 15B). This allows for symmetrical access to the terminal and helps to synchronize recorded currents, minimizing delay and distortion of responses. Neurons that appeared unhealthy during or immediately following the gathering data traces were not used in this study. Criteria for this decision included a transparency in the cell membrane, deterioration indicative of cell death surrounding the intended target neuron, and varicosity swellings along the axon visualized during the recording via fluorescent imaging. See Figure 15 for comparison of healthy and unhealthy neurons using post hoc fluorescent inspection.
Figure 15: Comparison of healthy and unhealthy neurons using fluorescent inspection
Healthy neurons (A, B, C) exhibit typical calyx morphologies both at the terminal and along the axon. Unhealthy neurons generally contain a large amount of blebs and deterioration around the terminal (D), and often contain varicosity swellings along the axon (D, E).

Gentle positive pressure (referred to as a "blowout") was applied to the pipette after the formation of a whole cell recording to clear residual membrane from the inside the pipette and improve overall access to the cell, reflected in the series resistance and cell capacitance in the form of an increase in the associated transient currents. Once cancelled as before, this process results in a significant reduction in the value of these parameters, indicating that residual membrane within the pipette after patch formation can function to reduce the access to the cell during recording. An example of this process can be found in Figure 16, where a fluorescent image of a patched presynaptic cell reveals some of the membrane and debris incorporated into the tip of the pipette during the process of
reaching a whole cell state, and how the tip looks following the careful application of pressure to clear out this excess.

Figure 16: Using the blowout technique to improve access to the cell.
A small amount of membrane in the pipette tip is shown after achieving a whole cell configuration (left). By applying a small amount of positive pressure through the pipette, this debris can be cleared (right). The improved access is apparent in the larger, faster voltage transients achieved (below).

Intracellular solutions were also formulated in a manner that maintained an osmolarity on the higher end of the acceptable range (roughly 320 mOsm); this was done to reduce obstructions at the pipette tip by generating a slight concentration gradient that would flow out of the pipette rather than in, serving to prevent the seal from occluding while promoting adequate perfusion of the pipette solution into the cell.
Stimulus Protocols and Data Analysis

As previously mentioned, calcium channels are commonly studied using prolonged step depolarizations as stimuli to assess facilitation, inactivation, and other channel properties, but studies using pAPs and other AP-like stimuli, such as small box wave pulses, have been more limited. Calcium channel dynamics on the scale of an action potential are quite different, and previous investigations have focused studying the effect of action potential repolarization and depolarization rates, as well as action potential shape in a more general sense. However, these works performed this analysis without regard for alterations in the overall amount of stimulus being provided. In studies conducted in this work, it was desired to normalize the protocols being injected to the integral of the provided stimulus to have a means to assess the elicited calcium response when the amount of channel activity is maintained relatively constant. This was performed using pAP stimuli and altering repolarization and depolarization profiles to determine how the $I_{Ca}$ was altered while the amount of stimulus was maintained. Since these waveforms are essentially geometric shapes, their integrals can be easily calculated by finding the area of the waveform.

Prolonged pulses are also useful for studying inactivation profiles of various channel activity. In this case, CDI is assessed in a comprehensive manner using box pulses on the order of 200 ms in conjunction with trains of action potential-like stimuli. Previous work has shown that action potential trains generate a greater amount of inactivity than similarly scaled long pulses in HEK cells. However, this preparation is highly artificial, and thus a similar analysis in a more physiological setting is warranted to better discern how CDI is modulated by different forms of stimulation. Inactivation of test pulses was
assessed using 5 ms box wave pulses to +30 mV before and immediately following test pulses. The integral and peak responses of these currents were measured and compared to determine the amount of reduction in activity that occurred as a result of the stimulus. This duration was long enough to allow the maximal activation of the calcium channels while short enough to prevent any appreciable level of inactivation.

Integrals and peaks of calcium responses were measured using Wavemetrics' Igor Pro with its built-in unipolar peak/area detection protocols. Data traces were loaded into the program from PatchMaster using Patcher's Power Tools, an expansion pack for Igor Pro written by Dr. Francisco Mendez and Frank Würriehausen of the Max-Planck Institute. To initiate the analysis, the baseline of the data trace is set by the experimenter; in the case of the experiments conducted in this work, most traces had a baseline at or very close to 0 nA, with some variance based on seal properties. Next, a peak threshold was set and used by the software to determine regions where peaks occurred, which in this case corresponded to calcium channel responses. Due to the fact that calcium activity takes for form of an inward currents, the polarity of the measurements were also set to a negative orientation such that the software would look for the minimum values for peaks rather than maximums. Finally, measurements were initiated and the software was used to determine where the peak threshold is exceeded and the beginning and end of the response is located by detecting where the data trace passes through the baseline before and after that peak. The minimal value below the peak threshold is measured and determined to be the peak amplitude of the response. The integral of the region from the peak of the response to the baseline between the start and end of the response is calculated using a trapezoidal rule method between each point contained in the region.
These outputs are generated in a spreadsheet, which is then exported to Microsoft Excel for further analysis. A depiction of this procedure is shown in Figure 17.

Figure 17: Peak and integral measurement using Igor Pro.
The calcium response to a 3 ms depolarization from -80 mV to 30 mV is used for this demonstration. The baseline of the response is detected on the base on activity surrounding the pulse. The peak threshold is set, and minimum values below that threshold are reported, along with points where the response crosses the baseline and the integral of the region under the curve to the baseline.

Once exported, the data for each experiment type was collected for comparative and statistical analysis using Microsoft Excel. Data values were expressed as the mean ± the standard error of the experimental sets being analyzed. Statistical significance was assessed using unpaired Student's t-test with p value cutoff of 0.01.
Results and Discussion

*Investigating calcium activity by modulating the action potential waveform*

Action potential waveforms can be used to establish a better understanding of how depolarization and repolarization kinetics of the action potential affect the influx of calcium into the nerve terminal through voltage-gate calcium channels (VGCCs)\(^{44,47}\). However, previous studies varied the depolarization and repolarization kinetics over a range of values, thus altering the durations of the stimulus templates. This makes it difficult to compare how changes in kinetics for a specified action potential duration affect the VGCC response. The kinetics and amplitude of an action potential exhibit significant effects on the resulting calcium response, and the duration of action potentials tend to be restricted to a limited range for a given type of neuron\(^{43}\). As previously mentioned, there is a non-linear relationship between the influx of calcium into the neuron across VGCCs and the corresponding release of neurotransmitter. Thus, even modest changes in the calcium current can generate significant differences in the postsynaptic response. Therefore, it’s informative to determine how action potential kinetics for a given action potential duration affect the calcium channel response.

To better understand how the amplitude and kinetics of the action potential waveform modulate calcium channel activity, an assortment of test pulses were generated and tested at stimulus amplitudes of 0, +30, and +60 mV, with +30 mV representing the near-physiological level. These waveforms at each amplitude tested were normalized by their stimulus area in order to maintain a consistent amount of overall current per pulse injected into the neurons. This allows for more meaningful comparisons between the elicited activity of each of the waveforms tested. These waveforms are shown in Figure
and included a symmetric triangular wave (Symmetric), a box wave (Box Wave), a triangular wave where depolarization occurs over the course of 1 ms followed by an instant repolarization to the baseline (1ms Depol), a triangular wave where depolarization is instant and repolarization occurs over 1 ms (1ms Repol), a triangular wave where the orientation is designed to mimic that of a typical action potential in the calyx (Physiological), and a wave with quick depolarization and repolarization where the stimulus is held briefly at its peak amplitude (Plateau). The timing information for these pulses can be found in Table 1.

Table 1: Parameters for defining shapes of stimulus waveforms

<table>
<thead>
<tr>
<th>Waveform</th>
<th>Depolarization Duration</th>
<th>Peak Duration</th>
<th>Repolarization Duration</th>
<th>Total Pulse Duration</th>
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</thead>
<tbody>
<tr>
<td>Symmetric</td>
<td>0.50 ms</td>
<td>0 ms*</td>
<td>0.50 ms</td>
<td>1.00 ms</td>
</tr>
<tr>
<td>Box Wave</td>
<td>0 ms*</td>
<td>0.50 ms</td>
<td>0 ms*</td>
<td>0.50 ms</td>
</tr>
<tr>
<td>1ms Depol</td>
<td>1.00 ms</td>
<td>0 ms*</td>
<td>0 ms*</td>
<td>1.00 ms</td>
</tr>
<tr>
<td>1ms Repol</td>
<td>0 ms*</td>
<td>0 ms*</td>
<td>1.00 ms</td>
<td>1.00 ms</td>
</tr>
<tr>
<td>Physiological</td>
<td>0.30 ms</td>
<td>0 ms*</td>
<td>0.70 ms</td>
<td>1.00 ms</td>
</tr>
<tr>
<td>Plateau</td>
<td>0.25 ms</td>
<td>0.25 ms</td>
<td>0.25 ms</td>
<td>0.75 ms</td>
</tr>
</tbody>
</table>

*Instantaneous (0 ms) shifts are not truly instant due to slight delays in the electronics and charging of the membrane.

The rationale behind the selection of these waveforms was to perform an initial assessment of various repolarization and depolarization kinetics while also comparing the frequently used box wave stimulus to pseudo-action potential (pAP) waveforms. Similar pAP waveforms have been used in previous studies\(^44,47\), but, as described earlier, the durations and overall current injection of these pulses has varied, making comparisons between the corresponding \(I_{Ca}\) responses difficult. The test pulses were injected at the
three different voltage amplitudes and compared. A representation of the stimulus waveforms and their corresponding $I_{Ca}$ response is shown in Figure 18.
Figure 18: Initial assessment of calcium response generated by a variety of different \( pAP \) waveforms of equivalent stimulus area

All test pulses were injected to peak voltage values of 0 (red), +30 (black), and +60 mV (blue) and are presented with their corresponding \( I_{Ca} \) response. Stimuli for a given peak voltage are area conserved. A, Symmetric \( pAP \) stimulus consisting of a 0.5 ms depolarization followed by a 0.5 ms repolarization. B, Box wave stimulus consisting of an instant depolarization to the desired voltage, maintained for 0.5 ms, followed by an instantaneous repolarization to baseline. C, 1 ms Depol template consisting of a 1 ms depolarization phase followed by an instantaneous repolarization to baseline. D, 1 ms Repol template consisting of an instant depolarization to desired voltage followed by a 1 ms repolarization to baseline. E, Physiological \( pAP \) stimulus consisting of a 0.3 ms depolarization followed by a 0.7 ms repolarization to baseline. F, Plateau wave stimulus consisting of a 0.25 ms depolarization to desired voltage, maintained for 0.25 ms, followed by a 0.25 ms repolarization to baseline.
The +30mv test pulses are closest to the physiological action potential amplitude typically observed in the calyx of Held. Therefore, additional comparisons between the $I_{\mathrm{Ca}}$ responses to these waveforms were made. By overlaying the calcium response from each of these waveforms, differences can be more easily inspected (Figure 19).

Figure 19: Comparison of calcium response to different stimulus waveforms at 30 mV
Overlay of the $I_{\mathrm{Ca}}$ response to each of the test pulse waveforms from Figure 18 for the +30 mV peak voltage case.

Often, a box wave of 1 ms is used as action potential-like stimulus to activate VGCCs because it produces a postsynaptic response that is close in amplitude to an action potential induced postsynaptic response. However, Figure 19 demonstrates that a box wave of half that duration still produces a much larger amount of calcium activity than the physiologically oriented pAP waveform. While this indicates that the calcium level achieved by an action potential waveform is less than the amount of calcium required to produce a full size postsynaptic response, it should be noted that the VGCC kinetics in response to a box wave are different from that of waveforms that more closely approximate an action potential. Future experiments will be done to test this directly by
using paired recordings to measure the postsynaptic response to the pAP waveforms described in this work. Interestingly, though previously the repolarization rate was thought to be the most significant contributor to the calcium response, the 1ms Repol waveform elicited the lowest peak $I_{\text{Ca}}$. This suggests that the relationship between the depolarization and repolarization rates of a fixed duration action potential may be more complicated in determining how it functions to influence calcium channel activity. In order to better understand the resulting $I_{\text{Ca}}$ responses of these various waveforms, the calcium response duration, peak amplitude, and integral were measured, averaged, and plotted; these results are shown in Figure 20A, Figure 20B, and Figure 20C, respectively.

The duration of the $I_{\text{Ca}}$ response remained relatively constant for a given waveform for each of the peak voltages tested (Figure 20A), with the exception of the 1ms Depol waveform, which showed a large change. The peak calcium amplitude activity for the different pAP waveforms shows an approximately linear increase as the peak amplitude increases from 0 to +30 mV (Figure 20B), with the exception of the Box Wave and Plateau waveforms. These two waveforms both reach a peak amplitude at lower peak stimulation voltages. Specifically, at the +30mV test pulse their response surpasses the peak amplitude reached by the other waveforms even when they are tested at +60mV. At a stimulus voltage of +60 mV, the Symmetric, Physiological, and 1ms Repol waveforms begin to elicit a larger calcium influx (Figure 20C) into the cell than the Box Wave and Plateau waveforms, despite the larger peak currents for the Box and Plateau waveforms. This suggests that at the higher peak voltage of +60 mV the pAP waveforms with a rapid depolarization (1ms Repol, Physiological, and Symmetric) along with a slower repolarization elicit the maximal calcium influx. This is likely due to the membrane
potential quickly reaching a voltage capable of causing near maximal VGCC opening, followed by a slow enough repolarization to maximize the duration of open channel activity.

Figure 20: Analysis of how different stimulus waveform effects calcium response at various voltages
All data is presented as the average across all neurons tested in the experiments shown in Figure 18 (n=5) for each of the tested waveforms. A. Duration (in ms) of the $I_{\text{Ca}}$ response corresponding to each of the test waveforms measured at the baseline. B. Peak amplitude (in nA) of the $I_{\text{Ca}}$ response corresponding to each of the test waveforms measured as the distance from the baseline. C. Calcium integral (in nA*ms) of the $I_{\text{Ca}}$ response corresponding to each of the test waveforms measured as the area beneath the curve using Igor Pro protocols.
To further analyze the effects of depolarization and repolarization kinetics on the calcium response, an additional set of protocols were constructed to assess this over an array of different action potential orientations. The first wave of this series was a pAP with an instant (0 ms) depolarization, followed by a 1 ms repolarization. The duration of the depolarization of each subsequent pulse was then increased by 0.1 ms, while the repolarization was decreased by the same amount, until the opposite orientation was achieved (1 ms depolarization followed by a 0 ms repolarization). This produced eleven pAP waveforms shifting through the range of possible orientations between a full 1 ms repolarization and a full 1 ms depolarization. By using the same total duration for each of these waveforms, the stimulus area is held constant for each wave in this series. These pAP waveforms were tested at 0, +30, and +60 mV as before, and are shown with the corresponding calcium responses in Figure 21.
Figure 21: Changes in the calcium channel response resulting from shifts in the orientation of an action potential-like stimulus

Test pulses are shown transitioning from a 0 ms depolarization followed by a 1 ms repolarization (lightest trace) to a 1 ms depolarization followed by a 0 ms repolarization (darkest trace). Stimulus waveforms are shown above, with the corresponding $I_{Ca}$ responses plotted below. Waveforms were injected to peak voltages of 0 mV (A), +30 mV (B), and +60 mV (C). Dashed traces indicate the approximately physiological waveform (0.3 ms depolarization, 0.7 ms repolarization) and the associated response.
In order to analyze the calcium response to these various pAP orientations, the peak amplitude and response integral were measured. To allow comparisons between the responses for the three different peak amplitudes tested, the calcium responses were normalized to the maximum response amplitude for each waveform, averaged, and plotted in Figure 22. Based on the peak amplitude of the responses, an initial inspection indicates that orientations with a longer depolarization duration and a shorter repolarization duration are favored at all three stimulus voltages (Figure 22A). However, the opposite effect is seen for the total calcium influx which was measured by taking the integral of the calcium channel response, and normalizing the data for each waveform to the maximum influx. As shown in Figure 22B, the calcium influx into the cell is actually maximized when the repolarization phase is longer than the depolarization phase. Though the driving force for calcium entering the cell is not maximized and the peak response is smaller, a longer repolarization phase allows more calcium to flow into the nerve terminal, perhaps by optimizing the number of open channels, increasing the duration each channel is open and providing sufficient driving force. These results agree with the data presented earlier (Figure 18 and Figure 20), in that a balance between the duration of the depolarization and the subsequent repolarization provides an optimal response.
Figure 22: Analysis of different pAP orientations on calcium response at various stimulus voltages

Normalization for each plot was performed by normalizing data sets for each cell to their maximum peak amplitude (A) and calcium integral (B). All data is presented as the average of these normalized values across all neurons tested in the experiments shown in Figure 21 (n=10) for each of the tested waveforms. Error bars indicate the standard error of the data set for each individual waveform. The dashed vertical line in each graph indicates the pAP orientation closely approximating the physiological action potential.

The results shown in Figure 22 further demonstrate the relationship between the duration of the depolarization and repolarization phases for a fixed action potential stimulation area and duration. The repolarization rate determines the driving force of calcium into the cell through open calcium channels. In this experiment, because the duration of the
stimulus wave is conserved, longer repolarizations are associated rapid depolarizations. This allows a rapid activation of VGCCs followed by long repolarization that continues to activate calcium channels while progressing toward voltages that have a higher driving force for calcium entry. During a long depolarization, channel activation is slower and the driving force for calcium entry is steadily decreasing as the membrane potential heads toward the reversal potential for calcium flow. Depolarization rates, peak voltage, and the duration that the voltage is maintained all function to modulate the amount of calcium channels opening and how long the channels remain open. Thus, a balance must be maintained between the two phases in order to produce an optimal influx of calcium current. The 0, +30, and +60 mV responses reveal that the physiological waveform orientation is near the maximum calcium influx for all three stimulus voltages tested. This suggests that the action potential waveform in the calyx of Held is shaped to elicit an optimal calcium response across a range of peak voltage stimuli.

**Studying the effect of the afterdepolarization on calcium activity**

The afterdepolarization (ADP) has been shown to improve action potential fidelity and reduce latency time in the calyx of Held, but no studies have been conducted to determine if this resurgence of sodium channel activity and the preceding fast afterhyperpolarization (fAHP) have an appreciable impact on the function of calcium channels. Due to the non-linear nature of calcium-mediated exocytosis of neurotransmitter filled vesicles, small changes in the calcium current ($I_{Ca}$) have been shown to cause significant changes in neurotransmitter release$^{48}$. To investigate if depolarizations of the membrane potential following the action potential affect the $I_{Ca}$, voltage clamp protocols were constructed to assess the calcium response to pAP stimuli with varying levels and types of
depolarizations immediately following the action potential wave for 10 ms. To allow comparison, we start with a normal symmetric pAP consisting of a 0.5 ms depolarization and 0.5 ms repolarization in the absence of any depolarization following the AP wave, which is referred to as the down-state (Figure 23A).

Next, the same pAP waveform was repolarized to a membrane potential starting at -75 mV, and this depolarization was maintained for 10 ms, then ramped back to the holding potential over a 50 ms period (Figure 23B). The depolarization period following the pAP waveform is referred to as an ADP phase, and these waveforms collectively are referred to as the up-state. This stimulus was repeated 14 more times with the amount of depolarization in the ADP phase increasing in 5 mV increments, such that the final waveform repolarized to -5 mV. Finally, the pAP was followed with a physiological ADP reconstructed from action potential recordings from P8-12 mice. This type of waveform, referred to as the ADP-state waveform, consisted of a fAHP that repolarized the membrane potential to -75 mV, followed by a four phase ADP. The first phase consisted of a steep 1.5 ms depolarization, followed by a 1.5 ms leveling-off phase to reach the peak ADP amplitude, then a fast repolarization phase for 15 ms, and terminating with a slow repolarization phase to follow the approximate time course of the ADP. This was also modulated over a range of depolarization potentials for the ADP to assess what potential would have an effect on the calcium tail current elicited by the pAP. (Figure 23C).
Figure 23: Effect of afterdepolarization potentials on calcium response elicited by pseudo-action potential stimulus. Test traces are shown (i) as the stimulus template (red) and the corresponding calcium current (black). Below (ii), the boxed region in i is expanded for closer inspection. Initial test pulse waveform consists of a symmetric pAP. A, Down-state waveform; symmetric pAP with no ADP activity. B, Up-state waveform; symmetric pAP followed by 10 ms elevated voltages modulated from -75 to -5 mV in 5 mV increments and a 50 ms gradual return to baseline. C, ADP-state waveform; symmetric pAP followed by fAHP to -70 mV and ADP constructed to better mimic the kinetics observed in the calyx of Held, modulated from -75 to -5 mV in 5 mV increments. Cyan traces represent the stimulus waveform for depolarizing potentials -30 mV and associated calcium response in which an onset of inward current was observed as a result of the after potentials, typically corresponding to a voltage between -35 and -30 mV.
It was surprising that only at very depolarized potentials, starting around -35 to -30 mV (highlighted in blue in Figure 23B), do we see an effect on calcium channel activity in the form of a prolongation of the $I_{Ca}$, well above the physiological range of the ADP. Interestingly, the data shown in Figure 23C reveal that the fAHP in the ADP-waveform stimulus effectively isolates the $I_{Ca}$ generated by the pAP from any activity produced during ADP potentials, even for ADP amplitudes well beyond the physiological range. Instead, a separate, reactivation of calcium channels occurs at depolarized potential starting around -30 mV, which is expected given the VGCC activation threshold. Thus, this experiment demonstrates that the fAHP drives the membrane potential to a sufficiently repolarized state to fully terminate VGCC activity.

Given that the driving force for calcium entry increases at more negative potentials, it was surprising that the ADP did not affect the $I_{Ca}$. To further explore this observation, a different test was constructed using a longer initial voltage jump to normalize the amount of channel activation. In addition, we tested the effect of repolarization rates of this stimulus on the calcium response seen across a series of potentials representative of a range of ADP amplitudes in the absence of the fAHP, referred to as afterpotentials from this point forward. This was performed to better understand the effect of the driving force of calcium influx into the cell as an action potential repolarizes to increasingly depolarized levels. In this experiment, a 10 ms depolarization to +30 mV was injected into the cell, followed by four different test repolarization rates to various afterpotentials, where the cell was held until 10 ms following the onset of the repolarization phase, then returned to the holding potential (Figure 24A). A 10 ms pulse was used in order to ensure that the majority of calcium channels were activated in order to achieve a consistent
amount of activity between the different test pulses. The afterpotentials used in these experiments were kept below the activation threshold of calcium channels in the range of a hyperpolarized value of -100 mV, up to just below the activation threshold at -40 mV, and the value was increased in 10 mV increments. Repolarization rates that were investigated include an instant repolarization to the holding potential (Instant Repol; Figure 24Bi), as well as ramped repolarizations of 0.5, 1.0, and 2.0 ms in duration (0.5 ms Repol; Figure 24Bii, 1.0 ms Repol; Figure 24Biii, and 2.0 ms Repol; Figure 24Biv, respectively). The corresponding $I_{Ca}$ responses are plotted below each stimulus template showing just the tail current response during the repolarization phase.
Figure 24: Effect of repolarization rate and afterdepolarization potential on calcium current response.

A. Test pulses of 10 ms box stimulus to +30 mV with varied repolarization rates followed by a 10 ms potential ranging from -100 to -40 mV in 10 mV increments. 0 ms repolarization, black traces; 0.5 ms repolarization, red traces; 1.0 ms repolarization, blue traces; 2.0 ms repolarization, green traces.

B. Region of interest for test pulses and the corresponding I_{Ca} response. i, Instant Repol; 0 ms repolarization. ii, 0.5 ms Repol. iii, 1.0 ms Repol. iv, 2.0 ms Repol.
This test reveals a rate-dependent stabilizing effect in the repolarization that effectively buffers the tail current of the I_{Ca} from the ADP, even in the absence of a fAHP. As can be seen in Figure 24Bi, the Instant Repol template exhibits a broadening of the tail current, as well as a slight reduction in the peak amplitude; the difference between the tail current elicited during the repolarization to -100 mV and that of the -40 mV (PeakDiff) was 0.289 ± 0.043 nA. However, as the repolarization rate is increased, less broadening is exhibited, and the PeakDiff is held more constant, with values of 0.253 ± 0.029 nA, 0.090 ± 0.017 nA, and 0.030 ± 0.010 nA for the 0.5ms Repol, 1.0ms Repol, and 2.0 ms Repol templates, respectively, as shown in Figure 25A.

The broadening of the response that occurs as the afterpotential in the instant repolarization increases is accompanied by a rightward shift in the peak response as the kinetics of the I_{Ca} appear to change from increasingly depolarized afterpotentials. This shift is significantly larger in the case of the Instant Repol (0.113 ± .011 ms) than in the case of the ramped repolarizations (0.5ms Repol: 0.040 ± 0.014 ms; 1.0ms Repol: 0.027 ± 0.005 ms; 2.0ms Repol: 0.038 ± 0.006 ms), shown in Figure 25B. The broadening of the I_{Ca} response was quantified using a stability assessment where the time required for the current to return to baseline was measured. As the peak response broadened, this return to baseline required more time, shifting the point where the baseline was crossed. These results are shown in Figure 25C for each afterpotential, with the progression of the reduction of the peak I_{Ca} as the afterpotential is increased depicted in Figure 25D. Peak amplitudes and stability of the I_{Ca} were normalized to the first response (-100 mV afterpotential) and plotted to depict the relative amount of drift between the different repolarization rates as the afterpotential is modulated.
Figure 25: Analysis of effects observed on calcium response from various repolarization rates
All data presented represent the average across all neurons tested in these experiments (n=8) with error bars denoting the standard error of the data sets. A, Peak amplitude difference between initial response (-100 mV afterpotential) and the final response (-40 mV afterpotential). When compared to the Instant Repol, the 0.5 ms Repol showed no significant different (p=0.54), while the 1.0 ms Repol and 2.0 ms Repol showed a statistically significant difference (p<0.01). B, Rightward shift in the peak timing between initial response (-100 mV repolarization potential) and the final response (-40 mV repolarization potential). Relative to the Instant Repol, the 0.5 ms Repol, 1.0 ms Repol, and 2.0 ms Repol tests all showed a statistically significant difference (p<0.01). Normalization was performed for each dataset to the first I_{Ca} response (-100 mV afterpotential) for the stability measurement (C) and peak amplitude (D) and plotted across the range of repolarization potentials.

These experiments reveal that the calcium influx generated by different repolarization rates is modulated by the afterpotential even for values below the deactivation threshold of calcium channels if the repolarization is sufficiently fast. However, as the repolarization duration increases, the I_{Ca} kinetics are less affected by the afterpotential, resulting in a stabilized response and preventing the broadening and reduction in the peak
amplitude of the tail current. This indicates that the action potential kinetics may be optimized in a manner that prevents the ADP from significantly effecting calcium activity while still functioning to promote action potential fidelity during bursts of activity. To test this hypothesis, a similar set of experiments was constructed using action potential-like stimuli to determine if this effect holds when the activity is on the scale of a single action potential. Stimulus templates included a 1ms box wave to assess the case of the fast repolarization, as well as a 1ms symmetric pAP and a 1ms physiological pAP constructed as described previously (see Table 1). The afterpotential was varied within the range of -100 to -40 mV in 10 mV increments for 10 ms following the repolarization phase as performed in the previous set of experiments. A representation of these stimulus templates and the resulting $I_{Ca}$ response can be seen in Figure 26.
Figure 26: Studying the repolarization of action potential like stimuli and how the afterdepolarizations alters the calcium response. Test pulses from previous section were used, including the Box wave (A), Symmetric pAP (B), and Physiological pAP (C) with all test pulses reaching a peak voltage of +30 mV, followed by repolarization to afterpotentials from -100 to -40 mV in increments of 10 mV, similar to that of Figure 24. Stimulus traces are shown above (red), with the corresponding $I_{\text{Ca}}$ response shown below (black).
In order to assess the I\textsubscript{Ca} response from these test templates, the integral of the calcium current was measured to determine how much calcium influx was occurring as a result of these stimuli. These values were normalized to the first response (afterpotential of -100 mV, as before) and plotted, as shown in Figure 27. Due to the broadening of the I\textsubscript{Ca} exhibited as the afterpotential is increased in the case of the box wave, the calcium integral shows a steady increase in the amount of calcium entering the cell, despite a slight reduction in the amplitude. This occurs at levels below the inactivation threshold of calcium channels, and the total current increases despite a decrease in the driving force, suggesting that the afterpotentials act to prolong the open state and/or increase the number of open calcium channels. However, both the symmetric and physiological pAP waveforms exhibit almost no modulation in the calcium integral with no appreciable broadening or amplitude shift in the I\textsubscript{Ca} until the afterpotential approaches the calcium deactivation threshold (-40 to -30 mV). The ramped repolarizations of these pAP waveforms (Figure 26B and Figure 26C) act to increase the duration of the calcium current, as well as decrease the variability in the kinetics and timing of the peak calcium current as the afterpotential voltage increases. Additionally, there might be a delay in the response time if voltage sensing regions of the VGCCs do not immediately move uniformly in response to an instantaneous voltage change, and therefore a gradual voltage change during the repolarization could act to reduce the variability in the closing probability, which is a function of time and voltage, during deactivation of the channel.
Figure 27: Calcium influx elicited by various pAP waveforms followed by depolarized potentials
All data presented represent the average across all neurons tested in these experiments (n=9) with error bars denoting the standard error of the data sets. The calcium integral for each test pulse was normalized in each data set to the first response (-100 mV repolarization potential) before averaging.

The experiments described thus far have examined the response to individual AP waveforms. However, as previously mentioned, typical AP activity in the calyx of Held consists of bursts of activity rather than individual pulses. To determine whether overall calcium influx and amount of calcium channel inactivation was varied by the presence and absence of the ADP during prolonged bursts of activity, trains of pAP were constructed to test this. Trains of 100 symmetric pAP pulses to +30 mV at a frequency of 300 Hz were constructed with physiological representations of the down-, up-, and ADP-state test stimuli (Figure 28A, zoomed for clarity in Figure 28B). Calcium channel inactivation is thought to occur through channel activation but due to the short duration, action potentials only open a fraction, ~30 to 40%, of the total population of calcium channel. Therefore, there is a significant population of channels that are not activated by an AP stimulus, thus making it difficult to determine the amount of channel inactivation.
using AP waveforms. To resolve this, a 5 ms test pulse was used before and after the train of AP waveforms to measure calcium channel inactivation.

The calcium integral produced in each $I_{Ca}$ response to the pAP waveforms is plotted in Figure 29A; for clarity, only every fifth response is shown. The percentage of inactivation was defined as the difference between the first 5 ms $I_{Ca}$ response that occurred before the train of AP waveforms and the 5 ms test pulse that occurred after the train of APs, divided by the first 5 ms $I_{Ca}$ response ($[I_{Ca,Before} - I_{Ca,After}] / I_{Ca,Before}$). This calcium inactivation was plotted for each test pulse and is shown in Figure 29B. Additionally, the average amplitude of the responses to each test pulse were plotted in Figure 29C. Interestingly, for all three waveforms, based on the change in the calcium integral between the 5 ms pulses before and after the test pulse, approximately 22% of the calcium channels became inactivated during the pAP stimulation train despite an increase in the calcium channel response to the pAP waveforms. However, there were no significant differences in inactivation between the different test pulses.
Figure 28: Investigating the effect of down-, up-, and ADP-state stimuli on calcium activity pseudo-action potential trains

A. Test pulses consisting of a train of 100 symmetric pAP pulses followed by down-state (black), up-state (red), and ADP-state (blue) afterpotentials, preceded and followed by 5ms box pulses to +30 mV for inactivation assessment. B. Boxed region in A magnified to demonstrate the difference in the stimulus trains. C, I_{Ca} response to the test pulses with corresponding color scheme. D, Boxed region in C magnified to demonstrate the similarity of each response. E, Boxed region in D magnified to further clarify the similarity of the I_{Ca} between the different test pulses for a single response.
Figure 29: Analysis of calcium response elicited by pAP trains with various ADP states
These data represent the average across all neurons tested in these experiments (n=12) with error bars denoting the standard error of the data sets. The peak amplitudes and integrals of each I_{Ca} response during the pAP trains were normalized to the first 5 ms pulse and averaged across all data sets. A, Calcium integral for each response during the pAP train (note every 5th response shown). B, Percentage of inactivation from each test pulse calculated, as described in the test, by the difference between the 5 ms pulses before and after the train of pAPs divided by the first 5 ms pulse; no significant difference was found between the down-, up-, and ADP-state tests (p>0.80). C, Average normalized peak calcium amplitude throughout the response; no significant difference was found between the down-, up-, and ADP-state tests (p>0.95).

Although no significant differences were found between the various afterdepolarization states, it is important to keep in mind that slight changes in calcium channel activity are capable of producing notable variances in the amount of neurotransmitter release and the resulting postsynaptic response\textsuperscript{46}. Thus, further testing of these test waveforms is desirable using simultaneous pre- and postsynaptic patch clamp recording techniques to determine whether there is any modulation in the postsynaptic response.
Understanding the correlation between stimulus properties and calcium channel inactivation

Calcium inactivation has previously been studied extensively using prolonged depolarizations, but investigation of inactivation generated by action potential trains has been more limited. One group has reported that VGCC inactivation generated by action potentials show greater inactivation than the amount of inactivation generated by a long pulse with a matched stimulation area\(^{41}\). However, this was performed in HEK cells transfected to express only one subtype of calcium channel at a time, and auxiliary subunits likely do not match those in the calyx, thus it was decided to test this finding in the calyx. To accomplish this, trains of 1ms and 2ms symmetric pAP stimuli were constructed (Figure 30B and Figure 30C, respectively) and normalized to the overall amount of stimulus provided by maintaining an equivalent stimulus area. Additionally, a long box wave (Long Pulse) was also manufactured with an equivalent stimulus area (Figure 30A). Parameters for these pulses can be viewed in Table 2. The injected currents and their associated calcium response are shown in Figure 30. For the pAP trains (pAPT), a frequency of 300 Hz was chosen, as it has been reported to be near the maximal firing frequency for prolonged activity of the calyx of Held\(^{21}\) and will thus portray inactivation at the upper threshold of physiological activity levels.

<table>
<thead>
<tr>
<th>Test Pulse</th>
<th>Depolarized Voltage (mV)</th>
<th>Stimulus Duration (ms)</th>
<th>Number of Stimuli</th>
<th>Overall Stimulus Area (mV*ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ms pAPT</td>
<td>+30 mV</td>
<td>1.00 ms</td>
<td>300</td>
<td>16,500</td>
</tr>
<tr>
<td>2 ms pAPT</td>
<td>+30 mV</td>
<td>2.00 ms</td>
<td>150</td>
<td>16,500</td>
</tr>
<tr>
<td>Long Pulse</td>
<td>0 mV</td>
<td>206.25 ms</td>
<td>1</td>
<td>16,500</td>
</tr>
</tbody>
</table>
Figure 30: Inactivation assessment of area normalized stimulus of long pulse and pAP trains

Test pulses are preceded and followed by 5 ms box depolarizations to +30 mV to measure inactivation. Stimulus templates are shown above (red), with the corresponding $I_{Ca}$ below (black). A, Long box wave test pulse consisting of a 206.25 ms box depolarization to 0 mV. B, 300 Hz train of 300 symmetric 1ms pAPs pulses with a peak voltage of +30 mV. C, 300 Hz train of 150 symmetric 2ms pAPs pulses with a peak voltage of +30 mV.

Inactivation was assessed as before, using 5ms depolarizations to +30 mV before and after the test pulses and comparing the difference; results are shown in Figure 31A. Additionally, the overall amount of calcium influx for each test pulse was found by calculating the integral of each current response. These data were plotted in Figure 31B. In the case of pAPTs, the total amount of activity was evaluated as the sum of activity of
each response, while for the long pulse (LP) it was taken as the integral over the course of the response to the stimulus. Inactivation of calcium channels from the pAPTs was found to be significantly less than that of the long pulse, with the 1ms pAPTs showing the least inactivation (29.4% ± 8.4%), followed by the 2ms pAPTs (44.8% ± 7.3%), while the long pulse demonstrated the largest percentage of inactivation (70.6% ± 7.2%). Interestingly, the amount of calcium influx into the cell showed no significant difference between the three test pulses (Figure 31B). It should be noted, however, that this is a difficult comparison to make because the calcium driving force into the cell shifts toward the maximal amount during the repolarization phase of a pAP waveform.

As seen in the previous set of experiments, the activity exhibited by the pAP trains during this experiment was not a good indicator of the amount of calcium channel inactivation. The 1ms pAP train exhibited no inactivation throughout the activity of the train itself, and in fact generally showed a small amount of facilitation. Conversely, the 2ms pAP train
demonstrated the initial calcium dependent facilitation (CDF), followed by a steady amount of inactivation throughout the test pulses. The peak amplitude of each response for the two cases is plotted in Figure 32. Since the 1ms pAP is a close approximation to real action potentials, these results suggest that the action potential activates less than 50% of the total activatable population which appears to reduce the amount calcium channel inactivation. This works to ensure a consistent and reliable calcium response from action potential stimulation and thus preserve a steady release of neurotransmitter.

![Figure 32: Peak response profile of calcium activity elicited by pAP trains](image)

For both sets of data, the peak amplitude of each $I_{Ca}$ response throughout the train was normalized to the amplitude of the 5 ms pulse that preceded. Values were averaged for each neuron and plotted.

In the process of analyzing this data, it became apparent that this comparison between a prolonged box pulse and action potential trains on the basis of stimulus area may not be an accurate comparison to make, which applies to this work and the work in at least one previous study\textsuperscript{41}. Since VGCC activation begins at approximately -40 mV, only portions of action potential stimulus above this threshold will cause an appreciable amount of calcium channel opening, as shown earlier (see Figure 23C for an example, as the ADP
waveform increases in amplitude). Furthermore, the driving force for calcium influx into the neuron is changing throughout the course of the action potential, while it remains constant in the case of the box wave. Therefore, it is difficult to compare the responses to the AP waveforms with the response to a prolonged box wave stimulus. As shown previously, regions of the action potential stimulus above around -40 mV are the only portion of the stimulus that have a large impact on calcium channel activity. Therefore the total area of the action potential stimulus may be an overestimation of the total amount of stimulation that results in calcium activity.

To resolve this, an additional experiment was constructed for a more meaningful comparison between long box waves and bursts of activity. Rather than using pAP trains, the test pulse was made up of a series of 2.5 ms box waves depolarized to 0 mV. Eighty three of these waves were included in the test pulse, such that the overall stimulus area would remain equivalent to that of the 0mV long pulse previously tested. This essentially breaks the long pulse apart into individual stimuli, allowing for a better comparison of the two types of stimulation. To determine how significant the space between each pulse was in modulating the amount of inactivation, two different sets of stimuli were constructed. The first had a short inter-pulse interval (IPI) of 1.5 ms (Figure 33A), while the second had a longer IPI of 10 ms (Figure 33B).
Figure 33: Inactivation resulting from repetitive box wave pulses normalized to stimulus area
Test pulses are flanked by 5 ms box depolarizations to +30 mV to measure inactivation. Stimulus templates are shown above (red), with the corresponding $I_{Ca}$ responses below (black). Trains of 83 box waves, each at 2.5 ms in duration with a peak voltage of 0 mV, were used. A, Repetitive box stimuli with a short (1.5 ms) IPI. B, Repetitive box stimuli with a long (10 ms) IPI.

By comparing the calcium channel inactivation of this experiment, as in previous experiments, it is clear that trains of activity function to inhibit calcium channels less than prolonged depolarizations. Furthermore, the amount of inactivation decreases as the space between these pulses increases, (see Figure 34A) such that long IPI trains show the least amount of inactivation, followed by the short IPI train, while the long pulse exhibits the largest amount of inactivation. Additionally, the amount of calcium influx into the cell increases as the IPI increases. This indicates that calcium channels are able to recover from inactivation between pulses during trains of activity. To correct for the tail current
produced as each pulse repolarized, the area of each tail current was measured and subtracted off from the total area of the response for that pulse to produce the adjusted calcium integrals that only account for the inward current of each pulse. Both of these conditions are shown in Figure 34B.

![Figure 34: Analysis of inactivation and overall calcium influx produced by various prolonged stimuli](image)

All data presented represent the average across all neurons tested in these experiments (n=12) with error bars denoting the standard error of the data sets. **A**, Percentage of calcium channel inactivation generated by each test pulse; the long pulse (LP), short interpulse interval (IPI) box wave train, and long IPI box wave train showed a statistically significant difference from one another (p<0.01). **B**, Total calcium response integral over the course of the test pulse, representing the sum of each individual pulse response in the case of box wave trains. The short and long IPI box wave trains exhibited a significant difference from the long pulse test stimulus (p<0.01). However, upon removing the area associated with the tail current from each response (adjusted), the difference between the three test pulses was not statistically significant (p>0.10).

These results demonstrate that as the time between pulses is increased, the amount of calcium channel inactivation is decreased without significantly effecting calcium influx into the neuron. Furthermore, this validates results shown in the previous experiment, suggesting that action potentials function to elicit enough calcium channel activity to release neurotransmitter in a manner that does not inactivate a large portion of the calcium channels. Since the action potential only activates less than 50% of the VGCCs,
this leaves a population of channels that are not activated following each action potential stimulation. This may serve as a safety factor for allowing a sufficient response later in the stimulation train when a higher number of VGCCs have been inactivated.

**Conclusions and Future Work**

The findings presented in this work allow us to better understand how various components of the action potential waveform function to effect calcium influx into the cell and modulate neurotransmitter release. In the physiological range of voltage stimulus, the kinetics of the action potential maximize the calcium influx into the cell when a fast depolarization phase is followed by a somewhat longer repolarization. If the depolarization is instantaneous it does not appear to activate as many calcium channels, thus a duration of 0.3 to 0.4 ms is optimal. Longer repolarization durations function to prolong calcium channel activity and steadily increase the driving force in order to allow more calcium into the cell. Thus, repolarization durations around 0.6 to 0.7 ms are optimal for VGCC activation. While these values pertain specifically to the assessment conducted in this work using standardized stimulus area and duration, the ratio of the relationship should hold in physiological settings. Thus, a repolarization-depolarization ratio between 1.5-2.3 appears to be the most ideal.

ADP activity following the action potential waveform was shown to alter calcium channel activity below the deactivation threshold of calcium channels when the repolarization phase was sufficiently fast. However, the presence of the fAHP appears to be sufficient to deactivate calcium channels at physiological repolarization rates, functioning to prevent the ADP from having a significant effect on calcium channel
activity. This was also shown to be the case for trains of action potential stimuli, which showed no significant difference in calcium channel inactivation or influx between waveforms with and without ADPs. It is important to recall that due to the non-linear relationship between calcium currents and neurotransmitter release, changes in calcium channel activity that are difficult and perhaps impossible to measure by direct electrophysiological recording of the calcium current may be capable of generating appreciable changes in postsynaptic activity.

Trains of pAP stimuli were shown to elicit significantly less inactivation that broader pAPTs and prolonged box waves when the test pulses were normalized to the stimulus area. These pulses also did not show a significant difference in calcium influx into the nerve terminal measured by electrical recordings. Due to complications in this comparison, however, an additional assessment was conducted using repetitive box wave stimulation as a means of more appropriately normalizing for stimulus area and compare trains of activity to a prolonged depolarization. This supported previous findings, showing that calcium channel inactivation was reduced when trains of stimuli were used to elicit activity, and decreased as the interval between the pulses was increased. These results demonstrate that the action potential waveform is sculpted to activate a calcium channels in a manner that produced minimal inactivation in order to improve consistency of the calcium channel response and calcium influx into the cell.

Due to nonlinearities in the correlation between calcium influx into the cell and neurotransmitter release, further studies are required to determine how the observation made in this work relates to postsynaptic activity. Future recording involving simultaneous pre- and postsynaptic recordings will be performed to study the effects of
these stimuli on activity in the postsynaptic neuron. Also, in order to more appropriately compare trains of action potential stimuli to prolonged depolarization pulses, additional normalizations between these different stimuli will be conducted using portions of the action potential waveform that are more representative of regions where calcium activity is elicited. Conductance must also be considered since it is constant throughout the prolonged depolarization pulses, while action potential waveforms function to shift the conductance of calcium into the cell throughout the pulse.
Reference


6 Sheng, Jiansong; He, Liming; Zheng, Hongwei; Xue, Lei; Luo, Fujun; Shin, Wonchul; Sun, Tao; Kuner, Thomas; Yue, David T; Wu, Ling-Gang (2012) Calcium-channel number critically influences synaptic strength and plasticity at the active zone. Nature Neuroscience 15(7): 998-1008.


29 Grieco, Tina M; Malhotra, Jyoti D; Chen, Chunling; Isom, Lori L; Raman, Indira M (2005) Open-Channel Block by the Cytoplasmic Tail of Sodium Channel β4 as a Mechanism for Resurgent Sodium Current. Neuron 45(2): 233-244.

30 Kaczmarek, Leonard K; Bhattacharjee, Arin; Desai, Rooma; Gan, Li; Song, Ping; von Hehn, Christian A A; Whim, Matthew D; Yang, Bo (2005) Regulation of the timing of MNTB neurons by short-term and long-term modulation of potassium channels. Hearing Research 206: 133-145.

31 Cladwell, John H; Schaller, Kristin L; Lasher, Robert S; Peles, Elior; Levinson, S Rock (2000) Sodium channel Na,1.6 is localized at nodes of Ranvier, dendrites, and synapses. Proceedings of the National Academy of Sciences of the United States of America 97(10): 5616-5620.

32 Leao, Ricardo M; Kushmerick, Christopher; Pinaud, Raphael; Renden, Robert; Li, Geng-Lin; Taschenberger, Holger; Spirou, George; Levinson, S Rock; von Gersdorff, Henrique (2005) Presynaptic Na⁺ Channels: Locus, Development, and Recovery from Inactivation at a High-Fidelity Synapse. Journal of Neuroscience 25(14): 3724-3738.

34Elezgarai, I; Diez, J; Puente, N; Azkue, J J; Benitez, R; Bilbao, A; Knopfel, T; Donate-Oliver, F; Grandes, P (2003) Subcellular localization of the voltage-dependent potassium channel Kv3.1b in postnatal and adult rat medial nucleus of the trapezoid body. Neuroscience 118(4): 889-898.


39Forsythe, Ian D; Tsujimoto, Tetsuhiro; Barnes-Davies, Margaret; Cuttle, Matthew F; Takahashi, Tomoyuki (1998) Inactivation of Presynaptic Calcium Current Contributes to Synaptic Depression at a Fast Central Synapse. Neuron 20: 797-807.


42Leao, R M; von Gersdorff, H (2009) Synaptic vesicle pool size, release probability, and synaptic depression are sensitive to Ca2+ buffering capacity in the developing rat calyx of Held. Brazilian Journal of Medical and Biological Research 42: 94-104.


45 Wu, Xin-Sheng; Sun, Jian-Yuan; Evers, Alex S; Crowder, Michael; Wu, Ling-Gang (2004) Isoflurane Inhibits Transmitter Release and the Presynaptic Action Potential. Anesthesiology 100: 663-670.

