Activation of sodium-activated potassium channels by sodium-influx

Travis Allen Hage
Washington University in St. Louis

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Activation of Sodium-Activated Potassium Channels by Sodium Influx

by

Travis Allen Hage

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT OF THE DISSERTATION

Activation of Sodium-activated Potassium Channels by Sodium Influx

By

Travis Allen Hage

Doctor of Philosophy in Neurosciences

Washington University in St. Louis, 2012

Professor Lawrence Salkoff, Chairperson

Sodium-activated potassium channels (K_{Na} channels) are a small family of high-conductance K^+-channels activated by increases in the intracellular sodium concentration. K_{Na} channels are broadly expressed in the nervous system, but the role, and even existence, of K_{Na} channels has been overlooked or doubted by neurophysiologists for many years. In the face of evidence to suggest a role for K_{Na} channels in normal physiology, resting Na^+ concentrations are lower than reported to be sufficient for activation of K_{Na} channels. This contradiction coupled with methodological shortcomings of some investigations into K_{Na} channels tempered acceptance of a contribution of K_{Na} channels in normal conditions. The goal of my dissertation research has been to determine if K_{Na} channels are participants in the normal physiology of neurons.

I have demonstrated the prominent activation of K_{Na} channels by sodium influx under normal physiological conditions in neurons of the rat central nervous system. Large K_{Na} currents were revealed by preventing sodium influx using multiple
experimental manipulations in neurons prepared from multiple brain regions.

Transfection of neurons with siRNA directed against Slack was used to demonstrate its contribution to the encoding of $K_{Na}$ channels.

Many of my experiments emphasize the importance of the persistent sodium current ($I_{NaP}$) as a source of sodium to activate $K_{Na}$ channels. One line of these experiments entailed the recording and measurement of the activity of individual $K_{Na}$ channel in membranes isolated from neurons. This work is the first demonstration of $K_{Na}$ channel activation by sodium-influx using single channel recordings. I also present results demonstrating that $I_{NaP}$ is active across a broad range of voltages, including membrane resting potentials, using both whole cell recordings as well as recordings of individual sodium-channels from isolated patches.

Finally, I’ve demonstrated that after blocking sodium influx, the resulting decrease in $K_{Na}$ channel activity is a slow process ($\tau \sim 13$ seconds). I developed a strategy to isolate $K_{Na}$ channel activity in neurons by taking advantage of this slow decrease in $K_{Na}$ channel activity. Isolation of neuronal $K_{Na}$ currents has not previously been possible due to a lack of specific antagonists for $K_{Na}$ channels.
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Chapter 1

Introduction
The fundamental property of neurons is electrical excitability. Differences in the electrical properties of neurons are evident when comparing neurons from different brain regions, developmental stages, and disease states. These differences arise from the unique expression and regulation of ion channels by a neuron. The sequencing of whole genomes has allowed identification of hundreds of ion channels, many of which have been well studied as isolated molecules. Despite this wealth of information, we are still far from a complete understanding of the electrical properties of neurons. One of the primary reasons for this knowledge gap is a relatively undeveloped understanding of how ion channels interact with one another and fit into the context of the variety of channel types expressed in a cell. My thesis work makes a significant step forward in this regard by demonstrating the activation of sodium-activated potassium channels by sodium-influx through the persistent sodium current. In this introductory chapter, I will provide a brief overview the study of ion channels in neuroscience, before describing the history of sodium-activated potassium channels, the relevant aspects of persistent sodium currents and measurements of the dynamics of sodium concentrations within cells.

**Ion channels and neurophysiology**

Hodgkin and Huxley’s experiments utilizing squid giant axon form the basis of our understanding of action potential generation (Hodgkin & Huxley, 1952). They described two currents in squid giant axon. The first was a transient, voltage-dependent sodium current responsible for the depolarizing phase of the action potential. The second, a voltage-dependent, sustained potassium current that is responsible for the repolarizing
phase of the action potential. This is now known to be a simple complement of ion channels. While the squid giant axon is highly efficient at propagating action potentials in an all-or-none manner across long distances, the system is not very dynamic. In response to a wide range of amplitudes of current injection, the frequency of action potentials generated by squid giant axon displays little variation (Guttman & Barnhill, 1970). By comparison, many other neurons are highly dynamic. The frequency and pattern of action potentials can vary greatly with stimulus strength and the properties of neuronal excitability are often plastic. Furthermore, in addition to the all-or-none, anterograde propagation of action potentials, many neurons all exhibit retrograde propagation of action potentials that can influence transmitter release at dendrite-to-dendrite synapses and synaptic plasticity. An amazing diversity of ion channels underlies the great complexities of neuronal behavior.

Ion channels are primarily grouped by the ions they are selective for, such as Na\(^+\), Ca\(^{2+}\), K\(^+\) and Cl\(^-\) channels. Ion channels also differ greatly in their gating properties and this serves as the most common means of classifying channel subtypes. The opening and closing of many channels is dictated by the transmembrane voltage, while other channels are gated by binding of specific compounds to the channel. Ion channels also differ in their single channel conductance, or the amount of current carried by an open channel at a give voltage, and this has also served as a means of distinguishing channels.

The largest and most diverse class of ion channels is the voltage-gated K\(^+\) channel (K\(_V\) channel). K\(_V\) channels are composed of 4 pore-forming proteins, called \(\alpha\)-subunits. Each protein contains 6 transmembrane domains (Figure 1). The voltage-sensitivity of
the channel is conferred by the 4th transmembrane helix (S4) of each α-subunit. KV channels vary in their sensitivity to changes in membrane voltage and kinetics of gating. The delayed-rectifier K⁺ current described by Hodgkin and Huxley, and present in most neurons, displays little decline in channel activity over the course of a sustained depolarization. 20 years after Hodgkin and Huxley characterized the delayed rectifier K⁺ current, transient K⁺ currents (IKA) were described (Connor & Stevens, 1971; Neher, 1971). In response to a depolarizing voltage step, IKA typically activates more rapidly than IKDR and subsequently inactivates. Through cellular physiology and genome sequencing it has been determined that the mammalian genome contains approximately 40 genes encoding pore-forming subunits of the KV family. KV channels have been grouped into physiological subfamilies based on their voltage-sensitivity, kinetics of activation, inactivation and deactivation. The 40 genes have been grouped into 12 classes based on sequence homology.

Many ion channels are gated by binding of chemical ligands to extracellular or intracellular domains. Regarding extracellular ligands, ionotropic glutamate and γ-aminobutyric acid (GABA) receptors mediate the majority of fast excitatory and inhibitory synaptic transmission in mammalian central nervous system, respectively. A number of channels are sensitive to intracellular factors such as Ca²⁺, pH, ATP or Na⁺ as described in detail below. Some ion channels are only sensitive to a ligand, such as the small-conductance Ca²⁺-activated K⁺ (SK, for small K⁺) channel, while other ion channels are also voltage-sensitive and the binding of a ligand modulates the voltage-sensitivity of the channel – such as the high conductance Ca²⁺-activated K⁺ (BK, for big
K⁺) channel. The BK channel (also called maxi-K or Slo1) was the first-identified member of the Slo family of potassium channel genes. The mammalian genome contains four members of the Slo family: Slo1 which encodes the BK channel, Slo2.1 and Slo2.2 which both encode sodium-activated potassium channels (K\textsubscript{Na} channels) and Slo3 which is only expressed in sperm and encodes a pH-sensitive K⁺ channel. The structure of K⁺ channels encoded by the Slo family grossly resembles K\textsubscript{V} channels (Figure 1). They are tetramers, and all Slo family members except Slo1 contain 6 transmembrane domains. Slo1 channels contain a 7\textsuperscript{th} transmembrane domain termed S0 which precedes S1-6. As a result, the N-terminal domain is extracellular. Unlike K\textsubscript{V} channels, Slo channels have large, cytoplasmic, carboxyl tails that are known or suspected to confer sensitivity to intracellular ligands (review by Salkoff et al., 2006) (Figure 1).

Voltage clamp recordings

An in-depth discussion of the history and principles of voltage-clamp recordings is beyond the scope of this introduction, but as the following sections of this chapter and subsequent chapters describe experiments performed using this technique, an overview is provided here. The voltage clamp allows an experimenter to record the current across a membrane at a desired command voltage. As ion channels open and close in response to a change in command voltage or chemical stimulus, the resultant voltage change is nearly instantaneously measured and a compensatory current is applied by way of a negative feedback mechanism. This compensatory current is equal to and opposite of the ionic current across the membrane. The patch clamp technique was developed by Sakmann
and Neher. With patch clamp, a glass micropipette is made to form a high-resistance seal with a cell membrane. This greatly improves the resolution of voltage clamp recordings and allows measurement of currents through single ion channels.

Most voltage clamp experiments are performed in the whole cell recording configuration. This is done by first obtaining a high resistance seal on the cell membrane and then gently rupturing the membrane patch. Ideally this technique provides a measure of the total current across a cell membrane. In practice, the elaborate morphology of neurons compromises the ability of a single electrode to control the voltage across distant regions of membrane. This is referred to as imperfect or inadequate space clamp. Many experimenters use acutely dissociated neurons or young dissociated cultures of neurons that lack elaborate axons and dendrites to limit potential space clamp errors.

**Patch clamp configurations to record activity of individual ion channels**

The following recording configurations allow an experimenter to resolve the activity of a single or a small number of ion channels. The ability to resolve the activity of individual biological molecules of interest in real time is extremely powerful and can be accomplished with very few other methods.

On-cell, or cell-attached, patches are made by obtaining a high resistance seal on the surface of a cell. If one or more channels are present in the mouth of the pipette, the activity of these channels can be recorded. A great advantage of this technique is that the cytosolic contents of the cell, including diffusible molecules or elaborate protein structures, that could greatly influence ion channel activity remain intact. Major
drawbacks of this technique include the inability to directly alter internal solutions or external solutions that contact the channels being recorded. Furthermore, the experimenter does not have absolute control of the membrane voltage as it will be the difference between the resting potential of the cell and the command voltage in the pipette. If one is confident in the resting potential of the cell being used, then the transmembrane voltage can be estimated based on this value. It has also recently been demonstrated that current flow through activated channels in the patch can alter the transmembrane voltage of a cell, particularly if the input resistance of the cell is high (Williams & Wozny, 2011).

With excised patches, one loses the intracellular milieu but gains greater experimental control. An inside-out patch may be obtained if one quickly draws the pipette away from a cell after obtaining an on-cell patch. The absolute voltage across the patch of membrane is now easily controlled and the gating of ion channels in the patch by intracellular ligands can be tested by changing the solution exposed to the cytosolic face of the patch. An outside-out patch may be obtained if one draws the pipette away from a cell after obtaining a whole cell recording configuration. The absolute voltage across the patch of membrane is again easily controlled and the effect of extracellular ligands and compounds on ion channel activity can be tested by altering the external solution. A nucleated patch is a variation of the outside-out patch in which a small amount of suction is applied as the pipette is withdrawn. This can result in drawing the nucleus of the cell toward the pipette. The nucleus can then act as a scaffold so that a larger patch of membrane can be recorded from.
Sodium-activated Potassium Channels

Sodium-activated potassium channels ($K_{Na}$ channels) are unique in many ways and as such present many important and challenging questions. They are a small family of $K^+$ channels that are sensitive to the intracellular $Na^+$ concentration – a property of very few ion channels or even enzymes. $K_{Na}$ channels display voltage-sensitivity, but lack the canonical S4 voltage-sensor found in voltage-gated $K^+$ channels. $K_{Na}$ channels are broadly expressed in the nervous system, but the existence of $K_{Na}$ channels has been overlooked or doubted by neurophysiologists for many years. There is accumulating evidence demonstrating the activity of $K_{Na}$ channels in normal physiology, but bulk $Na^+$ concentrations are significantly lower than thought to be sufficient for activation of $K_{Na}$ channels. In addition to $Na^+$, $K_{Na}$ channels are regulated by other factors including intracellular pH, chloride, metabolites, proteins and perhaps other elements yet to be identified. Furthermore, the surface expression and activity of $K_{Na}$ channels is regulated by multiple metabotropic signaling pathways. In this section I will discuss the history of $K_{Na}$ channels, beginning with early studies in which $K_{Na}$ channels were strictly physiologically defined. I will then discuss the discovery that $K_{Na}$ channels are encoded by the Slo2 gene family and new insights into the role $K_{Na}$ channels in neurophysiology that have come about since then.

$K_{Na}$ channels described in excised patches
$K_{Na}$ channels were first described over 25 years ago as high conductance $K^+$ channels activated by high concentrations of $Na^+$ applied to the cytosolic face of inside-out patches from guinea pig cardiomyocytes (Kameyama et al., 1984) (Figure 2a). The authors of this study reported a $K_D$ (dissociation constant) of 66 mM and minimal channel activity when $[Na^+] <30$ mM (Figure 2b). Noting that almost no channel activity was observed with physiological $[Na^+]$, the authors suggested that $K_{Na}$ channels strictly serve a protective role by limiting membrane depolarization during ischemia or hypoxia that can result from insufficient pumping of $Na^+$ out of a cell. The resulting increase in $[Na^+]_i$ was proposed to activate $K_{Na}$ channels which would then hyperpolarize the membrane and shorten action potential duration.

Following the discovery of $K_{Na}$ channels in cardiomyocytes, high-conductance $K^+$-channels sensitive to $[Na^+]$ were recorded in inside-out patches from a variety of neuronal preparations (Dryer et al., 1989; Haimann et al., 1990; Dryer 1991; Egan et al., 1992a; Egan et al., 1992b; Haimann et al., 1992; Dale, 1993; Dryer, 1993; Koh et al., 1994; Safronov & Vogel, 1996; Bischoff et al., 1998). Nearly all studies reported a $K_D$ in excess of typical cytosolic $Na^+$ concentrations as well as prominent subconductance states that serve as a hallmark of $K_{Na}$ channels. Some studies also noted decreased channel activity within several minutes of patch excision (Egan et al., 1992; Haimann et al., 1992; Dryer, 1993). Variability in the unitary conductance and voltage-dependence of gating of $K_{Na}$ channels is present across studies. This variability is likely due, in part, to various $[K^+]$ used on both sides of the membrane, which has been reported to affect the unitary conductance, and more recently, voltage-dependence of $K_{Na}$ channels (Dai et al.,
Other experimental variables, interspecies divergences, and differences in the precise molecular identity of $K_{Na}$ channels in different tissues or brain regions might also contribute to the variations in properties of $K_{Na}$ channel observed across studies.

Evidence for a $K^+$-current dependent on $Na^+$-influx

Shortly after the initial report of $K_{Na}$ channels in inside-out patches, fast, transient $K^+$-currents dependent on $Na^+$-influx in invertebrate (Hartung, 1985) and vertebrate (Bader et al., 1985; Dryer et al., 1989) neurons were reported (example in Figure 2c). In these studies, depolarizing voltage steps evoked transient $Na^+$ currents, followed by a transient $K^+$ current. Blockade of $Na^+$ currents with tetrodotoxin (TTX) (an alkaloid neurotoxin commonly used to block voltage-gated $Na^+$ channels) or reduction of external $Na^+$ removed both the $Na^+$ current and the transient $K^+$ current. It was therefore suggested that the transient outward currents were a consequence of $Na^+$ accumulation via the transient $Na^+$ current. These results can be partially attributed to experimental artifacts discussed in the following section.

There has also been evidence for a sustained $K_{Na}$ current dependent on $Na^+$-influx in whole cell recordings from neurons. Voltage-evoked sustained outward currents were reduced in the absence of external $Na^+$ in spinal neurons of *Xenopus* embryos (Dale, 1991). Voltage-evoked sustained and transient $K_{Na}$ currents have also been reported in *Drosophila* neuroblasts (Figure 2d) (Saito & Wu, 1991).

Consistent with the sustained $Na^+$-influx-dependent $K^+$ currents reported above, a slow, $K^+$-mediated, afterhyperpolarization lasting several seconds in mammalian cortical
neurons was found to be dependent on Na\textsuperscript{+}-influx (Schwindt et al., 1989; Foehring et al., 1989). Similar results were also obtained in neurons of the avian hyperstriatum ventrale (Kubota & Saito, 1991), rat spinal cord neurons (Safronov & Vogel, 1996), ferret perigeniculate neurons (Kim & McCormick, 1998), and more recently in rat neocortical neurons (Franceschetti et al., 2003) and rat paraventricular neurons (Zhang et al., 2010). In some cases, the afterhyperpolarizations lasted up to 15 seconds (Kim & McCormick, 1998). These studies were conducted in current-clamp and some are subject to the critique that manipulations to prevent or alter Na\textsuperscript{+}-influx, such as partial substitution of external Na\textsuperscript{+} with Li\textsuperscript{+} alter not only the afterhyperpolarization, but also the prior voltage trajectory of the membrane in response to current injection. Therefore, decreases in magnitude and duration of the afterhyperpolarization could be due, at least in part, to lower activity of voltage-gated K\textsuperscript{+} channels resulting from the altered voltage trajectory.

Afterhyperpolarizations following a single action potential or a burst of action potentials are observed in many neurons. Afterhyperpolarizations are important in determining the rate and pattern of subsequent action potentials. Brief afterhyperpolarizations are believed to be mediated by the Slo1 channel (Storm, 1987; Edgerton & Reinhart, 2003), while slower afterhyperpolarizations have been attributed to the SK channel (Stocker, 2004; Villalobos et al., 2004). In many neurons, the identity of the channels responsible for the afterhyperpolarizations remains unknown. While the experiments described above are imperfect, K\textsubscript{Na} channels are likely to contribute to afterhyperpolarizations in at least some neurons.
**Arguments against activation of K\textsubscript{Na} channels by Na\textsuperscript{+} influx**

While a number of studies suggested K\textsubscript{Na} channels were active due to Na\textsuperscript{+}-influx following the transient Na\textsuperscript{+} current, these studies were met with skepticism for reasons described here. Many early investigations of the activity of K\textsubscript{Na} channels in neuronal physiology focused on the transient Na\textsuperscript{+} current as a source of Na\textsuperscript{+}-influx due to its large amplitude and a nascent appreciation of the persistent Na\textsuperscript{+} current and other sources of Na\textsuperscript{+} permeability in neuronal membranes.

Experimental concerns centered on potential failure of the voltage-clamp, which can be compromised by several factors. These include the difficulty in accurately measuring currents originating from neuronal processes while recording at the soma. While this phenomenon of imperfect space clamp is a caveat of nearly all whole cell recordings of intrinsic and synaptic ion channels in neurons with intact processes, studies implicating Na\textsuperscript{+} influx as a means of K\textsubscript{Na} channel activation were particularly susceptible to this criticism. Na\textsuperscript{+} channels are most abundant in the axon hillock, initial segment and nodes of Ranvier while the vast majority of voltage-clamp recordings are made from the soma. Furthermore, the large amplitude and fast kinetics of transient Na\textsuperscript{+} currents push the limits of many voltage-clamp amplifiers to stably step to a depolarized voltage. Even when measuring isolated Na\textsuperscript{+} currents, these issues must be taken into account. This has lead many investigators to lower the temperature of their preparations, partially block Na\textsuperscript{+} channels with low concentrations of TTX, or lower the external Na\textsuperscript{+} concentration in an effort to slow the activation kinetics and/or decrease the amplitudes of Na\textsuperscript{+} currents. Some investigations have used alternative recording configurations such as on-cell,
outside-out or nucleated patches to supplement recordings of Na\(^+\) currents made in the more common whole cell configuration. Due to the potential for inadequate voltage-clamp, studies describing a transient K\(_{\text{Na}}\) current following the transient Na\(^+\) current appropriately were, and remain to be, met with scrutiny. The sustained K\(_{\text{Na}}\) currents described in several studies cannot as readily be attributed to experimental artifacts.

The previously mentioned report of transient and sustained K\(_{\text{Na}}\) currents in *Drosophila* neuroblasts illustrates the greater robustness of sustained Na\(^+\)-influx dependent K\(_{\text{Na}}\) currents (Saito & Wu, 1991) (Figure 2d). The permeability of voltage-gated Na\(^+\) channels for Li\(^+\) is nearly equal to that of Na\(^+\) (Hille, 1972). However, Li\(^+\) is a very weak activator of K\(_{\text{Na}}\) channels (Dale, 1994). This makes replacement of external Na\(^+\) with Li\(^+\) a useful experiment in that the limitations of the voltage-clamp are similar in each condition. In voltage clamp recordings from *Drosophila* neuroblasts, application of TTX removes a transient inward current, a transient outward current and a sustained outward current (Figure 2d, left panel). However, substitution of external Na\(^+\) for Li\(^+\) only removes a sustained outward current (Figure 2d, right panel). This demonstrates that while transient K\(_{\text{Na}}\) currents may, at least in part, be due to experimental artifacts, sustained K\(_{\text{Na}}\) currents generated by accumulation of intracellular Na\(^+\) appear to be a genuine phenomenon.

Quantitative modeling also seems to disparage the idea that Na\(^+\)-influx activates K\(_{\text{Na}}\) channels. Modeling of Na\(^+\)-influx through voltage-gated Na\(^+\) channels and subsequent diffusion in the cytosol suggested that Na\(^+\) concentrations would increase to values required for K\(_{\text{Na}}\) channel activation within a range of only a few nanometers of
voltage-gated Na$^+$ channels (Dryer, 1991). This is smaller than the size of the channels themselves and suggests that Na$^+$-influx is not capable of activating K$_{\text{Na}}$ channels. This modeling was done treating the cytosol as a semi-infinite space. However, barriers to diffusion are likely to exist in fine neural processes or even in the soma by way of organelles or cytoskeletal structures adjacent to the plasma membrane. Studies suggesting dramatic differences in ion concentrations near the plasma membrane relative to the bulk cytosol will be described below. Furthermore, the rundown of K$_{\text{Na}}$ channels following excision of inside-out patches has led to the suggestion that other compounds, such as NAD$^+$ may serve as co-activators of K$_{\text{Na}}$ channels and lower the K$_D$ of K$_{\text{Na}}$ channels for Na$^+$ (Tamsett et al., 2009). Therefore, the necessary proximity of K$_{\text{Na}}$ and Na$^+$ channels may not be as close as estimated.

The Slo2 family encodes K$_{\text{Na}}$ channels

The highly-conserved mammalian gene Slack (also known as Slo2.2 or Kcnt1) was found to encode K$_{\text{Na}}$ channels (Yuan et al., 2003). When expressed in heterologous systems, Slack encodes high-conductance K$^+$ channels that display Na$^+$-dependent activation similar to reports of K$_{\text{Na}}$ channels in native tissues. Channels encoded by Slack also exhibit prominent sub-conductance states characteristic of previously described K$_{\text{Na}}$ channels and are activated by intracellular Cl$^-$. Subsequent to this finding, Slick (also known as Slo2.1 or Kcnt2) was also found to encode K$_{\text{Na}}$ channels (Bhattacharjee et al., 2003). Slick channels display more rapid kinetics of activation than Slack channels and are inhibited by physiological concentrations ATP. Channels encoded by Slick are also
more sensitive to intracellular Cl\(^{-}\) than those encoded by Slack. Slack and Slick subunits form functional heteromultimers with unique properties (Chen et al., 2009). This molecular diversity of \(K_{Na}\) channel pore-forming subunits may be partially responsible for the variations in \(K_{Na}\) channels properties described throughout the nervous system and other tissues. Further diversification of \(K_{Na}\) channel properties may arise of alternative splicing of Slack (Brown et al., 2008) and Slick, association with accessory subunits or post-translational modifications. While differences in gating of channels encoded by Slack and Slick are noted in heterologous systems, there is currently no means of readily distinguishing the activity of the two channels types in neurons. Therefore, the term “Slo2 channels” will be used to refer to populations \(K_{Na}\) channels which could be comprised of either Slack, Slick, heteromultimers of the two genes or a composite of channel subtypes.

**Expression of Slack and Slick in central nervous system**

The expression of Slack and Slick mRNA and protein has been investigated using *in situ* hybridization by the Allen Mouse Brain Atlas, and immunohistochemistry (Bhattacharjee et al., 2002; Bhattacharjee et al., 2005). All investigations demonstrate broad and abundant expression of Slack and Slick throughout the brain. Expression of the two genes overlaps in many regions, and is consistent with the suggestion that Slack and Slick subunits form heteromultimers (Chen et al., 2009). More recently, it was determined that Slack is also expressed in neurons of the dorsal root ganglia (DRG) (Tamsett et al., 2009; Nuwer et al., 2010) consistent with a prior report of \(K_{Na}\) channels
in excised patches (Bischoff et al., 1998) of these cells. To emphasize the abundance of Slo2 channel expression, summaries of selected ion channel mRNA expression profiles from the Allen Brain Atlas are shown in Figure 3. Slack is more broadly expressed than the closely related Slo1 calcium-activated potassium channel – one of the most actively studied ion channels in neuroscience and biophysics. Notably, expression of Slack is comparable to that of the primary voltage-dependent delayed rectifier – \( \text{K}_V\text{2.1} \) (also called Shab1 or Kcnb1). Given the abundance of \( \text{K}_{\text{Na}} \) channels in the nervous system, it is surprising that, until recently, it was unclear if \( \text{K}_{\text{Na}} \) channels were active under normal physiological conditions.

*New investigations into Slo2/\( \text{K}_{\text{Na}} \) channels*

Identification of Slick and Slack as encoders of \( \text{K}_{\text{Na}} \) channels renewed interest in the physiological role of these channels. The fundamental issue was still whether or not these channels are active in normal physiology or only a reserve conductance. Chapter 2 of this thesis contains a publication from 2009 in which Slack channels were shown to carry a prominent current resembling a voltage-dependent delayed rectifier that was dependent on \( \text{Na}^+ \)-influx through TTX-sensitive \( \text{Na}^+ \) channels (Budelli et al., 2009). Following this publication, similar methods were used to reveal Slack/\( \text{K}_{\text{Na}} \) currents in neurons of the DRG (Nuwer et al., 2010), thalamic paraventricular neurons (Zhang et al., 2010) and mitral cells in acute slices and dissociated cultures of mouse olfactory bulb (Lu et al., 2010). This study also demonstrated that expression of the Slack channel and \( \text{Na}^+ \)-influx dependent \( \text{K}^+ \) current was up-regulated in mice in which the \( \text{K}_V\text{1.3} \) gene had been
deleted. While the same general phenomenon was observed in these studies, there were some notable differences in the results. Lu et al., reported that in mitral cells of the olfactory bulb, Na\(^+\)-influx through hyperpolarization-activated cyclic nucleotide-gated (HCN) channels must also be blocked (with ZD7288), in addition to blockade of Na\(^+\) channels with TTX, to reveal K\(_{Na}\) currents. Along similar lines, Nuwer et al. reveal K\(_{Na}\) currents by replacing Na\(^+\) in the external solution. Notably, DRG neurons carry idiosyncratic TTX-resistant sodium channels encoded by Na\(_V\)1.8 and Na\(_V\)1.9 that could act as a means of Na\(^+\) entry into the cell. It therefore seems likely that TTX-sensitive Na\(_V\) channels are not the exclusive source of Na\(^+\)-influx capable of activating K\(_{Na}\) channels. TTX-sensitive Na\(_V\) channels are emphasized in the studies reported as part of this thesis because we observed only a slightly larger effect of removal of external Na\(^+\) than application of TTX (Budelli et al., 2009).

The sustained activity of K\(_{Na}\) currents in our initial study (Budelli et al., 2009), suggested the persistent sodium current (I\(_{NaP}\)) to be an important source of Na\(^+\)-influx for activation of K\(_{Na}\) channels. Chapter 3 of this thesis contains a study published this year (Hage & Salkoff, 2012) demonstrating the coupling of I\(_{NaP}\) and K\(_{Na}\) channels. This study also provides the first measure of K\(_{Na}\) channel activation by Na\(^+\)-influx using excised patches to record and measure the activity of individual K\(_{Na}\) channels.

Metabotropic regulation of Slo2 channels

Changes in expression or regulation of non-synaptic channels - “intrinsic plasticity” - have gained attention as phenomena important for the normal functioning
and development of the nervous system and a component of some nervous system disorders (Review by Beck & Yaari, 2008). Slo2/K\textsubscript{Na} channels are attractive candidate molecules to mediate changes in intrinsic neuronal excitability given the widespread and abundant expression of these channels. Indeed, it appears likely that the activity and surface expression of Slo2 channels can be altered via metabotropic signaling pathways. The activity of Slo2 channels is altered by stimulation of protein kinase C (PKC) by phorbol esters or activation of G-protein coupled receptors (GPCRs) coupled to the Gq pathway (Santi et al., 2006). Intriguingly, channels encoded by Slick and Slack are regulated in opposing directions. The activity of Slack channels is increased by PKC activation, while the activity of Slick channels is decreased. In isolated Kenyon cells from cricket mushroom bodies, it has been reported that octopamine and dopamine are both capable of regulating K\textsubscript{Na} channel activity in a positive and negative manner, respectively, though the signaling pathways are unclear (Aoki et al., 2008).

In addition to regulation of ion channel activity by alteration of gating properties, ion channels can be regulated by changing the membrane trafficking and internalization rates. The most notorious instance takes place in long term potentiation (LTP) in which \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of glutamate receptors are inserted into the synaptic membrane as a means of increasing synaptic strength. This phenomenon is not limited to synaptic proteins, and regulation of membrane trafficking has been shown to take place with K\textsubscript{V}4 channels in an activity-dependent manner in neurons of rats and Drosophila (Kim et al., 2007; Ping & Tsunoda, 2012). Regarding K\textsubscript{Na} channels, the rapid internalization of Slack channels following
activation of protein kinase A (PKA) has been reported in dissociated cultures of DRG neurons (Nuwer et al., 2010). As a result of Slack channel internalization, neuronal excitability increased and was suggested by the authors as a potential mechanism of nociceptive sensitization during inflammation.

Nav channels are also prone to modulation by cell signaling pathways (reviewed by Cantrell & Catterall, 2001), raising the possibility that KNa channel activity could be altered as a primary or secondary consequence of metabotropic signaling. Specific examples of Nav channel modulation will be discussed later in this chapter.

**KNa channels as polymodal signal integrators**

Slack is the largest known K+ channel subunit. It is perhaps not surprising then, that there are multiple diverse means of regulating KNa channel activity. Early investigations of KNa channels using inside-out patches noted a dramatic decrease – or rundown – of KNa channel activity over varied time courses, even in the presence of high Na+ concentrations (Egan et al., 1992; Haimann et al., 1992; Dryer, 1993). This suggests other compounds are present in situ that either directly activate or alter the Na+ dependence of KNa channels. Such factors could become washed away following patch excision and perfusion. Cloning of the Slack and Slick genes has allowed investigation into such compounds. Both Slack and Slick are cooperatively activated by intracellular Cl− (Yuan et al., 2003; Bhattacharjee et al., 2003). Nicotinamide adenine dinucleotide (NAD+) increases the Na+-sensitivity of Slack channels expressed in heterologous systems and DRG neurons (Tamsett et al., 2009). Fragile X mental retardation protein
(FMRP) has been reported to bind to and directly activate Slack channels (Brown et al., 2010). Finally, the activity of Slack is decreased in low pH (Ruffin et al., 2008). The precise cytosolic concentrations of the above compounds, and perhaps other cytosolic factors, could result in the Na⁺-sensitivity of K_{Na} channels being greater in the presence of the cytosolic milieu than in excised patches. This may partially explain how K_{Na} channels can contribute to neuronal physiology even when bulk internal Na⁺ concentrations are not elevated.

**K_{Na} channel pharmacology**

Despite recent investigations into pharmacological regulation of K_{Na}/Slo2 channels (Yang et al., 2006; Biton et al., 2012), there are no known specific blockers of K_{Na} channels. High concentrations of tetra-ethyl ammonium (TEA), quinidine and bepridil have all been reported to block K_{Na}/Slo2 channels (Yang et al., 2006). Bithionol, niflumic acid and loxapine have been reported activators of Slo2 channels (Yang et al., 2006; Dai et al., 2010; Biton et al., 2012). The low specificity of these compounds limits their potential use as tools to investigate the physiological role of K_{Na}/Slo2 channels. However, such compounds could serve a potential therapeutic role, and will likely be investigated as such in the future.

**Persistent sodium current**

The persistent sodium current (I_{NaP}) will be focused on as a source of Na⁺ influx important for the activation of K_{Na} channels in chapters 2 and 3. An overview of the
properties of $I_{\text{NaP}}$ most relevant to these studies is provided here. $\text{Na}^+$ currents are essential for action potential generation and propagation. The well known transient sodium current ($I_{\text{NaT}}$) is responsible for the upstroke of the action potential. A fraction of this current is non-inactivating, or slowly inactivating, and responsible for $I_{\text{NaP}}$ (reviewed by Crill, 1996). $I_{\text{NaP}}$ is active at subthreshold voltages and can be an important determinant in the frequency and pattern of which neurons generate action potentials.

*Role of persistent sodium current in neuronal firing*

The amplitude of $I_{\text{NaP}}$ is typically only 0.5-5% of the peak amplitude of the $I_{\text{NaT}}$. However, the high input resistance of neurons at subthreshold voltages allows a small current to have dramatic effects on neuronal excitability. While $I_{\text{NaT}}$ and the depolarizing phase of the action potential are grossly similar among neurons, the amplitude and kinetics of $I_{\text{NaP}}$ are more variable. Despite its diminutive amplitude, the presence of $I_{\text{NaP}}$ – and its behavior - is increasingly appreciated as a key determinant of the electrical identity of a neuron.

Many neurons generate bursts of action potentials in which multiple spikes are rapidly generated then followed by a quiescent period. Properties of bursting behavior such as spike frequency within the burst, burst duration and time between bursts can vary greatly among neurons. Bursting neurons may be particularly important in central pattern generators and aberrant bursting is a characteristic of epilepsy. $I_{\text{NaP}}$ has been implicated in burst generation in many types of neurons (Brumberg *et al.*, 2000; Wu *et al.*, 2005; Enomoto *et al.*, 2006; Golomb *et al.*, 2006). The termination of bursts can be mediated
by slowly activating $K_V$ currents as well as $Ca^{++}$-activated potassium currents that are activated by accumulation of $Ca^{++}$ via voltage-gated $Ca^{++}$ channels during the burst (Goldberg & Wilson, 2005). $Na^+$ may also accumulate during neuron bursting by way $I_{NaP}$ (Rose & Ransom, 1997; Mittman et al., 1997; Fleidervish et al., 2010). This could lead to $K_{Na}$ channel activation which could then to contribute to burst termination.

In the absence of synaptic input some neurons display rhythmic, spontaneous generation of action potentials, called pacemaking. This neuronal behavior is widespread and can be driven by multiple currents including $I_H$ and voltage-gated $Ca^{++}$ channels (Pape, 1996; Puopolo et al., 2007). $I_{NaP}$ has been implicated as a critical component of pacemaking in subthalamic neurons (Bevan & Wilson, 1999; Do & Bean, 2003), tuberomammillary neurons (Taddese & Bean, 2002), suprachiasmatic nucleus neurons (Jackson et al., 2004), and dopaminergic ventral tegmental area neurons (Khaliq & Bean, 2010). Our understanding of neuronal pacemaking is far from complete, particularly regarding repolarizing currents that could balance depolarizing drive from $I_{NaP}$ or $I_{Ca}$ (but see Khaliq & Bean, 2008). By way of their functional coupling, $I_{K_{Na}}$ could provide feedback in pacemaking neurons in which $I_{NaP}$ has been demonstrated to contribute to rhythmic firing.

Subthreshold oscillations in membrane voltage have been reported in many neurons of the central nervous system and could play an important role in synchronizing activity among neurons. $I_{NaP}$ is required for subthreshold oscillations in mitral cells of the olfactory bulb (Desmaisons et al., 1999) and cortical neurons (Alonso & Llinas, 1989; Gutfreund et al., 1995; Hutcheon et al., 1996). In other classes of neurons, subthreshold
oscillations are mediated by voltage-gated calcium channels and calcium-activated potassium channels (Llinas & Yarom, 1986; Bal & McCormick, 1997). While a repolarizing current may not be strictly necessary for $I_{\text{NaP}}$-mediated subthreshold oscillations, it is intriguing to speculate that $K_{\text{Na}}$ channels could play a complimentary role in generation of such oscillations.

It is somewhat surprising that something as fundamental as the resting potential of neurons is poorly understood. The proximity of the membrane resting potential to the equilibrium potential of $K^+$ ($E_K$) and steep dependence on external $[K^+]$ suggests it is largely determined by non-voltage-gated, or weakly-voltage-gated $K^+$ channels. However, many neurons rest at a voltage $>10$ mV depolarized from $E_K$. Therefore, there must be a contribution from depolarizing currents as well. While $I_{\text{NaP}}$ is often described as turning on at voltages between the resting potential and action potential threshold, ion channel activity is probabilistic and even a tiny amount of activity could influence the resting membrane potential given the high input resistance of many neurons at rest. A TTX-sensitive $I_{\text{NaP}}$ has been shown to contribute to the resting potential of the calyx of Held (Huang & Trussell, 2008), large diameter DRG neurons (Baker & Bostock, 1997) and in chapter 3 of this thesis, I demonstrate activity of $I_{\text{NaP}}$ at resting potentials in cultured olfactory bulb neurons (Hage & Salkoff, 2012). Depolarizing drive at resting membrane potentials is also provided by TTX-insensitive channels such as NALCN (for Na$^+$ leak channel, non-selective) (Lu et al., 2007) and HCN channels. The constant influx of Na$^+$ into neurons suggests that elevated neuronal activity may not be required for $K_{\text{Na}}$ channels to contribute to neuronal physiology. In some neurons, $K_{\text{Na}}$ channels
appear to lack strong voltage-sensitivity. It is therefore possible that $K_{Na}$ channels themselves could contribute to the resting potential.

Finally, $I_{NaP}$ is important for dendritic integration of synaptic potentials. $I_{NaP}$ amplifies excitatory post-synaptic potentials (EPSPs) and can compensate for attenuation of EPSP amplitude by dendritic cable properties (Magee et al., 1995; Schwindt & Crill, 1995; Stuart & Sakmann, 1995; Andreasen & Lambert, 1999). $I_{NaP}$ can also effectively amplify inhibitory post-synaptic potentials (IPSPs). If the membrane potential is sufficiently depolarized to activate $I_{NaP}$, an IPSP will hyperpolarize the membrane and reduce the activity of $I_{NaP}$, resulting in a net outward current (Stuart, 1999).

Immunocytochemical staining for Slo2 channels suggests expression in dendrites, but a contribution to dendritic integration is currently unclear. Depending on the specific properties of such channels, they could serve to shunt synaptic potentials, or act to sharpen EPSPs compensating for the broadening that takes place due to dendritic cable properties. The latter would compliment the proposed role of $I_{NaP}$ in amplifying synaptic potentials to compensate for the attenuation of EPSP amplitude by cable properties.

Molecular determinants of the persistent sodium current

The precise molecular identity of $I_{NaP}$ is unknown and not likely to be identical across mammalian neurons. The same family proteins which carry $I_{NaT}$ are believed responsible for $I_{NaP}$. Indicative of this, both the transient and persistent components of $I_{Na}$ are blocked by TTX, and while many compounds preferentially inhibit $I_{NaP}$, none are completely selective. Similar to other classes of ion currents, the properties of $I_{NaP}$ vary
across cell types, subcellular compartments and developmental phases (McCormick & Prince 1987; Alzheimer et al., 1993b; Crill, 1996) indicative of the idea that there may be multiple molecular bases for $I_{\text{NaP}}$.

$I_{\text{NaP}}$ may be partially explained by the properties of $I_{\text{NaT}}$. In some neurons, it appears that behavior of $I_{\text{NaT}}$ can completely account for $I_{\text{NaP}}$. A physiologically uniform population of voltage-gated $\text{Na}^+$ channels underlies the spike-generating $\text{Na}^+$ current as well as the subthreshold $\text{Na}^+$ current responsible for the spontaneous firing of tuberomammillary neurons (Taddese & Bean, 2002). The overlap of the activation and inactivation curves of $I_{\text{NaT}}$ – often described as a “window current” has been suggested as a source of $I_{\text{NaP}}$. Sustained window currents are not unique to $\text{Na}^+$ currents, but are also generated by some inactivating voltage-gated $\text{K}^+$ currents. In the case of the $\text{Na}^+$ current, the window current predicted by activation and steady-state inactivation curves of $I_{\text{NaT}}$ does not display the same voltage-dependence as $I_{\text{NaP}}$ directly recorded from neurons (Strafstrom et al., 1985; French et al., 1990). While some component of $I_{\text{NaP}}$ may simply be a result of the behavior of $I_{\text{NaT}}$, there must also be alternative molecular bases of $I_{\text{NaP}}$.

It has been suggested that $I_{\text{NaP}}$ could be carried by a unique population of $\text{Na}_V$ channels that do not inactivate (Strafstrom et al., 1985; French et al., 1990). This is in some ways supported by distinct immunological staining for $\text{Na}_V$ subunits between subcellular compartments (Westenbroek et al., 1989; Westenbroek et al., 1992; Gong et al., 1999; Caldwell et al., 2000; Krzemien et al., 2000; Schaller & Caldwell, 2003; Lorincz & Nusser, 2008; Fryatt et al., 2009; Lorincz & Nusser, 2010). To my knowledge, there has been only one published report of a population of $\text{Na}_V$ channels that
display no inactivation (Masukawa et al., 1991). Using cultured hippocampal neurons, the authors report that on-cell patches of dendrites contain putative Na\textsubscript{V} channels that display no inactivation, while on-cell patches from the soma contain both inactivating and non-inactivating sodium channels. The cloning and heterologous expression of Na\textsubscript{V} channels has, not yet, identified a TTX-sensitive Na\textsubscript{V} channel that displays no fast inactivation. That is, cells expressing Na\textsubscript{V} channel subunits display I_{NaT} as well as I_{NaP} (to varying extents and with varying properties), but never only I_{NaP}.

It has instead been suggested that Na\textsuperscript{+} channels exhibit modal gating in which Na\textsubscript{V} channels enter a structural state that increases the propensity of the channel to generate I_{NaP}. On-cell recordings made from cortical pyramidal neurons display prominent transient channel activity upon depolarization followed by late openings that display similar unitary conductance as the transient channel openings at the onset of the voltage step (Alzheimer et al., 1993a). This led the authors to conclude that I_{NaP} was due to periodic failures of I_{NaT} inactivation. Similar results have been obtained in other studies (Brown et al., 1994; Fleidervish & Gutnick, 1996), though the extent of structural change associated with the modal gating of Na\textsuperscript{+} channels is unclear.

Association with accessory subunits influences the persistent gating behavior of Na\textsubscript{V} channels. In heterologous systems and cultured hippocampal neurons, β-subunits have been shown to alter the voltage-dependence of activation and inactivation as well as the amplitude of I_{NaP} (Aman et al., 2009). In dissociated cultures of cerebellar granule neurons, knockdown of the β4 subunit with RNAi has been shown to alter several properties of the sodium currents, including a decrease in I_{NaP} amplitude (Bant and
Raman, 2010). The association of β4 with NaV1.6 has been suggested to alter the window current predicted to be generated by the channel, but this study did not confirm or disaffirm this prediction (Zhao et al., 2011).

Clinical importance of $I_{NaP}$

$I_{NaP}$ plays an important role in the treatment and pathophysiology of epilepsy (review by Strafstrom, 2007). Many anti-epileptic compounds are preferential inhibitors of $I_{NaP}$. Mutations in NaV channel α-subunits associated with epilepsy have been shown to alter the magnitude of $I_{NaP}$. Blockade of persistent influx of Na$^+$ through NaV channels has been proposed as a treatment for multiple sclerosis (Waxman, 2008). Increased activity of $I_{NaP}$ underlies heightened cortical neuron excitability in a genetic model of Amyotrophic Lateral Sclerosis (Pieri et al., 2009). This hyperexcitability is attenuated by riluzole, which preferentially inhibits $I_{NaP}$. Mutations in human Na$v_1.1$ linked to familial hemiplegic migraine have been shown to generate an increase in the amplitude $I_{NaP}$ (Kahlig et al., 2008).

Many disease-associated mutations of voltage-gated sodium channels have only recently begun to be investigated. As the consequences of these mutations are considered in the context of neurons, the downstream effect of altered $I_{NaP}$ on $K_{Na}$ channel activity will be an important subject of investigation.

Metabotropic regulation of $I_{NaP}$
While there are many studies demonstrating neurotransmitter regulation of Na\textsubscript{V} channels (reviewed by Cantrell & Catterall, 2001) only a fraction of these studies describe an effect on I\textsubscript{NaP}, and instead report changes in behavior of I\textsubscript{NaT}. This is may be due in part to the small amplitude of I\textsubscript{NaP} and inherent difficulties in measuring fractional changes of tiny signals. However, as emphasized above, small alterations of subthreshold currents can have dramatic impacts on neuronal firing. Because I\textsubscript{NaP} is thought to be generated by the same population, or a similar population, of channels responsible for I\textsubscript{NaT}, it feasible that signaling events which alter I\textsubscript{NaT} will also affect I\textsubscript{NaP}.

Dopamine is reported to reduce significantly the amplitude of I\textsubscript{NaP} in striatal neurons (Cepeda et al., 1995). Similarly, activation of muscarinic acetylcholine receptors inhibits I\textsubscript{NaP} in neocortical pyramidal neurons (Mittman & Alzheimer, 1997). Activation of PKC is reported to induce a hyperpolarizing shift in the voltage-dependence of activation of I\textsubscript{NaP}, and consequentially an increase in cell excitability, in somatosensory cortex, prefrontal cortex and neocortex (Astman et al., 1998; Gorelova & Yang, 2000; Franceschetti et al., 2000). Serotonin is reported to inhibit I\textsubscript{NaP} via PKC signaling in prefrontal cortex and via PKA signaling in mesencephalic trigeminal neurons (Carr et al., 2002; Tanaka & Chandler, 2006).

**Sodium as a second messenger**

Sodium is not typically thought of as a likely second messenger. While basal levels of calcium (the most common second messenger) are maintained at very low levels, sodium is much more abundant and structurally more similar to potassium, the
most abundant cytosolic cation. It has long been appreciated, and often emphasized in
text books, that the magnitude of sodium currents responsible for a single action potential
is unlikely to alter the total cytosolic [Na+] given the relatively large volume of neurons.
Recent evidence has demonstrated that sustained neuronal spiking or activity of I_{NaP} can alter the bulk [Na+] and that changes in more confined domains can be rather dramatic.
 Intriguingly, elevations in [Na+] are slow to decay.

Sodium imaging

The fluorescent dye sodium-binding benzofuran isophtalate (SBFI) is commonly
used to investigate changes in [Na+]_{i}. In cultured hippocampal neurons, spontaneous
increases in bulk [Na+]_{i} of 5 mM have been reported (Rose & Ransom, 1997). In more
confined cellular domains such as dendritic shafts and spines, tetanic stimulation results
in [Na+]_{i} exceeding 100 mM (Rose & Konnerth, 2001). I_{NaP} has been implicated as a key
source of Na^{+}-influx underlying elevations in [Na+]_{i} (Mittman et al., 1997; Fleidervish et
al., 2010). In experiments utilizing SBFI, the time constant of decay of [Na^{+}]_{i} in the
soma and dendrites is as high as 10 seconds – much slower than expected by simple
diffusion of Na^{+}. Such slow changes could be attributed to artifacts such as the buffering
capability of SBFI – except that the decay of [Na^{+}]_{i} transients measured in the axon are
rapid, with time courses less than 1 second (Fleidervish et al., 2010). Elevated [Na^{+}]_{i}
could therefore have long-lasting (10s of seconds) effects on neuronal excitability by way
of K_{Na} channel activation. The reasons for the slow decay of elevations in [Na^{+}] are not
well understood.
Unique submembrane concentration

While it seems clear that \([\text{Na}^+]_i\) within confined domains such as dendritic shafts and spines can, and does, dramatically increase, \(K_{\text{Na}}\) channels appear to be abundant in patches excised from the soma of neurons. This is further supported by immunological staining for Slick and Slack on the soma of neurons. Studies reporting increases in \([\text{Na}^+]_i\) in the soma of voltage-clamped neurons using SBFI did not include calibration to estimate the absolute \([\text{Na}^+]_i\) (Mittmann *et al*., 1997; Fleidervish *et al*., 2010), but as expected, changes appear to be much lower than in the confines of neural processes. However, evidence for a submembrane accumulation of \(\text{Na}^+\) in cardiac myocytes in a so-called “fuzzy space” or “unstirred layer” has been investigated for years (reviewed by Barry *et al*., 2006). Physiological investigations suggest \(\text{Na}^+\)-influx through \(\text{NaV}\) channels influences the activity of the sodium-calcium exchanger, resulting in entrance of \(\text{Ca}^{++}\) into the cell via the exchanger (Leblanc & Hume, 1990; Lederer *et al*., 1990). One study investigated this phenomenon using electron probe microanalysis (EPMA) (Wendt-Gallitelli *et al*., 1993). With EPMA a sample is bombarded with an electron beam similar to that of a scanning electron microscope and the resulting emitted X-rays are detected and analyzed to identify wavelengths characteristic of elements of interest - in this case \(\text{Na}^+\). EMPA was used with ventricular myocytes to show that while the bulk \([\text{Na}^+]_i\) was essentially unchanged following voltage-clamp stimulation, \([\text{Na}^+]_i\) within 20 nanometers of the membrane increased to approximately 40 mM from a resting concentration of approximately 17 mM. \([\text{Na}^+]_i\) within 20 nanometers of the membrane remained elevated
8 seconds after stimulation, but returned to baseline levels after 3 minutes consistent with the slow decay of [Na$^+$]$_i$ measured using SBFI. Furthermore, this study described a “microheterogeneity” of [Na$^+$]$_i$ in which [Na$^+$]$_i$ varied from 0-90 mM within 20 nanometers of the membrane - perhaps suggesting clustering of channels laterally in the membrane.

In the following chapters I present data demonstrating that $K_{Na}$ channels are activated by Na$^+$ influx via $I_{NaP}$. These results show that $K_{Na}$ channels are active during normal physiology and likely contribute to the repolarization of action potentials. While the two widespread components of neuronal excitability have been previously studied as isolated currents, their interrelatedness was unappreciated and is a novel finding in the field of neurophysiology.
Figure 1 Structures of a voltage-gated potassium channel and a sodium-activated potassium channel. Dotted lines represent the phospholipid bilayer of the plasma membrane. “N” and “C” denote the amino- and carboxyl- terminus of each protein, respectively. “P” denotes the pore-loop that forms the lining of the pore of the ion channels and is critical for $K^+$-selectivity. “S1-S6” denote transmembrane segments 1-6. Notably S4 of the voltage-gated $K^+$ channel is often referred to as the voltage-sensing helix. It contains positively charged amino acids that are critical to the voltage-sensitivity of the channel.
Figure 2 Early investigations of $K_{Na}$ channels a & b from Kameyama et al., 1984. a) An inside-out patch from a cardiac myocyte contains a potassium selective channel that displays increased activity with exposed to high [Na$^+$] on the cytosolic side of the patch. b) Sodium-dependence of $K_{Na}$ channels in inside-out patches quantified by plotting channel open probability against [Na$^+$]. c) from Bader et al., 1985. (top) Currents were evoked from cultured avian neurons by a voltage step from -100 mV to 0 mV. Control currents are shown in solid line indicated by 1. Other dotted and solid lines represent currents evoked by same voltage command following application of TTX. (bottom) TTX-sensitive currents revealed by subtracting steady-state currents in TTX from control currents and currents partially blocked by TTX. Dotted line indicates 0 current level. The authors noted a correlation between the amplitude of the transient inward current and transient outward current. d) from Saito & Wu, 1991. Sodium and potassium currents recorded from a dissociated Drosophila neuroblast. Left) Upon application of TTX, transient sodium currents are blocked and transient and sustained potassium currents decreased in amplitude. Right) In contrast to TTX, replacement of external Na$^+$ with Li$^+$ only decreased amplitude of sustained potassium currents.
**Figure 3** Summaries of mRNA expression of selected K⁺ channels

Data was taken from online Allen Brain Atlas. Relative expression of transcripts of 3 K⁺ channel genes was measured by automated *in situ* experiments and quantified in terms of expression level (proportional to number of transcripts) and expression density (proportional to fraction of cells expressing transcript) in indicated regions of mouse brain. Top – data for K⁺Na channel, *Slo2.2*. Middle – data for high-conductance calcium-activated K⁺ channel, *Slo1*. Bottom – data for primary voltage-dependent delayed rectifier *Kv2.1*.
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Preface to Chapter 2

This chapter is taken from a paper which appeared in the journal *Nature Neuroscience*. The complete citations is Na+-activated K+ channels express a large delayed outward current in neurons during normal physiology. Budelli G, Hage TA, Wei A, Rojas P, Jong YJ, O'Malley K, Salkoff L. *Nat Neuro* 2009 12:745-750. Some adjustments have been made for the thesis format. Gonzalo Budelli and I were co-first authors on the paper. My experimental contributions were primarily all of the recordings from olfactory bulb neurons. All authors participated in experimental design, data interpretation, and composition of the manuscript.
Chapter 2

Na⁺-activated K⁺ channels express a large delayed outward current in neurons during normal physiology
ABSTRACT

One of the largest components of the delayed outward current that is active under physiological conditions in many mammalian neurons, such as medium spiny neurons of the striatum and tufted-mitral cells of the olfactory bulb, has gone unnoticed and is the result of a Na\(^+\)-activated K\(^+\) current. Previous studies of K\(^+\) currents in mammalian neurons may have overlooked this large outward component because the sodium channel blocker tetrodotoxin (TTX) is typically used in such studies. We found that TTX also eliminated this delayed outward component in rat neurons as a secondary consequence. Unexpectedly, we found that the activity of a persistent inward sodium current (persistent \(I_{Na}\)) is highly effective at activating this large Na\(^+\)-dependent (TTX sensitive) delayed outward current. Using siRNA techniques, we identified SLO2.2 channels as being carriers of this delayed outward current. These findings have far reaching implications for many aspects of cellular and systems neuroscience, as well as clinical neurology and pharmacology.
INTRODUCTION

The original discovery of high-conductance sodium-activated potassium channels (K$_{Na}$ channels) in the heart (Kameyama et al., 1984) and brain (Dryer et al., 1989; Egan et al., 1992) presented a conundrum, as studies of single channel properties in inside-out patches showed that they respond to very high levels of Na$^+$, far exceeding those that are present in the normal intracellular bulk cytosol (Kameyama et al., 1984; Dryer et al., 1989; Egan et al., 1992; Dryer, 1991; Koh et al., 1994). Thus, it was suggested that this channel class represents a reserve conductance that can be activated during times of stress resulting from ischemia or hypoxia, when sodium ions accumulate in cells (Kameya et al., 1984; Dryer, 1994). However, other studies have indicated that K$_{Na}$ channels may be active under normal physiological conditions (Dryer, 1994; Bhattacharjee & Kaczmarek, 2005; Wallen et al., 2007; Yang et al., 2007) and the effectiveness of sodium entry through voltage-dependent sodium channels in activating K$_{Na}$ channels remains in dispute (Dryer 1994; Bhattacharjee & Kaczmarek 2005). To explore these questions, we undertook a study of the action of TTX on outward currents in several types of rat neurons. Unexpectedly, we found that many neuronal cell types had a large TTX-sensitive delayed outward current that decayed only slightly over a time course of a second.
METHODS

Cell preparation and recording methods.

Dissociated MSNs and tufted-mitral cells were cultured from postnatal day 2 rat pups and recorded from 4–7 d post-plating. Recording solutions used an internal (pipette) \([\text{Na}^+]\) solution as indicated in the figure legends and an external (bath) \([\text{Na}^+]\) solution of 150 mM or as indicated in the figure legends. Experiments requiring the removal of external \(\text{Na}^+\) replaced sodium with choline in the extracellular solution. Experiments that elevated \(\text{Na}^+\) in the internal pipette solution removed equimolar \(\text{K}^+\). The 140 or 150 mM \(\text{Na}^+\) bath solution was composed of 150 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), 10 mM dextrose, 10 mM HEPES, pH 7.4 with NaOH. The 0 mM \(\text{Na}^+\) bath solution contained 150 mM choline chloride, 5 mM KCl, 2 mM MgCl\(_2\), 10 mM dextrose, 10 mM HEPES, pH 7.4 with KOH. Data were acquired using an Axopatch 200A or Axopatch 200B amplifier (Molecular Devices), digitized at 10 kHz using a Digidata 1440A (Molecular Devices), filtered at 5 kHz and collected using pCLAMP 10. Recording pipettes had tip resistances of 3–6 M\(\Omega\). In experiments using TTX or riluzole, drugs were added to external solutions at concentrations of 1 and 20 \(\mu\)M, respectively. Sodium-dependent potassium currents sometimes showed 'rundown' in the whole-cell or detached patch-recording modes. We did see rundown during the initial phase of recording currents in some cells. However, we applied test voltage steps over time, before the application of TTX or other treatments, and only initiated experiments after test pulses produced
delayed outward currents that were stable over time (in general, the current stabilized within \( \sim 2 \) min).

*Statistical analysis.*

We performed statistical analyses using previously described tools (Kirkman, T.W. Statistics to use. [http://www.physics.csbsju.edu/stats/]; 1996). For comparison between two groups, we used paired Student's \( t \) tests for the same procedures before and after applied treatments.

*Animal welfare.*

It is the policy of Washington University Medical School that all research involving animals be conducted under humane conditions, with appropriate regard for animal welfare. Washington University Medical School is a registered research facility with the United States Department of Agriculture (USDA) and is committed to complying with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services), the provisions of the Animal Welfare Act (USDA) and all applicable federal and state laws and regulations. At Washington University Medical School an Animal Care Committee has been established to insure compliance with all applicable federal and state regulations for the purchase, transportation, housing and research use of animals. Washington University Medical School has filed appropriate assurance of compliance with the Office for the Protection of Research Risks of the US National Institutes of Health.
RESULTS

We assumed that the observed TTX sensitivity of the delayed outward current was a secondary consequence of the block of inward Na\(^+\) current by TTX, and found this to be the case in a variety of experiments (Fig. 1). To demonstrate the effectiveness of Na\(^+\) entry through TTX-sensitive sodium channels in activating the delayed outward current, we adjusted the intracellular concentration of Na\(^+\) to very low levels by removing Na\(^+\) from the intracellular pipette recording solution; thus, any intracellular Na\(^+\) would be a minor residual. Under these conditions, we applied voltage step pulses to voltage clamped neurons and compared the delayed outward current before and after the addition of TTX (examples are shown of a tufted/mitral cell, a medium spiny neuron of the striatum (MSN) and a cortical pyramidal cell; Fig. 1a–c). We plotted the delayed outward current component as the average current during the interval of 150–250 ms after the initiation of step pulses. The addition of TTX reduced the delayed outward current by 43.3% \(\pm\) 2.5% \((n = 14, P \leq 0.01)\) in MSNs and 57.2% \(\pm\) 3.6% \((n = 21, P \leq 0.01)\) in tufted/mitral cells. In cortical pyramidal cells, the reduction was smaller, but still represented a substantial component in about half of the cells (Fig. 1c). To further validate that the TTX-sensitive outward current was evoked by the influx of sodium, we repeated the experiment, removing extracellular Na\(^+\) instead of adding TTX, and the results were similar to those obtained by adding TTX, with the delayed outward current being reduced by 49% (Figs. 1d and 2a). The effect of eliminating extracellular Na\(^+\) was readily reversible, although perhaps not totally (Fig. 2a). As an additional method of showing the selective activation of the delayed outward current by Na\(^+\) entry, we
substituted equimolar lithium ion for external Na\(^+\). Although Li\(^+\) is carried by voltage-gated sodium channels, prior studies (Dryer, 1994) have shown that K\(_{Na}\) channels are insensitive to Li\(^+\). We found that substituting Li\(^+\) for Na\(^+\) had a similar effect to adding TTX, reducing the delayed outward current in MSNs by 41.6\% versus a 43\% reduction by TTX (Fig. 2b). This result not only shows the specificity of Na\(^+\) influx for activation of the delayed outward current, but also eliminates the possibility that the change in the current is an artifact resulting from changing space-clamp conditions by TTX addition or Na\(^+\)_\(o\) removal.

Tufted/mitral cells of the rodent olfactory bulb had been used in classic studies (Egan et al., 1992a; Egan et al., 1992b) to determine the properties of Na\(^+\)-activated potassium channels in mammalian neurons because they are abundant in these cell types. We previously found that the \textit{Kcnt1} (also known as \textit{Slo2.2} and \textit{Slack}) gene encoded a Na\(^+\)-activated potassium channel (Yuan et al., 2003) and it has been demonstrated by immunocytoLOGY and \textit{in situ} techniques that Slack channels are expressed in those cell types (Bhattacharjee et al., 2002). Thus, the \textit{Slack} gene is a prime candidate to carry the Na\(^+\)-dependent delayed outward current that we observed. An immunocytochemical survey of many areas of the rodent brain found that the \textit{Slack} gene was widely expressed in both cortical and subcortical regions (Bhattacharjee et al., 2002). In contrast with olfactory bulb cells, Slack channel expression in MSNs has not been well studied. As we had previously detected that a substantial fraction of the delayed outward current was Na\(^+\) dependent, we sought to verify that Slack channels are expressed in MSNs and that Slack channels carry the large Na\(^+\)-dependent delayed outward current in those cells.
positively identified Slack channel expression in MSNs using a variety of techniques, including western blot and reverse transcription PCR (Supplementary Fig. 1). The expression of Slack channels in MSNs was also independently verified in another study by in situ hybridization (Berg et al., 2007). To investigate whether Slack channels actually carried the Na\(^+\)-dependent delayed outward current in MSNs, we designed siRNA primers to knockdown Slack expression in these cells, with the expectation that Slack-siRNA treatment would remove or reduce the Na\(^+\)-dependent outward current that is present in MSNs (siRNAs were designed on the basis of a previous study (Pei & Tuschi, 2006). As a control, we used a HEK cell line stably transfected with the Slack gene (Supplementary Fig. 2). We observed the efficient knockdown of Slack channel expression by siRNA to Slack by both immunocytological staining and physiological recordings (Supplementary Fig. 2). We then used the siRNAs validated by these experiments to knockdown Slack expression in primary cell cultures of MSNs (Fig. 1e). In MSNs transfected with Slack-siRNA (and a green fluorescent protein (GFP)-expressing vector), TTX reduced the delayed outward current by only 16.8% \(\pm\)3.2% \((n = 8)\), whereas it reduced the current in MSNs transfected with a control siRNA (Slick) (and a GFP-expressing vector) by 34.0% \(\pm\)3.9% \((n = 8, P \leq 0.01)\).

*A persistent Na\(^+\) current activates outward current*

One indication that persistent Na\(^+\) entry might be involved was the observation that the sodium-dependent delayed outward current persisted long after the transient sodium current was fully inactivated. Indeed, the sodium-dependent delayed outward
current showed only a minor decay even during step pulses lasting for 1,000 ms (Fig. 1b).

We examined the importance of the persistent $I_{Na}$ component relative to the transient $I_{Na}$ component in activating the sodium-dependent delayed outward current using two separate methods. First, we employed the pharmacological agent riluzole, which preferentially blocks the persistent $I_{Na}$ over the transient component of the sodium current (Hebert et al., 1994). Riluzole is known to block the persistent sodium current by stabilizing the inactivated state of the sodium channel, thereby preventing re-openings (flickering) of the channel (Hebert et al., 1994). A large fraction of the delayed outward current was reduced by the application of riluzole (20 μM; Fig. 3a), but the transient sodium current was not reduced (Fig. 3b). We examined the I-V relationship for the subtracted component and found that the subtracted (riluzole sensitive) component also included a persistent inward current, as well as the delayed outward component (Fig. 3c).

Note that the currents represent average values at 150–250 ms, long after the cessation of the transient inward component. Thus, riluzole removed the sodium-dependent outward current and the persistent $I_{Na}$, but not the transient $I_{Na}$. This indicates that the persistent $I_{Na}$ is the more important inward component coupled to the Na$^+$-dependent delayed outward component. Control experiments in our stably Slack-transfected HEK cell line showed no reduction of the Slack delayed outward current by riluzole (Fig. 3). In MSNs, riluzole (20 μM) was slightly less effective than TTX in reducing the delayed outward current, reducing it 30.1% (±3.09%, n = 9, P < 0.01). Thus, because a large fraction of the transient $I_{Na}$ remained after the application of riluzole, the transient sodium current does
not appear to be the major factor in activating the sodium-dependent delayed outward current.

Our second method for examining the importance of the persistent $I_{Na}$ was to use a relatively depolarized holding potential (-50 mV) to inactivate the transient $I_{Na}$ component. In these experiments, the TTX-sensitive delayed outward current was still present, as determined by the substantial reduction of the delayed outward current after the addition of TTX (Fig. 3d). The TTX-sensitive outward component was larger than the remaining outward current. At a holding potential of -50 mV, persistent sodium currents are active in most neuronal cell types, including MSNs (Alzheimer et al., 1993; Taddese & Bean, 2002; Chao & Alzheimer, 1995). The amplitudes of persistent sodium currents are often only a fraction of peak transient sodium currents, but are active over a broader voltage range (Alzheimer et al., 1993; Taddese & Bean, 2002; Chao & Alzheimer, 1995; Huang & Trussell, 2008). It is now widely accepted that several of the voltage-dependent sodium channel types that carry fast transient Na$^+$ currents, also carry persistent sodium currents (Alzheimer et al., 1993; Taddese & Bean, 2002), but we have not established the genetic identity of the persistent Na$^+$ currents in the cells that we have been studying. Our experiments here indicate that the persistent Na$^+$ current is the largest factor in activating the Na$^+$-dependent delayed outward current, but we cannot rule out the possibility that the transient Na$^+$-current is a contributing factor.

Experiments to measure the persistent Na$^+$ current in MSNs (Fig. 4) revealed a current that was a small fraction of the peak transient Na$^+$ current, as has been previously reported (Chao & Alzheimer, 1995). Also, as previously reported, we found the persistent
Na⁺ current to be active over a wider voltage range than the transient Na⁺ current, with some current being seen at negative potentials of at least -70 mV. Note that we adjusted the Na⁺ equilibrium potential in these experiments to approximately +23 mV to improve voltage control by raising internal [Na⁺] (Fig. 4). The lower driving force on Na⁺ resulted in a smaller sodium current than would be seen under normal physiological conditions and showed a reversal potential close to \( E_{\text{Na}} \). Experiments raising \( E_{\text{Na}} \) by lowering internal [Na⁺] indicated little inactivation of the persistent \( I_{\text{Na}} \) at voltages exceeding +23 mV.

**Na⁺-activated \( K^+ \) channels and Na⁺ channels may be clustered**

Given the high [Na⁺]i requirements for Na⁺-activated \( K^+ \) channel activation (Kameya et al., 1984; Koh et al., 1994; Yuan et al., 2003), it seems unusual that such a small Na⁺ current could effectively activate these \( K^+ \) channels. However, during a depolarizing step pulse, the transient \( I_{\text{Na}} \) is only maximal for 1 to 2 ms, whereas the persistent Na⁺ current, although smaller, is active indefinitely as long as sufficient depolarization is maintained. It may also be continuously active at or near cell resting potentials, albeit to a smaller degree. Conceivably, both Na⁺-activated \( K^+ \) channels and sodium channels might be tightly clustered in a microdomain that permits an increased concentration of Na⁺ relative to the bulk cytosol, similar to that seen for Ca²⁺ microdomains, in which calcium channels and Ca²⁺-dependent \( K^+ \) channels are clustered (Fakler & Adelman, 2008). Considering the high diffusion constant for Na⁺, we considered the possibility that a Na⁺ microdomain might consist of an ‘unstirred layer’
(Abriel & Horisberger, 1999; Pohl et al., 1998) or 'fuzzy space' (Barry, 1993; Carmeliet 1992) that restricts the diffusion of Na\(^+\), but such a space would also restrict the movement of K\(^+\), thus lowering the K\(^+\) conductance of Na\(^+\)-activated K\(^+\) channels. However, an alternative mechanism for the creation of a Na\(^+\)-rich microdomain might be an electrostatic environment that concentrates Na\(^+\) at a level that is higher than at the bulk cytoplasm. Such an electrostatic microdomain for K\(^+\) enrichment is present at the entrance to the intracellular vestibule of the BK (SLO1) channels, which have a ring of eight negatively charged glutamate residues (Brelidze et al., 2003; Nimigean et al., 2003). This ring of charge has been shown to double the conductance of SLO1 channels by increasing the local concentration of K\(^+\) in the vestibule through an electrostatic mechanism. Notably, the concentration of K\(^+\) in the vestibule by the ring of charge has been calculated to be equivalent to that achieved by increasing the K\(^+\) in the bulk intracellular solution from 150 to 500 mM (Brelidze et al., 2003; Nimigean et al., 2003). Such a simple electrostatic mechanism might function alone or in conjunction with an unstirred layer or fuzzy space to raise the local concentration of Na\(^+\) to a higher concentration than that of the bulk cytoplasm.

Our experiments (Figs. 1, 2, 3) were designed to determine the activation of delayed outward current by Na\(^+\) influx into a cell in which [Na\(^+\)]\(_{i}\) had been adjusted to minimal levels. However, we also undertook experiments to determine the activation of delayed outward current by Na\(^+\) influx into a cell in which [Na\(^+\)]\(_{i}\) had been adjusted to higher levels. Thus, we raised the internal [Na\(^+\)] by filling whole cell–patch recording electrodes with intracellular recording solutions containing 20, 30 and 40 mM Na\(^+\). In
these experiments, we found that, even though \([\text{Na}^+]_i\) was elevated, additional \(\text{Na}^+\) influx during voltage-clamp step pulses and/or at rest produced an incremental increase in the delayed outward current, noted as a TTX-sensitive component (Fig. 5a). This was true even though sodium entry during voltage-clamp step pulses was unlikely to appreciably raise the concentration of bulk \([\text{Na}^+]\).

One possible explanation for the effectiveness of sodium entry into \(\text{Na}^+\)-loaded cells in activating additional \(\text{Na}^+\)-dependent outward current is that persistent sodium entry may occur in close proximity to Slack channels. Hypothetically, if an inward \(\text{Na}^+\) current in close vicinity to Slack channels can raise the local \([\text{Na}^+]\) to a higher level than the bulk intracellular solution, then an outward \(\text{Na}^+\) current in close proximity to the Slack channels might deplete the local \([\text{Na}^+]\)_i sensed by Slack channels. A prediction of this hypothesis was that removing extracellular \(\text{Na}^+\) would remove a larger component of \(\text{Na}^+\)-dependent outward current than by simply adding TTX. This is because the removal of extracellular \(\text{Na}^+\) from \(\text{Na}^+\)-loaded neurons should result in an outward \(\text{Na}^+\) current in close proximity to Slack channels, whereas adding TTX should simply block the \(\text{Na}^+\) current. To test this, we raised \([\text{Na}^+]_i\) in a cell by filling the intracellular pipette with 40 mM \(\text{Na}^+\) (Fig. 5a). After applying a series of control voltage-clamp step pulses to a maximum of +90 mV, we removed extracellular \(\text{Na}^+\). After approximately 1.5 min, we repeated the series of voltage-clamp step pulses and observed a substantially diminished delayed outward current. Finally, we added TTX to the extracellular solution containing 0 \(\text{Na}^+\), and, after approximately 1.5 min, we again repeated the series of voltage-clamp step pulses. At this final condition, the delayed outward current was larger than the current
with 0 mM [Na\(^+\)]\(_o\). Our interpretation of this result is that Na\(^+\) entry in the first series of control voltage-clamp step pulses raised the [Na\(^+\)] in the vicinity of Slack channels to a higher level than that present in the bulk intracellular solution. However, after removing extracellular Na\(^+\), sodium moved in the outward direction across the membrane, diluting the intracellular Na\(^+\) in the immediate vicinity of Slack channels to a lower concentration than would be present if the outward flow of Na\(^+\) was blocked by TTX. Finally, after adding TTX to the extracellular solution containing 0 Na\(^+\), the outward flow of Na\(^+\) was reduced and the local intracellular concentration of Na\(^+\) in the vicinity of Slack channels rose to a level that was intermediate between control conditions, when the Na\(^+\) current was inward and the condition in which the Na\(^+\) current was outward.

We also determined the residual delayed outward currents obtained by subtracting the currents recorded after removal of external Na\(^+\) from the control currents recorded in normal [Na\(^+\)]\(_o\), and the residual currents obtained by subtracting the currents recorded after addition of TTX to 0 mM external Na\(^+\) from the control currents recorded in normal [Na\(^+\)]\(_o\) (Fig. 5a). Notably, these residual currents declined at higher voltages. This is probably a result of the fact that Slack channels at higher voltages are particularly vulnerable to block by [Na\(^+\)]\(_i\)\(_t\) (Dryer, 1994; Yuan et al., 2003). This could also be a result of the fact that the sodium current was outward at the higher voltage steps for the control currents recorded in normal [Na\(^+\)]\(_o\), and the outward flow of Na\(^+\) could therefore partially deplete the local concentration of Na\(^+\) sensed by SLO2 channels. However, we noted that the reduction of outward current that occurred after TTX was added (as in Fig. 1a–c) was not immediate and required approximately 1 to 2 min before
the current was stabilized at the lower level. Thus, the local concentration of Na\(^+\) sensed by SLO2 channels may only be partially changed during shorter voltage-clamp step pulses.

These results clearly suggest that, even if the internal bulk concentration of Na\(^+\) is raised to as high as 40 mM, there appears to be an additional rise in the Na\(^+\) concentration in the vicinity of Slack channels as long as the driving force on Na\(^+\) is inward. To explore this further, we conducted a series of experiments on cells 'loaded' with different intracellular concentrations of Na\(^+\) and subjected to a series of voltage-clamp step pulses. We then plotted the incremental rise in the Na\(^+\)-dependent delayed outward conductance resulting from Na\(^+\) influx augmenting the level of Na\(^+\) already present in the presumed microdomain (Fig. 5b). These incremental conductance-voltage curves were constructed from the residual currents obtained by subtracting the currents recorded after removal of external Na\(^+\) from the control currents recorded in normal [Na\(^+\)]\(_o\) (as in Fig. 5a). An examination of the normalized incremental conductance curves for 0, 20 and 30 mM [Na\(^+\)]\(_i\) (Fig. 5b) revealed two important features: as [Na\(^+\)]\(_i\) increases, the incremental conductances shifted leftward to more negative voltages, and the incremental conductances had a steeper slope. This seems to be consistent with the hypothesis that, at higher concentrations of bulk [Na\(^+\)]\(_i\), the sodium influx is augmenting the local Na\(^+\) concentration to an even higher level. These observations also seem to be consistent with earlier studies of the Na\(^+\) sensitivity of cloned Slack channels in inside-out patches, which found that there is a steep and accelerating relationship between Slack channel activity and [Na\(^+\)]\(_i\) (Yuan et al., 2003). That study showed that raising [Na\(^+\)]\(_i\) from 10 to 30 mM
increased Slack channel activity by only 20%, but raising [Na\(^+\)], from 30 to 50 mM increased channel activity by 70%. In the neurons that we studied, we asked how high the concentration of Na\(^+\) reached in the local region sensed by Slack channels. To answer this question, we loaded neurons with increasing concentrations of internal Na\(^+\) by including Na\(^+\) in the internal pipette solution. We found that, as long as the driving force on Na\(^+\) was inward, we could still detect a TTX-sensitive delayed outward current until the internal pipette solution contained approximately 70 mM Na\(^+\). This suggests that the TTX-sensitive Na\(^+\) flux could still substantially increase the local concentration of Na\(^+\) in the vicinity of Na\(^+\)-activated K\(^+\) channels until the bulk intracellular [Na\(^+\)] approached 70 mM. This, however, could be an underestimate, as loading cells with high intracellular concentrations of Na\(^+\) causes a substantial block of most K\(^+\) channels (Bezanilla & Armstrong, 1972).
DISCUSSION

Na\(^+\)-activated K\(^+\) channels are difficult to study because their activity is influenced by many factors, including their Na\(^+\) sensitivity, weak voltage sensitivity, channel run-down, modulation by second messenger systems and, not least of which, the mechanism and kinetics of Na\(^+\) delivery to the channels themselves. Nevertheless, although the precise mechanisms that are responsible for the local rise of [Na\(^+\)] have not been fully established, our data strongly suggest that Na\(^+\) entry through TTX-sensitive Na\(^+\) channels, most likely carrying a persistent Na\(^+\) current, activates a Na\(^+\)-dependent delayed outward current that is a large component of outward conductance in some neurons under normal physiological conditions. This result further suggests a very special relationship between Na\(^+\)-activated K\(^+\) channels and TTX-sensitive sodium channels. Even at relatively high internal bulk concentrations of Na\(^+\), for example, 40 mM, an inward Na\(^+\) current can still activate a substantial incremental component of delayed outward current. Thus, local sodium entry appears to be highly effective at raising the local [Na\(^+\)]\(_i\), regardless of the concentration of the internal bulk solution. Although a diffusion barrier model might permit a local increase in Na\(^+\), an electrostatic microdomain model might explain not only a local rise in [Na\(^+\)], but a unique requirement for persistent sodium entry as well. A putative electrostatic microdomain would most likely be nonselective and would elevate both K\(^+\) and Na\(^+\) in relative proportion to their bulk intracellular concentrations. However, closely related persistent Na\(^+\) entry might displace a substantial amount of K\(^+\), elevating the local [Na\(^+\)] to a higher
level. Notably, Slack channels are similar to SLO1 channels in having a double ring of negative charges surrounding the inner vestibule of the channel (Brelidze et al., 2003). They also have other cytoplasmic domains with high net negativity, but the relevance of these features, if at all, to the mechanism of Na\(^{+}\)-dependent gating of Slack channels must await further studies. Regardless of the exact nature of the Na\(^{+}\)-rich microdomain, our results suggest that Na\(^{+}\) is normally maintained at a higher concentration in the microdomain than in the bulk cytoplasm. TTX-dependent persistent Na\(^{+}\) entry appears to occur over a wide range of voltages and there appears to be sufficient entry of Na\(^{+}\) at a holding potential of -70 mV such that neurons maintained at that holding potential for long periods still show a TTX-sensitive component of outward current even when test steps are taken to the Na\(^{+}\) reversal potential. Thus, the possibility that a small contribution of persistent inward Na\(^{+}\) current operates continuously at cell resting potentials and maintains the local intracellular concentration of Na\(^{+}\) at a higher level than that of the bulk cytoplasm at the resting state cannot be excluded.

The potential role of the sodium channel–Na\(^{+}\)-activated K\(^{+}\) channel–coupled system may differ widely in different neuronal cell types, but in some, its effect may be profound. The relative contribution of the sodium-dependent delayed outward current relative to the total outward current may increase as membrane resting potential becomes more positive because some voltage-dependent K\(^{+}\) currents inactivate (for example, A-type currents (Connor & Stevens, 1971), whereas Na\(^{+}\)-activated K\(^{+}\) currents and persistent sodium currents do not. These findings of a major, but previously unseen, K\(^{+}\) conductance have far reaching implications for many aspects of systems and cellular
neuroscience, as well as clinical neurology and pharmacology. In systems and cellular neuroscience, studies of 'up-down' states of neuronal excitability, spike adaptation, synaptic integration and other aspects of neuronal physiology may have to be reexamined taking this system into consideration. In clinical and pharmacological studies, this previously unseen current system that is active during normal physiology represents a new and promising pharmacological target for drugs dealing with seizure and psychotropic disorders.
Figure 1 - The TTX-sensitive (Na⁺-dependent) delayed outward current and its elimination by Slack-siRNA.

(a–c) The TTX-sensitive delayed outward current in tufted/mitral cell (a), MSN (b) and cortical pyramidal cell (c), respectively. The top traces show the family of control currents evoked from a holding potential of -70 mV. The middle traces show the outward current remaining after addition of TTX (1 μM). The bottom traces show the TTX-sensitive current (TTX-sensitive K⁺ current) that is the difference between the control currents before TTX and the remaining currents after the addition of TTX. The current values shown in the I-V are average values measured in the interval of 150–250 ms after the initiation of the voltage step. The intracellular pipette solution in these whole-cell patch-clamp experiments contained no Na⁺ and no Ca²⁺ was present in the extracellular recording solutions. Some current traces in our experiments showed unusual kinetics at the initiation of the voltage-clamp step pulse. It is likely that these were anomalies resulting from the fact that TTX was not present, and very rapid inward Na⁺ currents therefore are opposing rapid transient outward currents at the initiation of the pulse in a cell in which the space clamp is not perfect (which is why TTX is so often used in studies of outward currents). (d) The removal and subsequent replacement of extracellular Na⁺ revealed the Na⁺-dependent delayed K⁺ current in an MSN. Control indicates the family of control currents evoked from a holding potential of -70 mV, -Na⁺ indicates the outward current remaining after removal of external Na⁺, Na⁺-dependent K⁺ current indicates the difference between the control currents before Na⁺ removal and the remaining currents after Na⁺ removal, and recovery indicates the current after
reintroduction of extracellular Na\(^+\). The intracellular pipette solution in these whole-cell patch-clamp experiments contained no Na\(^+\). (e) An example of an MSN treated with Slack-siRNA that had a smaller TTX-sensitive outward component. This supports the hypothesis that the TTX-sensitive delayed outward current is carried by Slack channels. See Figure 2 for statistical information.
Figure 2 - Inhibition of Na\(^+\)-dependent delayed outward current by the removal of extracellular Na\(^+\) and the substitution of external Li\(^+\) for Na\(^+\).

(a) The removal of extracellular Na\(^+\) reduced the delayed outward current by 49.9\% \(\pm\) 2.3\% \((n = 8, P \leq 0.01)\) in MSNs. Recovery after reintroduction of external Na\(^+\) was 85.7\% \(\pm\) 3.4\% \((n = 7, P = 0.047)\) compared with starting control currents. This indicates that recovery after reintroduction of [Na\(^+\)]\(_o\) may not have been quite complete. (b) The substitution of external Li\(^+\) for Na\(^+\) reduced the delayed outward current by 41.6\% \(\pm\) 3.1\% \((n = 11, P \leq 0.001)\). Recovery after reintroduction of external Na\(^+\) was 103\% \(\pm\) 8.0\% \((n = 6, P = 0.78)\) compared with starting control currents. Error bars represent standard errors.
Figure 3 - Evidence that a persistent Na⁺ current activates the sodium-dependent delayed outward current.

Riluzole removed the persistent $I_{Na}$, but not the transient $I_{Na}$, and also removed a delayed outward current. (a) Currents before and after the application of riluzole (20 µM) and the subtracted (riluzole sensitive) component recorded from a tufted-mitral neuron. The currents shown on the left are plotted with respect to voltage. Plotted currents were average amplitudes measured during the 150–250-ms interval after initiation of the step pulse. (b) Transient sodium currents in a at higher resolution showed no reduction by riluzole. (c) The base of the plotted current curves from the graph in a is shown at higher resolution. The arrow indicates a riluzole-sensitive persistent inward current. Also note the TTX-sensitive persistent inward current in Figure 1e. Note that we did not find any block of Slack currents in control experiments by recording delayed outward currents before and after applying riluzole (20 µM) to our stably Slack-transfected HEK cell line. The addition of 20 µM riluzole to the cell line resulted in a slight statistically insignificant increase in delayed outward current (4.83% ± 3.02%, $n = 3$). Currents were measured 200 ms after voltage step to +40 mV. The effect of riluzole was similar across all voltages tested (-90 mV to +80 mV). (d) The TTX-sensitive current shown from a holding potential of -50 mV. Except for the depolarized holding potentials, other experimental details are as described in Figure 1a. No transient inward sodium current was noted in this cell, but the TTX-sensitive delayed outward current was well over half the total outward current. (e) The base of the plotted current curves from the graph in d is shown at higher resolution. The arrow indicates a TTX-sensitive persistent inward current.
Figure 4 - Transient and persistent Na\(^+\) currents in MSNs.

(a,b) Transient Na\(^+\) current. (c,d) Persistent inward Na\(^+\) current. The persistent Na\(^+\) current was plotted as the mean current during the 150–250-ms interval after the transient inward Na\(^+\) current, as indicated in c \((n = 3)\). We used 100 mM cesium ion in the internal pipette solution and 40 mM tetraethylammonium chloride (TEA) in the extracellular saline to block the potassium conductances. To isolate the TTX-sensitive components, we recorded currents before and after applying TTX. We then subtracted the residual currents after TTX application from the currents recorded before TTX application. Thus, the currents that are shown represent both the TTX-sensitive transient and persistent components. The internal pipette solution contained 40 mM Na\(^+\) to reduce the driving force of Na\(^+\) and to gain better voltage control. This also reduced the amplitudes of the currents. The approximate Na\(^+\) equilibrium potential was +23 mV. Error bars represent standard errors.
Transient sodium current

Persistent sodium current

\( E_{\text{Na}} = +23 \text{ mV} \)
Figure 5 - Experiments in cells loaded with high [Na$^+$].

(a) A greater amount of delayed outward current was blocked by removing extracellular Na$^+$ than by blocking Na$^+$ flux with TTX. Current-voltage plots are shown from a cell in which the intracellular pipette solution contained 40 mM Na$^+$. A series of control voltage-clamp step pulses were applied at 10-mV intervals to a maximum of +90 mV. Control indicates the delayed outward currents plotted from the cell with normal [Na$^+$]o. -Na$^+$o indicates the outward currents plotted from the same cell after the removal of external Na$^+$. -Na$^+$o + TTX indicates the outward currents plotted from the same cell after subsequently adding TTX to the -[Na$^+$]o condition. This plot shows the delayed outward current in response to the same series of voltage-clamp step pulses after reducing the outward movement of Na$^+$. These experiments were repeated seven times in MSNs and three times in tufted/mitral cells with similar results. (b) Conductance/voltage relationships showing the normalized incremental conductance increases in the delayed outward current resulting from Na$^+$ influx in cells loaded with different levels of [Na$^+$]i. Tufted/mitral cells loaded with different intracellular concentrations of Na$^+$, as indicated, were subjected to a series of voltage-clamp step pulses. The incremental conductance/voltage curves were constructed from residual currents obtained by subtracting currents recorded after removal of external Na$^+$ from control currents recorded in normal [Na$^+$]o (as in a, 1–2). As [Na$^+$]i increased, the incremental conductance curves shifted leftward to more negative voltages and also had steeper slopes. This seems consistent with the hypothesis that, at higher concentrations of bulk [Na$^+$]i, the sodium influx substantially increases the local internal Na$^+$ concentration to a
level above that of the bulk cytoplasm. Conductances ($g$) were calculated for individual cells at the three indicated concentrations, normalized to the maximum value and then averaged. Regions of the curves to the left of maximum conductance ($g_{\text{max}}$) were fit by a Boltzmann equation (red). For 0 mM [Na$^+$]$_i$, $V_{1/2} = 11.8$ mV, $n = 3$; 20 mM [Na$^+$]$_i$, $V_{1/2} = 1.40$ mV, $E_{\text{Na}} = 50.8$ mV, $n = 3$; 30 mM [Na$^+$]$_i$, $V_{1/2} = -11.0$ mV, $E_{\text{Na}} = 40.5$ mV, $n = 4$. 

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Supplementary Figure 1. SLO2.2 (Slack) expression in striatal neurons as shown by Western Blots (left) and rtPCR (right) from primary cultures of rat striatal neurons. **Left.** Western blots showing Slack protein expression in indicated developmental stages of striatum (P1-P15). The antibody used in these Western studies was previously characterized (15). Western blots also showed Slack expression in adult striatum (not shown). **Right.** Slack transcripts detected by rtPCR in striatal primary neuronal cultures (rat) and whole brain (mouse). Slack-specific primers were designed to amplify a 390bp fragment. Control rtPCRs were performed with β-actin primers (772bp product) and with primers alone. First-strand syntheses were performed using 2.0 µg of total RNA isolated from rat striatal primary neuronal cultures, and 5.0 µg of total RNA from whole mouse brain. A fraction of each first strand reaction served as template for rtPCRs; rat striatal neuronal cultures (~5.0%) and mouse whole brain (~2.0%). rtPCR assays were cycled 35 times, and the products electrophoresed on a 1.5% agarose gel with molecular weight markers.
Supplementary Figure 2. siRNA knock-down of Slack channel expression in a HEK cell line stably transformed with Slack. a. Slack (stained red with an anti-Slack antibody) is abundantly expressed in all the cells of this line. b. Knock-down of Slack expression after transfection with anti-Slack siRNA. Transfection of siRNAs into cells included co-transfection of a plasmid expressing GFP as a fluorescent marker (green) to identify transfected cells and assess transfection efficiency. Upper left, Nomarski image of cells in culture. Upper right, transfected GFP expressing cells (green) lack expression of Slack (red) as seen in the lower left panel. Lower left, Slack (red) expressing cells which have not been transfected. Lower right, composite image of upper left, upper right, and lower left panels. If siRNA-transfected cells expressed Slack they would be yellow in this image. Lipofectamine (Invitrogen) was used to transfect HEK cells, and a high efficiency electroporation method was used to transfect neurons in primary cell culture (Mouse Neuron Nucleofector Kit from Amaxa, Inc.). Transfection efficiency of siRNAs was monitored by co-transfection with a plasmid expressing green fluorescent protein. c. Anti Slack siRNA effectively knocks down Slack expression in a stably transfected HEK-Slack cell line. Left traces, representative current records from a control HEK cell that has not been transfected with Slack. Middle traces, current records from a HEK-Slack stably transfected cell. Right traces, current records from a HEK-Slack cell after transfection with siRNA. All experiments had 10 mM Na+ in the intracellular pipette solution. Control cells 217.3 +/- 30.8 pA; n=8. [Control non-transfected HEK-Slack cells 730.5 +/- 89.9 pA; n=3. pairwise with Control HEK-Slack cells (+GFP) 798 +/- 95 pA; n=4. *P = 0.43*]; [HEK-Slack cells (+GFP + Slack siRNA) 272

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+/- 30 pA, n=10 pairwise with Control HEK-Slack cells (+GFP). \( P = \lt .01 \). Measured step pulse = +40 mV.
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Bhattacharjee, A. & Kaczmarek, L.K. For K⁺ channels, Na⁺ is the new Ca²⁺. Trends Neurosci. 28, 422–428 (2005).


Preface to Chapter 3

This chapter is taken from a paper which appeared in the *Journal of Neuroscience*. The complete citation is Sodium-activated potassium channels are functionally coupled to persistent sodium currents. Hage TA, Salkoff L. *J Neurosci* 2012 32:2714-21. Some adjustments have been made for the thesis format. All data was generated and analyzed myself in close consultation with my advisor Lawrence Salkoff.
Chapter 3

Sodium-activated potassium channels are functionally coupled to persistent sodium currents
ABSTRACT

We report a novel coupled system of sodium-activated potassium currents ($I_{KNa}$) and persistent sodium currents ($I_{NaP}$), the components of which are widely distributed throughout the brain. Its existence and importance has not been previously recognized. Although $I_{KNa}$ was known to exist in many cell types, the source of Na$^+$ which activates $I_{KNa}$ remained a mystery. We now show in single membrane patches generated from the somas of rat neurons that sodium influx through $I_{NaP}$ is sufficient for activation of $K_{Na}$ channels, without substantial contribution from the transient sodium current or bulk [Na$^+$]. $I_{NaP}$ was found to be active at cell membrane resting potentials, a finding that may explain why $I_{KNa}$ can be evoked from negative holding potentials. These results show an unanticipated role for $I_{NaP}$ in activating a negative feedback system countering the excitable effects $I_{NaP}$; the interrelatedness of $I_{NaP}$ and $I_{KNa}$ suggests new ways neurons can tune their excitability.
INTRODUCTION

Sodium-activated potassium channels (K\textsubscript{Na} channels) were first described over 25 years ago as high conductance potassium channels activated by high concentrations of internal sodium (Kameyama et al., 1984). Despite many reports of their expression throughout the nervous system and other tissues (for review, see Dryer, 1994; Bhattacharjee and Kaczmarek, 2005), it was unclear whether K\textsubscript{Na} channels were active under normal physiological conditions. Experiments with inside-out patches appeared to show that K\textsubscript{Na} channel activity requires bulk sodium concentrations in excess of that normally present in bulk cytosol. Such experiments were taken as evidence against the participation of I\textsubscript{KNa} during normal electrophysiological activity. Rather, it was suggested that K\textsubscript{Na} channels might represent a type of “reserve conductance” active only under conditions of hypoxia when [Na\textsuperscript{+}]\textsubscript{i} might substantially increase. However, recent studies have shown that K\textsubscript{Na} channels produce large outward currents in several types of neurons under normal physiological conditions, and appear to be dependent on sodium influx rather than high bulk [Na\textsuperscript{+}]\textsubscript{i} (Budelli et al., 2009; Lu et al., 2010; Nuwer et al., 2010).

I\textsubscript{KNa} is a tetrodotoxin (TTX)-sensitive delayed outward current that lasts for seconds but the transient sodium current (I\textsubscript{NaT}) is over within a few milliseconds; I\textsubscript{NaT} thus seems an unlikely source of Na\textsuperscript{+} to activate I\textsubscript{KNa}. Here we explore the possibility that the TTX-sensitive persistent sodium current (I\textsubscript{NaP}) is the source of Na\textsuperscript{+} activating I\textsubscript{KNa}. Amplitudes of I\textsubscript{NaP} are tiny compared with peak amplitudes of I\textsubscript{NaT}, and even prolonged I\textsubscript{NaP} activity will not quickly increase bulk [Na\textsuperscript{+}]\textsubscript{i}. Therefore a mechanism permitting I\textsubscript{NaP}
to deliver sufficient cytosolic Na\(^+\) to activate \(I_{\text{KNa}}\) would require that channels underlying both currents be present together in single membrane patches. We show that the activity of \(I_{\text{KNa}}\) indeed depends on the activity of \(I_{\text{NaP}}\) by recording single-channel \(I_{\text{NaP}}\) and \(I_{\text{KNa}}\) currents in membrane patches isolated from the soma of olfactory bulb mitral cells. Single-channel currents recorded from outside-out patches revealed that treatments that eliminated \(I_{\text{NaP}}\), also removed \(I_{\text{KNa}}\); conversely, enhancing \(I_{\text{NaP}}\) resulted in increased \(I_{\text{KNa}}\) activity. This dependence of \(I_{\text{KNa}}\) on \(I_{\text{NaP}}\) was demonstrated under conditions where \(I_{\text{NaT}}\) was absent, and bulk \([\text{Na}^+]_i\) was 0, thus verifying the importance of \(I_{\text{NaP}}\). These results focus further attention on \(I_{\text{NaP}}\) which is increasingly being recognized for its role in controlling many aspects of cell excitability (Crill, 1996; Vervaeke et al., 2006; Huang and Trussell, 2008). Although the action of \(I_{\text{NaP}}\) is usually thought of as producing an increase in cell excitability (Stafstrom et al., 1984) our results suggest an unanticipated role for \(I_{\text{NaP}}\) in activating a negative feedback system, \(I_{\text{KNa}}\), which provides an opposing inhibitory current.
MATERIALS AND METHODS

Primary neuronal culture preparations.

Primary cultures of dissociated olfactory bulbs were prepared from either sex of neonatal Sprague Dawley rats (postnatal day 0). Dissected olfactory bulbs were incubated in 0.25% trypsin in PBS for 30 min at 37°C. For the last 5 min, trypsin solution was supplemented with 0.05% DNase. Enzyme digested tissue was then triturated in PBS containing soy bean trypsin inhibitor using Pasteur pipettes of decreasing diameter. Cells were plated on poly-lysine-coated glass coverslips in Dulbecco's Modified Eagle Medium/F-12 medium (Invitrogen) with 10% FBS and 2% B-27 supplement (Invitrogen). Three days after plating, medium was replaced with Neurobasal (Invitrogen) + 2% B-27 supplement and 5 μm arabinofuranosyl cytidine to inhibit division of glia. Whole-cell and excised patch recordings were made up to 4 and 10 d after plating, respectively.

Voltage-clamp recordings.

Tufted/mirital cells were identified on the basis of their pyramidal shape and large size (Trombley and Westbrook, 1990). Recordings were made with borosilicate glass pipettes with tip resistances of 3–6 MΩ. Outside-out patches were obtained by steadily moving the pipette away from the cell after establishing a whole-cell patch at the soma. Voltage-clamp recordings were made with an Axoclamp 200B, filtered at 2 kHz and digitized at 50 kHz with a Digidata 1440A. Internal pipette solution contained the
following (in mm): 140 KCl, 10 HEPES, 5 EGTA, 1 MgCl₂, pH 7.40 with KOH. Bath solutions contained the following (in mm): 150 NaCl, 10 HEPES, 10 dextrose, 5 KCl, 2 MgCl₂, pH 7.30 with NaOH. For recordings of $I_{Na}$, the internal solution contained 130 CsCl, 10 NaCl, 1 MgCl₂, 10 HEPES, 5 EGTA, pH 7.40 with KOH. The external solution for recording $I_{Na}$ was the same as the standard external recording solution with 10 mm tetraethylammonium (TEA) · Cl, 2 mm 4 aminopyridine (4-AP) · Cl, and 0.2 mm CdCl₂ replacing equimolar NaCl. Zero Na⁺ external solutions replaced all NaCl with choline · Cl or LiCl as indicated. TTX and veratridine were used at 1 and 50 µm concentrations, respectively. Veratridine was stored as a 50 mm stock frozen in DMSO. Experiments using veratridine included 0.1% DMSO in control external solution. No effect of this concentration of DMSO on channel activity was noted. In control experiments examining the effect of TTX on Slack-transected human embryonic kidney cells, 10 mm NaCl replaced equimolar KCl in the internal solution.

*Single-channel analysis.*

Data were acquired and analyzed with pClamp 10.0 and Microsoft Excel. Single $K_{Na}$ channel activity was analyzed and $P_o$ calculated using the Single Channel Search tool in pClamp 10.0. $K_{Na}$ channel activity was measured during repeated 3 s sweeps to +60 mV from a holding potential of −70 mV, using a 10 s intersweep interval. Under differing experimental conditions, no changes in the mean amplitude of detected events or distribution of peaks in histograms of event amplitude were found. The number of channels in a patch was estimated by the maximum number of simultaneously active
channels (additive steps) observed over the course an experiment. The minimum estimate of channel number provided by this method was used to determine $P_0$. In the majority of experiments, patches analyzed were estimated to contain 4–6 channels. Single $I_{\text{NaP}}$ channel activity was measured using Threshold Search tool in pClamp 10.0. Many of the persistent TTX-sensitive openings were so brief that we could not reliably measure a mean channel open time, even when digitizing at high rates. Thus, we used the number of detectable events, and the Threshold Search tool as a means of quantification rather than channel open probability. Threshold levels were determined by measuring unitary current levels from selected “box-like” open channel events. The brevity of many events prevented use of an all points histogram to determine unitary current level. Identical threshold searches were performed for control sweeps and sweeps evoked in TTX. A small number of events crossed threshold in TTX and those values were subtracted from corresponding control values to generate the plot in Figure 5B (see Results).

*Estimation of $[\text{Na}^+]_i$ increase.*

The volume of pipette solution was estimated to be 7.26 µl using the inner diameter of the recording pipettes (0.68 mm) and an approximation of internal solution fill height starting at the tapering of the pipette (20 mm). The number of Na$^+$ molecules entering the patch was estimated based on a high approximation of average $I_{\text{Na}}$ throughout the experiment: $-50$ pA. Using the Faraday constant, this corresponds to $5.18 \times 10^{-16}$ mol/s. Given this rate of Na$^+$ influx and the volume of pipette solution, the
concentration of sodium would increase by 0.257 µm over the course of an hour-long experiment.

This is likely an overestimate, the fill height was usually >20 mm, and there is additional volume in the tapered portion of the pipette not considered here. In most excised patches, the peak amplitude of $I_{Na}$ was much smaller than ~50 pA and even smaller at the holding potential that patches were clamped at for the majority of the experiment.

**Statistical analysis.**

We performed statistical analyses using T.W. Kirkman's Statistics to Use (http://www.physics.csbsju.edu/stats/) software. For comparison between two groups, we used paired Student's $t$ tests for the same procedures before and after applied treatments.

**Animal welfare.**

It is the policy of Washington University Medical School that all research involving animals be conducted under humane conditions, with appropriate regard for animal welfare. Washington University Medical School is a registered research facility with the United States Department of Agriculture (USDA) and is committed to complying with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services), the provisions of the Animal Welfare Act (USDA), and all applicable federal and state laws and regulations. At Washington University Medical School an Animal Care Committee has been established to insure compliance with all
applicable federal and state regulations for the purchase, transportation, housing and research use of animals. Washington University Medical School has filed appropriate assurance of compliance with the Office for the Protection of Research Risks of the National Institutes of Health.
RESULTS

In whole-cell recordings of olfactory bulb tufted/mitral cells (T/M cells) (Fig. 1) or striatal medium spiny neurons (Budelli et al., 2009), blockade of voltage-gated sodium (Nav) channels with TTX (1 µm) reduces the amplitude of sustained outward currents. This portion of TTX-sensitive outward current removed represents a sodium-activated potassium current (IKNa) (Budelli et al., 2009). As shown in Figure 1, the kinetic properties of the TTX-sensitive outward current (IKNa) do not appear to depend on INaT; IKNa is a long-duration delayed outward current showing very little inactivation, while INaT is over in a few milliseconds. Since a key feature of IKNa is its sensitivity to TTX, an alternative to its activation by the TTX-sensitive INaT component could be its activation by the TTX-sensitive INaP which is known to accompany INaT in most neurons (Crill, 1996). Supporting this hypothesis are our observations showing that IKNa persists even when INaT is eliminated or greatly reduced by a depolarized holding potential of −50 mV (Fig. 1b).

IKNa in detached outside-out patches

Notably, the whole-cell experiments shown in Fig. 1 were undertaken with the internal bulk concentration of sodium ion reduced to virtually 0 mm. This means that the activation of IKNa by INaP has to be accomplished by sodium ion crossing the membrane into the cell. This also implies that the local internal concentration of Na+ in the vicinity of IKNa must be raised to a sufficiently high level without any contribution from the bulk
internal \([\text{Na}^+]\). Because of the low conductance of \(I_{\text{NaP}}\) such a scenario might seem unlikely, but might be possible if channels carrying \(I_{\text{KNa}}\) and \(I_{\text{NaP}}\) were closely associated. A rigorous test of the hypothesis of the close association and functional coupling of these two channel types would be demonstrating the dependency of \(I_{\text{KNa}}\) on the activity of \(I_{\text{NaP}}\) in an isolated membrane patch.

To test this hypothesis, we recorded \(I_{\text{KNa}}\) currents in outside-out patches excised from the soma of T/M cells and showed their dependence on \(I_{\text{NaP}}\) as we had in whole-cell configuration. Figure 2 shows an outside-out macro patch containing a prominent potassium current resembling those recorded in the whole-cell configuration, but lacking any indication of \(I_{\text{NaT}}\) (Fig. 2a). As we showed for whole-cell currents, outward currents were substantially reduced when external sodium was removed (Fig. 2b) but returned when \([\text{Na}^+]_o\) was restored (Fig. 2c). In these experiments, no sodium was included in the internal pipette solution so that activation of \(K_{\text{Na}}\) currents would be minimally dependent on bulk sodium and primarily determined by sodium influx. The component of \(K^+\) current dependent on \(Na^+\) influx was revealed by subtraction, and closely resembles that seen in whole-cell recordings (Fig. 2d) (amplitude of sustained outward current in external choline was reduced to 63.5 ± 3.01% of control values \(p < 0.001, n = 5\); upon restoration of external \(Na^+\), amplitude of sustained outward current returned to 88.4 ± 7.39% of control values, \(p < 0.05\) compared with choline, mean ± SEM, \(n = 5\), \(V_m = +60\) mV). We also applied TTX to macropatches and, as in whole-cell recordings, saw a significant reduction in the amplitude of total delayed outward current, revealing a large component of \(I_{\text{KNa}}\) (amplitude of sustained outward current in TTX was reduced to 47.3 ± 12.4% of
control values $p < 0.05$, mean ± SEM, $n = 4$, $V_m = +60$ mV). As a control for a direct effect of TTX on $K_{Na}$ channels, we measured whole-cell currents from a line of human embryonic kidney cells stably transfected with the Slack gene which is known to encode $K_{Na}$ channels, and found no change in $I_{KNa}$ current amplitude following application of 1 µm TTX (amplitude of sustained outward current in TTX was 101% ± 2.62% of control value, $n = 3$, measured at +60 mV $p = 0.6$). Furthermore, replacing external sodium with the NaV-channel-permeant cation, lithium, also resulted in a significant decrease in the amplitude of sustained outward currents in excised patches (amplitude of sustained outward current in external Li$^+$ was reduced to 56.2 ± 3.67% of control values $p < 0.001$, $n = 4$; upon restoration of external Na$^+$, amplitude of sustained outward current returned to 78.8 ± 3.01% of control values, $p < 0.01$ compared with Li$^+$ mean ± SEM, $n = 4$, $V_m = +60$ mV). These results support the hypothesis that both $I_{KNa}$ and $I_{NaP}$ currents were present in macropatches.

Only trace amounts of $I_{NaT}$ were evoked by step pulses from excised patches held at −70 mV (although patches held at −90 mV exhibited an obvious $I_{NaT}$). Thus, it is unlikely that $I_{NaT}$ had any substantial role in evoking the Na$^+$-dependent outward current seen in Figure 2d. Since internal Na$^+$ was not included in the pipette solution in these experiments, the results imply that persistent Na$^+$ influx was responsible. However, because of the low amplitude of $I_{NaP}$ it is unlikely that there would be a sufficient influx of Na$^+$ during the first few milliseconds of a depolarizing step pulse to substantially raise the local [Na$^+$], near $K_{Na}$ channels to a sufficiently high level to permit $I_{KNa}$ activation. However, as will be shown in a following section, $I_{NaP}$ was observed at all holding
potentials and thus, the most likely mechanism is that a small but persistent influx of Na$^+$ at holding or resting potentials maintains Na$^+$ at a higher concentration at the site of K$_{Na}$ channels than in the bulk cell interior. Since K$_{Na}$ channels resemble Ca$^{2+}$-activated BK channels in having a voltage-dependent component to their activation, current amplitudes increase with depolarizing step pulses, as seen in Figure 2.

**Single-channel $K_{Na}$ currents coupled to Na$^+$ influx**

To examine the functional association of K$_{Na}$ channels with $I_{NaP}$ at higher resolution, we used smaller outside-out patches where we could distinguish the activity of individual K$_{Na}$ channels (Fig. 3a). These channels resemble K$_{Na}$ channels in inside-out patches from T/M cells as previously reported to be abundantly expressed in this cell type (Egan et al., 1992). K$_{Na}$ channels from these native cells also resembled sodium-activated SLO2 channels expressed in heterologous systems (Bhattacharjee et al., 2003; Yuan et al., 2003; Santi et al., 2006). The excised patch in Figure 3a contains at least 8 active high conductance channels, and even though single-channel openings are easily discerned, the evoked current as a whole resembles that seen in macropatches as in Figure 2. When TTX (1 µm) was perfused on the outside of this patch and others like it, there was a substantial decline in channel activity (Fig. 3a) ($P_o$ decreased to 57.3 ± 3.00% of control value after application of 1 µm TTX, $p < 0.001$ $n = 5$, measured at +60 mV). These results suggest that, in addition to the high conductance K$_{Na}$ channels in the patch which are obvious, the patch also contains TTX-sensitive Na$_V$ channels which are not. We will
show in a following section that single-channel $I_{NaP}$ can be revealed when measures are taken to block $K^+$ channels.

Like macroscopic $I_{KNa}$ seen in whole-cell experiments and in macropatches, TTX-sensitive single $K_{Na}$ channel activity was observed without the addition of $Na^+$ to our pipette solution (Fig. 3a). Thus, even at the single-channel level it appears that TTX-sensitive sodium influx and not bulk internal $Na^+$ was activating $I_{KNa}$. This seems especially persuasive in these experiments with excised outside-out patches because the volume of solution inside the pipette is enormous relative to the miniscule fluid volume in contact with the inner surface of the membrane patch. In these experiments, bulk $[Na^+]_i$ is not likely to be significantly altered (see calculations in Materials and Methods). Yet, these experiments clearly show the $Na^+$ dependence of single $K_{Na}$ channels. Such activity implies that local $[Na^+]_i$ near $K_{Na}$ channels at the intracellular membrane surface was being raised to a higher level than bulk internal $Na^+$ by persistent TTX-sensitive $Na^+$ influx. As previously reported in whole-cell experiments (Budelli et al., 2009), replacement of external $Na^+$ with $Li^+$, decreased observed single $K_{Na}$ channel activity in outside-out patches (Fig. 3b). ($P_o$ decreased to $66.0 \pm 4.16\%$ of control value after replacement of $Na^+$ with $Li^+$, $p < 0.001$, mean $\pm$ SEM $n = 3$, measured at $+60$ mV).

*External veratridine increases local internal $[Na^+]_i$*

After demonstrating that removing external $Na^+$ or blocking $Na^+$ influx decreases $K_{Na}$ channel activity in isolated patches, we hypothesized that increasing $Na^+$ influx would increase $K_{Na}$ channel activity. We applied veratridine (50 $\mu$m), which prolongs the
open state of the sodium channel (Barnes and Hille, 1988), to outside-out patches containing active $K_{Na}$ channels, and observed a significant increase in $K_{Na}$ channel activity (Fig. 4). The activating effect of veratridine on single $K_{Na}$ channel activity could be abolished by the removal of external $Na^+$ after application of veratridine. ($K_{Na}$ channel $P_o$ increased to 171% ± 9.44% of control value after application of 50 µm veratridine, $p < 0.001$, $n = 10$, and decreased to 55.7 ± 12.7% value following subsequent removal of external $Na^+$, $p < 0.001$, mean ± SEM, $n = 10$, measured at +60 mV). Thus, our interpretation of these results is that veratridine increases $K_{Na}$ channel activity by increasing sustained $Na^+$ influx via $NaV$ channels. We emphasize that the effect of veratridine must be via increasing the local $[Na^+]$ in the vicinity of $K_{Na}$ channels and cannot be due to changing the internal bulk $[Na^+]$. Given the small amplitude of $I_{NaP}$ in excised patches (see below) and the volume of internal solution used in recording pipettes, increases in bulk $[Na^+]_i$ are estimated to be well below 1 µm in experiments using outside-out patches (Materials and Methods), which is orders of magnitude below $[Na^+]$ necessary for $K_{Na}$ channel activation. Therefore, changes in activity of $K_{Na}$ channels upon enhancement of $I_{NaP}$ by veratridine must be due to local, subcellular changes in $[Na^+]$.

Persistent $Na^+$ currents in patch recordings

While many of the recordings described above had some indication of macroscopic and single-channel $I_{NaP}$ currents, accurate recording of $I_{NaP}$ currents in outside-out patches required blockade of $K^+$ channels. This is due to the small single-
channel conductance of Na\textsubscript{V}-channels, simultaneous activity of Na\textsuperscript{+} and K\textsuperscript{+} currents and prominent subconductance states of K\textsubscript{Na} channels. We found that both transient and persistent TTX-sensitive sodium currents were present in outside-out patches from the soma (Fig. 5\textit{a,b}). We observed that \(I_{\text{NaP}}\) at a single-channel level consists of brief openings occurring over a wider voltage range than \(I_{\text{NaT}}\) and displaying no significant inactivation over the course of 1 s voltage steps. Many of these persistent TTX-sensitive openings were so brief that we could not reliably measure a mean channel open time, even when digitizing at high rates. Thus, we used the number of detectable events as a means of quantification rather than channel open probability (Fig. 5\textit{b}). Because of the brevity of openings it is likely that we failed to detect many of them. Although TTX-sensitive openings of \(I_{\text{NaP}}\) occurred less frequently at hyperpolarized voltages, they were clearly present even at −90 mV (Fig. 5\textit{b}). \(I_{\text{NaP}}\) with similar voltage-sensitivity was identified in whole-cell recordings using voltage steps and slow voltage ramps (Fig. 5\textit{c,d}). These results suggest \(I_{\text{NaP}}\) is a determinant of the resting potential in T/M cells. Consistent with this, the resting potential of mitral cells undergoes subthreshold oscillations in membrane voltage known to be mediated by TTX-sensitive \(I_{\text{Na}}\) (Desmaisons et al., 1999). This finding was consistent with our earlier whole-cell experiments that seemed to indicate that at cell resting potentials K\textsubscript{Na} channels were maintained in a “primed” state under normal physiological conditions, which, given the small voltage-sensitive component to K\textsubscript{Na} channel gating, produces a delayed outward current upon sufficient membrane depolarization. Such a “primed” state is likely to be the result of a tiny but persistent replenishment of Na\textsuperscript{+} in the local vicinity of K\textsubscript{Na} which
maintains local [Na$^+$]$_i$ at a higher level than bulk [Na$^+$]$_i$. $I_{NaT}$ in excised patches displayed an unusually hyperpolarized steady-state inactivation curve ($V_{1/2} = -81.7 \pm 1.3$ mV, peak current measured at −20 mV following 1 s prepulses of −120 to −30 mV, $n = 6$ patches). Consistent with the lack of apparent $I_{NaT}$ in our recordings of $K_{Na}$ channel activity, only ∼10% of the sodium channels contribute to $I_{NaT}$ evoked from a holding potential of −70 mV. Atypically hyperpolarized sodium channel inactivation (of the transient component) has recently been reported in other mammalian neurons as well (Grimes et al., 2010; Scott et al., 2010). To confirm that $I_{Na}$ in excised patches was sensitive to veratridine, we applied veratridine to an outside-out patch in which $I_{Na}$ was isolated. Upon application of 50 µm veratridine, we observed substantially increased $I_{NaP}$ activity during voltage steps and a prominent, slowly decaying tail current upon repolarization (Fig. 6a,b) consistent with previous reports of its action on $I_{Na}$ (Barnes and Hille, 1988).
DISCUSSION

Upon initial identification in cardiac myocytes, the suggested role for $K_{Na}$ channels was that of providing a reserve conductance that would protect cells during hypoxia when an elevation in $[Na^+]_i$ occurred as a result of blocking the $Na^+/K^+$ ATPase (Kameyama et al., 1984). This idea arose as a result of observations of $K_{Na}$ channels in inside-out patches which showed that $K_{Na}$ channel activation required a much higher $[Na^+]_i$ than that normally found in bulk cytoplasm. However, we have uncovered an unexpected phenomenon by which $Na^+$ entering the cell via a small but persistent sodium current is surprisingly effective in activating $I_{KNa}$, even in the total absence of bulk internal $Na^+$. The seeming implausibility of such a mechanism may be partially responsible for keeping this major and widespread component of delayed outward conductance unappreciated by electrophysiologists for many years. These observations suggest a mechanism by which $I_{KNa}$ can commonly participate in providing a large outward conductance in many neuronal types. $K_{Na}$ channels are expressed throughout the brain (Bhattacharjee et al., 2002, 2005). The Allen Brain Atlas shows that one of two genes which encode $K_{Na}$ channels (KCNT1, also known as Slack and Slo2.2) is as widespread and abundantly expressed as KCNB1, the gene encoding the major voltage-dependent delayed rectifier (http://mouse.brain-map.org/).

The functional coupling of $I_{KNa}$ to $I_{NaP}$ that we observe at the single-channel level in isolated outside-out patches suggests a highly specialized relationship between $K_{Na}$ and $Na_v$ channels perhaps similar to the colocalization between calcium channels and the
Slo1 calcium-activated potassium channel (BK) (Marrion and Tavalin, 1998). Although the coupling of $K_{Na}$ to Na channels may be analogous, the two orders of magnitude difference in the required concentrations of $Na^+$ and $Ca^{2+}$ needed to activate the respective $K^+$-channels suggests some differences. Hence, we are unlikely to observe a correlation between the opening of single $Na_V$ and $K_{Na}$ channels as is observed between calcium channels and single BK channels. Nevertheless there are may parallels between the two systems. In both systems the channels are activated by two major factors, an intracellular ion ligand, and voltage (albeit $K_{Na}$ channels have lower voltage sensitivity). In both systems, the effect of increasing the concentration of the intracellular ion ligand is to shift the conductance-voltage relation leftward to more hyperpolarized voltages. We previously demonstrated this for $I_{KNa}$ by loading cells with higher concentrations of $Na^+$ and noting a leftward shift in the conductance-voltage relation of $I_{KNa}$ (Budelli et al., 2009). Significantly, those experiments showed that cells loaded with higher concentrations of $[Na^+]_i$ still retained an obvious delayed outward component of $I_{KNa}$ due to the influx of $Na^+$. Thus, the TTX-dependent influx of $Na^+$ appears to be additive to the $[Na^+]_i$ contributed by the bulk solution.

A possible mechanism for the functional coupling of $K_{Na}$ and $I_{NaP}$ is their presence in a region of limited diffusion near the plasma membrane, sometimes described as a fuzzy space or unstirred layer. Physiological evidence of such a region in cardiac myocytes has been accumulating for many years (for review, see Barry, 2006). Microheterogeneity of sodium concentrations in internal submembrane space has been inferred many times (Barry, 2006), and has even been directly measured (Wendt-
Gallitelli et al., 1993). Measurements using electromagnetic pulse analysis shows $[\text{Na}^+]$ near the inner membrane surface can be several fold higher than the bulk $[\text{Na}^+]$, and heterogeneous within a cell, ranging from 0 to 80 mm at the inner membrane surface. Thus, it is possible that a small but constant influx of $\text{Na}^+$ through $I_{\text{NaP}}$ is responsible for increasing $[\text{Na}^+]$ in some submembrane regions where $K_{\text{Na}}$ channels are located, and that limited diffusion as well as colocalization of $I_{\text{NaP}}$ and $I_{\text{KNa}}$ both contribute to activation of $I_{\text{KNa}}$ by $I_{\text{NaP}}$.

This study focused on $I_{\text{NaP}}$ as a major source of sodium for the activation of $K_{\text{Na}}$ channels. While our data suggest $I_{\text{NaT}}$ is not necessary for $K_{\text{Na}}$ activation as a delayed outward current, we have not ruled out a contribution of $I_{\text{NaT}}$ in all circumstances. The $\text{NaV}$-channels that carry $I_{\text{NaP}}$ are likely to also carry $I_{\text{NaT}}$, and it is difficult to imagine a mechanism by which only persistent $\text{Na}^+$-channel activity would activate $K_{\text{Na}}$ channels. However, our study indicates that the $\text{Na}^+$-channels present in somal membrane may be largely inactivated at resting membrane potentials with regard to their capacity to carry a transient sodium current on depolarization, while maintaining the ability to carry $I_{\text{NaP}}$ at most voltages. Activation of $I_{\text{KNa}}$ by $I_{\text{NaT}}$ has been previously examined in outside-out patches of neurons from chick brainstem (Dryer, 1991). That study found that while TTX blocked $I_{\text{NaT}}$ it had no effect on potassium currents. However, those experiments observed the effects of $I_{\text{NaT}}$ on outward currents at $-25$ to $-10$ mV, near peak $I_{\text{NaT}}$ amplitude. We point out that, under conditions of low $[\text{Na}]$, loading, we also do not detect net outward $I_{\text{KNa}}$ at those voltages, which we ascribe to the voltage-dependent component.
of $I_{\text{KNa}}$ activation. Thus, Dryer's experiments must be repeated at higher voltages before ruling out a role for $I_{\text{NaT}}$ in $K_{\text{Na}}$ activation.

The activity of $K_{\text{Na}}$ channels has been shown to be sensitive to intracellular Cl$^-$ (Bhattacharjee et al., 2003; Yuan et al., 2003). We have found the magnitude of TTX-sensitive outward current in whole-cell recordings to be accordingly affected by [Cl$^-$]). TTX-sensitive potassium currents in recordings using a physiological [Cl$^-$]$_i$ of 20 mm comprise ~30% of the sustained outward current in mitral cells, compared with 57% in 150 mm [Cl$^-$]$_i$ (Budelli et al., 2009; our unpublished data). Our current study used an elevated [Cl$^-$]$_i$ to increase the likelihood of observing $K_{\text{Na}}$ channels and to more precisely measure channel $P_{\circ}$. $K_{\text{Na}}$ channels have been shown to be activated by the ubiquitous enzyme cofactor nicotinamide adenine dinucleotide (NAD$^+$) (Tamsett et al., 2009). The presence of NAD$^+$ may increase the activity of $K_{\text{Na}}$ channels at physiological concentrations of internal bulk Na$^+$.

The contribution of $I_{\text{NaP}}$ to the activation of a prominent potassium conductance may seem counterintuitive given the established role of $I_{\text{NaP}}$ in increasing neuronal excitability. We point out that the $I_{\text{NaP}}$-$I_{\text{KNa}}$ coupled system produces a net inward current in mitral cells between typical resting potentials and action potential thresholds (Figs. 1c,d, 2e). Nevertheless, within those voltages the activation of $I_{\text{KNa}}$ may mitigate depolarization and while it does not overcome $I_{\text{NaP}}$, could provide some negative feedback against the excitatory role of $I_{\text{NaP}}$, in the form of membrane accommodation. It may also act as a repolarizing current that shortens action potential duration. In addition, there is evidence for neuromodulatory regulation of both $I_{\text{NaP}}$ (for review, see Cantrell

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and Catteral, 2001) and \( I_{\text{KNa}} \) (Santi et al., 2006; Nuwer et al., 2010). Hence, the discrete or combined regulation of either component of the \( I_{\text{NaP}}-I_{\text{KNa}} \) coupled system could provide a mechanism for long-lasting changes in excitable properties over a wide range of both subthreshold and depolarized voltages.

\( I_{\text{NaT}} \) and the upstroke of action potentials are grossly similar across neurons. In contrast, the behavior of \( I_{\text{NaP}} \) varies and contributes to diverse physiological properties such as amplification of synaptic potentials, repetitive firing of action potentials, spike timing, and even membrane resting potential (Crill, 1996; Vervaeke et al., 2006, Huang and Trussell, 2008). Indeed, \( I_{\text{NaP}} \) may be a significant determinant of some of the most subtle excitable membrane behavior such as electrical resonance generating theta frequency firing (Hu et al., 2002). In all of these electrical phenomena in which \( I_{\text{NaP}} \) participates, \( I_{\text{KNa}} \) may now be found to be a coparticipant. Many inherited epilepsies are due to gain- or loss-of-function mutations in voltage-gated sodium channels which alter \( I_{\text{NaP}} \). Accordingly, several antiepileptic drugs primarily target \( I_{\text{NaP}} \); however, understanding of pathophysiological mechanisms of \( I_{\text{NaP}} \) toward epilepsy and other disorders is incomplete (Stafstrom, 2007; Saint, 2008; Waxman, 2008). For all these reasons, \( I_{\text{NaP}} \) is a topic of great research interest for clinical and basic neuroscientists. The functional coupling of \( I_{\text{NaP}} \) to \( K_{\text{Na}} \) channels must now also be considered as the contribution of \( I_{\text{NaP}} \) to neuronal physiology continues to be investigated.
Figure 1. The TTX-sensitive delayed outward current, $I_{KNa}$, does not depend on the transient sodium current.

a, Whole-cell currents before and after TTX (1 µm) and TTX-sensitive currents evoked by voltage steps (−90 to +40 mV; 10 mV intervals) from a holding potential of −70 mV. Note the large component of inward Na$^+$ current present in a (arrows) but absent in b. b, Currents evoked from the same neuron as in a from a holding potential of −50 mV. Note that despite the substantial diminution of the transient sodium current by holding at −50 mV, TTX-sensitive outward currents and persistent inward currents were largely unchanged. c, d, $I$–$V$ plots of sustained currents (mean current during final 50 ms of voltage step) from −70 mV and −50 mV holding potentials, respectively. Recordings were made from the soma of dissociated tufted/mitral cells of the olfactory bulb.
Figure 2. $I_{KNa}$ depends on sodium influx into an outside-out macropatch.

a, Currents were evoked from a holding potential of $-70$ mV by steps from $-90$ to $+80$ mV. b, Removal of external sodium dramatically reduced magnitude of outward currents. c, Currents returned to previous levels upon restoration of external sodium. d, Delayed outward $K_{Na}$ currents, revealed by subtracting $0 \ [Na^+]_o$ currents from control currents, resemble those recorded in whole-cell configuration. e, $I–V$ plot of currents in a–d (mean current of final 50 ms of voltage step). Note that sodium ion was absent from the internal bulk solution.
outside-out macropatch from soma

a

control

b

$[\text{Na}^+]_o$

[Na$^+$]$_o$

restored

d

[Na$^+$]$_o$ - dependent K+ current

e

$pA$

$-80$ $-60$ $-40$ $-20$ $20$ $40$

$mV$

1500

1000

500
Figure 3. Single-channel $K_{Na}$ activity depends on TTX-sensitive persistent sodium influx into an outside-out patch.

*a*, Currents evoked by a family of voltage steps in control conditions and in the presence of TTX (1 $\mu$m). $K_{Na}$ channel activation was stimulated by TTX-sensitive entry of sodium ion from the external medium; sodium ion was absent from the internal bulk solution. Upper traces show currents evoked by step pulses from −90 to +60 mV in 10 mV intervals from a holding potential of −70 mV. Lower traces display distributed traces at indicated voltages to allow resolution of individual $K_{Na}$ channel events. *b*, Currents evoked by steps to indicated voltages from a holding potential of −70 mV in control conditions and after complete replacement of external Na$^+$ with Li$^+$. 
**Figure 4. Enhancement of $I_{NaP}$ increases $K_{Na}$ channel activity.**

**a,** Application of veratridine, which prolongs the open state of the sodium channel, increases $K_{Na}$ channel activity. Single-channel $K_{Na}$ currents were evoked by steps to indicated voltages from a holding potential of $-70$ mV in control conditions (left), after application of veratridine ($50$ µm; center), and after removal of external $Na^+$ in the continued presence of veratridine (right). **b,** The effect of veratridine on $K_{Na}$ channel activity in the presence and absence of external $Na^+$ was quantified by calculating the $P_o$ of the channels, as described in Materials and Methods, in each condition ($n = 10$ patches). For each experiment, measured $P_o$ during individual sweeps was normalized against mean $P_o$ in control conditions. Bars represent relative $P_o$ in indicated conditions. Error bars represent SE. $p < 0.001$ comparing control to veratridine and veratridine to $0 Na^+$. 
$K_{\text{Na}}$ activity in outside-out patch

(a) control + veratridine + veratridine $-\left[\text{Na}^+\right]_o$

(b) relative $P$

30 pA
100 ms

control veratridine veratridine, 0 Na$^+$
Figure 5. Voltage-gated sodium currents present in the soma of T/M cells include a persistent component.

Sodium currents were isolated by using Cs\(^+\) in the patch pipette and TEA and 4-AP in the bath to block potassium channels.  

\(a–d\), Recordings were made in outside-out patches (\(a, b\)) and whole-cell configuration (\(c, d\)) with the internal sodium concentration set to 10 mm to obtain better voltage control of inward current and allow measurement of channel reversal potential.  

\(a\), Single-channel sodium currents evoked by a voltage step from −90 to −40 mV have both early occurring, high open probability, transient openings, as well as low probability, persistently recurring, brief openings (asterisks). Both components were blocked by TTX (1 \(\mu\)m).  

\(b\), Plot of the number of channel opening events versus voltage. Channel events were counted from 50 ms after the initiation of a 1 s voltage step to its end.  

\(c\), Whole-cell, TTX-sensitive persistent sodium currents evoked by 500 ms steps to indicated voltages from a holding potential of −90 mV. Mean current is plotted during the last 100 ms of the pulse (arrow). Error bars represent SEM.  

\(n = 5\) cells.  

\(d\), Whole-cell, TTX-sensitive sodium currents evoked by a voltage ramp from −100 to +20 mV, 40 mV/s. In \(c\) and \(d\), TTX-sensitive currents were isolated and plotted by subtracting residual currents evoked in TTX (1 \(\mu\)m) from currents evoked before TTX application.
Figure 6. Veratridine enhances $I_{NaP}$ in outside-out excised patches.

Sodium currents were isolated by using Cs$^+$ in the internal pipette solution and TEA and 4-AP in the bath solution to block potassium channels. Recordings were made in macroscopic outside-out patches with the internal sodium concentration set to 10 mm. $a$, Application of 50 µm veratridine to outside-out patches increased $I_{NaP}$ activity during voltage steps, and induced a prominent, very slowly decaying tail current upon repolarization. Inset shows same recordings at higher time resolution. Only a minor difference is seen in the amplitude of $I_{NaT}$ before and after veratridine. Dashed lines represent 0 current level. $b$, Veratridine-induced $I_{Na}$ was revealed by subtracting control current from current recorded in veratridine.
WORKS CITED


Preface to Chapter 4

Work contained in this chapter has not been published. All data was generated and analyzed myself in close consultation with my advisor, Lawrence Salkoff.
Chapter 4

$K_{Na}$ channels contribute to action potential repolarization
ABSTRACT

The functional coupling of sodium-activated potassium channels (K$_{\text{Na}}$ channels) to Na$^+$-influx has recently been demonstrated using whole cell and excised patch recordings. Here we report further on this phenomenon by noting the slow rate at which K$_{\text{Na}}$ channel activity decreases after blocking Na$^+$-influx through voltage-gated Na$^+$-channels (Na$_V$ channels) with tetrodotoxin (TTX). This rate is much slower than the direct effect on TTX on Na$_V$ channels. The same slow rate of decrease in K$_{\text{Na}}$ channel activity was observed when Na$^+$ was removed from the external solution; however, the rate at which K$_{\text{Na}}$ channel activity was recovered upon restoration of external Na$^+$ was much faster. We use this new assay of K$_{\text{Na}}$ channel activity to demonstrate the activation of K$_{\text{Na}}$ channels by Na$^+$-influx through Na$_V$ channels in neurons cultured from the cerebellum, cortex, hippocampus, olfactory bulb and striatum. Finally, the slow decrease in K$_{\text{Na}}$ channel activity relative to Na$_V$ channel blockade by TTX allowed us to measure K$_{\text{Na}}$ currents unmarred by Na$_V$ currents and determine the activity of K$_{\text{Na}}$ channels during action potential waveforms. In doing so, we find that K$_{\text{Na}}$ channels can act as the primary repolarizing current in tufted/mitral cells of the rat olfactory bulb.
INTRODUCTION

Sodium-activated potassium channels (K\textsubscript{Na} channels) have gained increased attention in recent years as a conductance that contributes to the normal physiology of neurons (Budelli \textit{et al.}, 2009; Tamsett \textit{et al.}, 2009; Nuwer \textit{et al.}, 2010; Hage & Salkoff 2012). This is in contrast to the protective role proposed for K\textsubscript{Na} channels upon their discovery in cardiomyocytes (Kameyama \textit{et al.}, 1984). In spite of the increased attention toward K\textsubscript{Na} channels, there is still much to be learned about the specific contributions K\textsubscript{Na} channels make to neuronal excitability. K\textsubscript{Na} currents (I\textsubscript{KNa}) have been revealed in voltage-clamp experiments in which physiologists have blocked Na\textsuperscript{+}-influx with TTX or removed external Na\textsuperscript{+} to remove I\textsubscript{KNa}. Such manipulations are not amenable to current clamp studies traditionally used to measure the firing properties of neurons because Na\textsuperscript{+} influx is necessary for action potential generation. A specific blocker of K\textsubscript{Na} channels would allow investigation into the role of K\textsubscript{Na} channels in action potential firing, but no such compound is available.

Some experimenters have used partial or complete replacement of external Na\textsuperscript{+} with Li\textsuperscript{+} as a means to test a role of K\textsubscript{Na} channels in neuronal excitability. Li\textsuperscript{+} can permeate Na\textsubscript{V} channels almost as well as Na\textsuperscript{+}, but does not activate K\textsubscript{Na} channels (Hille, 1972; Dryer, 1994). Therefore, it is proposed that Li\textsuperscript{+} is capable of mediating the depolarizing phase of the action potential, but any chemical effect of Na\textsuperscript{+} will be lost. While it is accurate that the relative permeability of Li\textsuperscript{+} through the Na\textsuperscript{+} channel is high (0.93) (Hille, 1972), even in this first study, it was noted that the amplitude of Li\textsuperscript{+}
currents is smaller than predicted by this value. Peak inward Li$^+$ currents were nearly 30% smaller than Na$^+$ currents. Therefore, the depolarizing current is not as large and replacement of external Na$^+$ with Li$^+$ should not be viewed as a completely selective means of inhibiting $K_{Na}$ channel activity in current clamp experiments.

In this study, we have characterized the time course of the effect of removing Na$^+$-influx on $K_{Na}$ channel activity. The rate of decrease of $K_{Na}$ channel activity is well fit by a single exponential decay equation with a time constant ($\tau$) of $\sim$13 seconds (s). Direct blockade of Na$^+$ or K$^+$ channels and recovery of $K_{Na}$ channel activity were all determined to be much more rapid ($\tau$ $\sim$ 3 s), suggesting the slow time constant of $K_{Na}$ channel activity decline was not due to slow solution exchange in our recording chamber.

Action potential clamp (also described as “waveform clamp”) is a variation of voltage-clamp experiments in which a previously recorded action potential is used as the command voltage. Using this technique Na$^+$-influx can be altered by TTX or removal of external Na$^+$, but the voltage trajectory will be unchanged. Action potential clamp was used in this study to determine the activity of $K_{Na}$ channels during a naturally occurring action potential waveform. We find that in neurons of the olfactory bulb, $K_{Na}$ channels are often the primary repolarizing current, especially if the spike is preceded by a slow subthreshold depolarization that inactivates many voltage-gated K$^+$ channels.
METHODS

*Primary neuronal culture preparations.*

Primary cultures of dissociated olfactory bulb, striatum, cortex, hippocampus and cerebellum tissue were prepared from either sex of neonatal Sprague Dawley rats (postnatal day 0). Dissected tissues were incubated in 0.25% trypsin in PBS for 30 min at 37°C. For the last 5 min, trypsin solution was supplemented with 0.05% DNase. Enzyme digested tissue was then triturated in PBS containing soy bean trypsin inhibitor using Pasteur pipettes of decreasing diameter. Cells were plated on poly-lysine-coated glass coverslips in Dulbecco's Modified Eagle Medium/F-12 medium (Invitrogen) with 10% FBS and 2% B-27 supplement (Invitrogen). Three days after plating, medium was replaced with Neurobasal (Invitrogen) + 2% B-27 supplement and 5 μm arabinofuranosyl cytidine to inhibit division of glia.

*Voltage-clamp recordings.*

Voltage clamp recordings were made 1-5 days after plating of neurons. For experiments described in Figures 5 & 6, only cells plated within the previous 48 hours with minimal neurite extension were used. Internal pipette solution contained, in mM: 115 K-methanesulfonate, 20 KCl, 10 Na-methansulfonate, 10 hepes buffer, 1 MgCl₂, 0.5 EGTA. External solution contained, in mM: 150 NaCl, 3 KCl, 2 MgCl₂, 10 hepes buffer, 10 dextrose pH adjusted to 7.4 with NaOH. TTX (final concentration 1 μM) was added to external solutions the day of experiments form stock solution in citrate buffer. In
experiments using tetraethylammonium-chloride and or 4 amino-pyridine, the concentration of NaCl was reduced in an equimolar fashion. In experiments in which external Li\(^+\) replaced external Na\(^+\), 150 mM LiCl completely replaced NaCl. Recordings were made with borosilicate glass pipettes with tip resistances of 3–6 M\(\Omega\). Voltage-clamp recordings were made with an Axoclamp 200B, filtered at 2 kHz and digitized at 50 kHz with a Digidata 1440A using pClamp v 10.0 software.

**Animal welfare.**

It is the policy of Washington University Medical School that all research involving animals be conducted under humane conditions, with appropriate regard for animal welfare. Washington University Medical School is a registered research facility with the United States Department of Agriculture (USDA) and is committed to complying with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services), the provisions of the Animal Welfare Act (USDA), and all applicable federal and state laws and regulations. At Washington University Medical School an Animal Care Committee has been established to insure compliance with all applicable federal and state regulations for the purchase, transportation, housing and research use of animals. Washington University Medical School has filed appropriate assurance of compliance with the Office for the Protection of Research Risks of the National Institutes of Health.
RESULTS

Deactivation of $K_{Na}$ channels occurs at a much slower rate than blockade of $I_{Na}$

In the course of previous studies (Budelli et al., 2009; Hage & Salkoff, 2012), we noted that the blockade of $I_{Na}$ by application of TTX was very rapid, while the decline in amplitude outward $I_{KNa}$, previously shown to be mediated by $K_{Na}$/Slo2 channels, appeared to take place at a slower rate. This is consistent with our prior conclusions, that $K_{Na}$ channel activity decreased as a secondary consequence of TTX application – blockade of Na$^+$-influx decreased [Na$^+$], near $K_{Na}$ channels. This apparent difference in time course of the direct and indirect effect of TTX was not quantifiable in previous studies because $I_{Na}$ and $I_{KNa}$ were measured by evoking currents with a complete family of voltage steps which requires 1 minute or more to complete. In this study we have measured this phenomenon by using a 500 millisecond (ms) voltage ramp from -90 mV to 0 mV that allows measurement of both $I_{Na}$ and $I_{KNa}$. As tufted/mitral cells of the olfactory bulb have been previously noted by ourselves and others to be the cell type in which $K_{Na}$ channels are most abundant (Egan et al., 1992a; Budelli et al., 2009), we used dissociated cultures of olfactory bulb neurons for most of the experiments conducted in this study. Upon initiation of the voltage ramp, whole cell current became gradually positive until activation of $I_{Na}$ overcame the leak conductance of the cell and currents displayed an inward sag. As the depolarizing ramp proceeded, multiple populations of K$^+$ channels ($K_V$ and $K_{Na}$) were activated and relatively large outward currents were recorded (Figure 1a, black trace). By repeating this depolarizing voltage ramp every 3
seconds (holding potential = -90 mV) we could demonstrate the rapid blockade of $I_{Na}$ by TTX (1µM) and slow deactivation of $I_{KNa}$ due to loss of Na$^+$-influx.

Examples of a control current trace (black), a trace ~6 seconds after application of TTX (blue) and a trace ~30 seconds after TTX (green) from the same cell are shown in Figure 1a. Note that only the inward sag corresponding to $I_{Na}$ is removed ~6 seconds after application of TTX (compare black to blue). ~30 seconds after application of TTX, a dramatic decrease in outward $I_{K}$ amplitude is obvious, with no further reduction in $I_{Na}$. For each current sweep evoked by the voltage ramp, the amplitude of $I_{Na}$ was measured as the average current amplitude between -65 and -45 mV. The amplitude of $I_{K}$ was measured as the average current amplitude between -20 and 0 mV (the end of the ramp). $I_{Na}$ and $I_{K}$ were then normalized to their respective maxima and minima over the course of the experiment. $I_{Na}$ values were inverted so that the value 1 would correspond to maximum current amplitude for both inward $I_{Na}$ and outward $I_{K}$. The averaged results from 7 olfactory bulb neurons are plotted in Figure 1b. The rates of decline of $I_{Na}$ and $I_{K}$ were well fit with a single exponential decay function (shown in red). The rate of decline of $I_{Na}$ amplitude (2.72 ± 0.67 s, n=7) was much faster than the rate of decline of $I_{K}$ amplitude (12.43 ± 1.52 s, n=7). This is consistent with our previous studies demonstrating a direct effect of TTX on blocking $I_{Na}$ and an indirect effect on $I_{KNa}$ – deactivation of the current resulting from loss of Na$^+$-influx. The phenomenon was similar using modifications of the voltage protocol including different holding potentials, voltage ramp lengths, magnitudes and rates. The paradigm described above was used for the majority of experiments in this study because it was empirically determined to
provide reliable measurement of both $I_{Na}$ and $I_{KNa}$ with little cumulative inactivation of either $I_{Na}$ or $I_{KV}$. The rapid and complete blockade of $I_{Na}$ suggests the slow reduction in $I_{K}$ amplitude following application of TTX is not a consequence of the perfusion system utilized in this study. To further confirm this, we examined the rate of decline of $I_{K}$ when the $K^{+}$ channels were directly blocked. When $I_{K}$ was directly blocked with 30 mM tetraethylammonium (TEA), decline of $I_{K}$ amplitude was rapid with a rate comparable to block of $I_{Na}$ by TTX (Figure 2). This high concentration of TEA will block both $K_{V}$ and $K_{Na}$ channels. No decline in outward current amplitude following application of TTX was observed if all $K^{+}$ channels were blocked at the outset of an experiment with a cesium-based internal pipette solution (not shown).

In most experiments of our previous studies of the activation of $K_{Na}$ channels by $Na^{+}$-influx, we used internal solutions which did not include $Na^{+}$ to strictly test the ability of $Na^{+}$-influx to activate $K_{Na}$ channels, without any contribution from bulk $Na^{+}$ (Budelli et al., 2009; Hage & Salkoff, 2012). The aim of this study was to examine the contribution of $K_{Na}$ channels to physiological activity. Therefore, a more typical internal pipette solution containing 10 mM $Na^{+}$ and 22 mM $Cl^{-}$ was used in experiments presented here (see Methods). However, the time course of decay was similar in experiments using either solution (0 $Na^{+}$ internal solution not shown) suggesting the mechanism of coupling between $Na^{+}$-influx and $K_{Na}$ channels is the same in this and previous studies.

*Decay and recovery rates of $I_{KNa}$ following removal and restoration of external $Na^{+}$*
As an alternative to TTX, $I_{\text{KNa}}$ can be revealed by removing external Na$^+$ (Budelli et al., 2009; Nuwer et al., 2010; Hage & Salkoff, 2012) and replacing it with an impermeant cation, such as choline, or Li$^+$ which can permeate Na$^+$ channels, but is a very weak activator of K$_{\text{Na}}$ channels. We measured the rate of decay of $I_{\text{KNa}}$ amplitude following replacement of external Na$^+$ with choline or Li$^+$ using the same protocol as described for TTX. The rate of decay of $I_{\text{KNa}}$ amplitude is comparable to the rate of decay following application of TTX (Li$^+$, 13.37 ± 1.14 s, $n=10$; choline, 12.25 ± 1.26 s, $n=4$; TTX, 12.43 ± 1.52 s, $n=7$) (Figure 3, compare to Figure 1). Intriguingly, upon restoration of external Na$^+$, recovery of $I_K$ amplitude is relatively rapid (Li$^+$ 1.92 ± 0.23 s, $n=10$; choline 3.26 ± 0.99 s, $n=4$) (Figure 3). Consistent with previous results (Budelli et al., 2009; Hage & Salkoff, 2012), recovery of $I_{\text{KNa}}$ following restoration of external Na$^+$ is incomplete. This may be due to rundown of K$_{\text{Na}}$ channels (Egan et al., 1992b). Data presented in Figure 3 was taken from experiments in which external Na$^+$ was replaced by Li$^+$ for ~48 seconds. Similar decay and recovery rates were observed when Li$^+$ solution replaced the control external solution for shorter or longer periods of time. Changes in activity of $I_{\text{KNa}}$ upon exchange of Na$^+$ and Li$^+$ could be repeated multiple times over the course of a single recording, though magnitude of recovery would typically decrease upon each trial. The results from experiments in which we replaced external Na$^+$ are consistent with the hypothesis that the Na$^+$-influx-dependent outward currents recorded by depolarizing ramps are similar to K$_{\text{Na}}$ currents identified in previous studies. This also supports the hypothesis that the effect of TTX on outward currents is due to loss of Na$^+$-influx rather than a direct effect on K$_{\text{Na}}$ channels. Furthermore, the rapid recovery of $I_K$,
relative to the decay suggests the slow decay is not a consequence of the perfusion system, but a biological phenomenon.

\( \text{Na}^+ \)-influx-dependent \( K_{\text{Na}} \) currents are expressed in many mammalian neurons

Use of the voltage ramp paradigm was extended to dissociated neuron cultures generated from several different brain regions (cerebellum, cortex, hippocampus, olfactory bulb and striatum). Examples of currents evoked from different neuron subtypes are shown in Figure 4. Consistent with the previously reported widespread expression of Slack and Slick (Bhattacharjee et al., 2002; 2005), TTX-sensitive outward currents were observed in all brain regions examined. The currents recorded before and after blockade of \( \text{Na}^+ \)-influx varied amongst cells in terms of amplitude and kinetics of activation (Figure 4). The magnitude of reduction in outward currents following application of TTX also varied between cell types (percentage decrease in mean current amplitude between -20 and 0 mV ± standard error of the mean: olfactory bulb, 32.2 ± 4.9%; striatum 49.9 ± 5.9%; cortex 25.5 ± 6.6%; hippocampus 34.6 ± 5.7%; cerebellum 35.3 ± 9.0%). While the amplitudes of both \( I_{\text{Na}} \) and \( I_{\text{KNa}} \) varied within and between cultures of different brain areas, we consistently observed rapid block of inward \( I_{\text{Na}} \) followed by slow decay of outward \( I_{\text{KNa}} \). Notably, a broad range of \( I_{\text{KNa}} \) amplitudes was observed in cortical pyramidal neurons. Some cortical pyramidal neurons displayed almost so reduction in outward current following application of TTX while in others, the amplitude of outward current measured between -20 and 0 mV decreased by up to 40%.
This is in agreement with the variable levels of expression of Slack in the cortex observed in the Allen Brain Atlas.

**Different rates of TTX effect on $I_{Na}$ and $I_{KNa}$ can be used to reveal individual currents**

In prior studies utilizing families of voltage steps, $I_{KNa}$ was isolated post hoc by subtracting currents recorded in the presence of TTX or absence of external Na⁺ from control currents (Budelli et al., 2009; Hage & Salkoff, 2012). Currents measured in this manner contain both $I_{Na}$ and $I_{KNa}$. While the sustained behavior and large amplitude of $I_{KNa}$ compared to $I_{NaP}$ minimized any distortion present in this analysis, it would be advantageous to have a technique of revealing only $I_{KNa}$. Lacking a specific pharmacological blocker to do so, we instead sought to take advantage of the slow, secondary effect of TTX on $K_{Na}$ channels. Figure 5 illustrates the principle of how this can be done. Whole cell currents were evoked by voltage steps from -90 mV to -20 mV every 3 seconds. In control conditions we observed the fast activation of transient $I_{Na}$ followed by activation of outward currents including $I_{KV}$ and $I_{KNa}$ (Figure 5a, black trace). TTX was then perfused onto the cell. As with the voltage ramps used in previous figures, $I_{Na}$ was rapidly blocked by TTX (Figure 5a, blue trace; $\tau = 3.41 \pm 0.36$ s, measured as peak negative current). This was followed by a slower, steady decline of $I_{K}$ (Figure 5a, green trace; $\tau = 14.70 \pm 1.66$ s, measured as mean current amplitude during final 5 ms of the voltage step). By subtracting currents recorded soon after application of TTX from control currents, we find transient $I_{Na}$, with some indication of $I_{NaP}$, but no outward current (Figure 5b, blue trace). By subtracting currents recorded ~30 seconds
after application of TTX from currents recorded soon after application of TTX, we find only sustained outward currents - $I_{\text{KNa}}$ - with minimal distortion from $I_{\text{Na}}$ (Figure 5b, green trace). By subtracting currents recorded ~30 seconds after application of TTX from control currents, we reveal the sum of $I_{\text{Na}}$ and $I_{\text{KNa}}$ (Figure 5b, black trace). This is equivalent to what was done in previous studies (Budelli et al., 2009; Hage & Salkoff, 2012). Recent studies of $I_{\text{KNa}}$ describe it as a sustained, delayed-rectifier like current. However, a transient component to $I_{\text{KNa}}$, could not be ruled out because it was possible that such a current could be masked by a simultaneously active transient inward $I_{\text{Na}}$ that was necessarily removed to reveal $I_{\text{KNa}}$. Using the strategy outlined above to isolate $I_{\text{KNa}}$, we find no evidence for transient $I_{\text{KNa}}$ activity evoked by a sustained voltage step.

This strategy of analysis was extended to experiments in which an action potential voltage waveform was used in place of a voltage step in olfactory bulb neurons. In some experiments, an additional condition in which voltage-gated $K^+$ channels were blocked with TEA (150 mM) and 4 aminopyridine (4AP) (5 mM) was added to the end of the experiment. This allowed us to reveal the voltage-gated potassium current ($I_{\text{KV}}$) during the action potential waveform by subtracting currents evoked in TEA and 4AP from currents evoked in TTX. The net sum of active currents, including $I_{\text{Na}}$, $I_{\text{KNa}}$ and $I_{\text{KV}}$, is revealed by subtracting currents recorded in the final condition (with all conductances blocked) from control currents.

Accurate measurements of currents evoked by rapid voltage changes, like that of an action potential, require a high quality of voltage control. For this reason, action potential clamp recordings were limited to cells plated within the previous 48 hours.
exemplary cell is shown in Figure 6. As expected, by examining the net currents (black trace) an inward current is observed during the depolarizing phase of the action potential and an outward current is observed during the repolarizing phase with a 0 current level observed near the peak of the action potential - where there is momentarily no change in voltage. This suggests that while the space clamp of neurons in culture may be imperfect, this technique can be used to obtain a reasonable approximation of currents underlying the action potential. Estimation of \( I_{Na} \) by subtracting currents recorded soon after TTX application from control currents reveals only an inward current that is most active during the depolarizing phase of the action potential (Figure 6, green trace). Approximation of \( I_{KNa} \) by subtracting currents recorded ~30 seconds after TTX application from those recorded soon after TTX application, reveals a prominent outward current active during the repolarizing phase of the action potential. Time constants of decay of \( I_{Na} \) and \( I_{KNa} \) following application of TTX were similar to previous experiments (\( I_{Na} \): \( \tau = 3.12 \pm 0.75 \) s; measured as peak negative current during depolarizing phase of action potential waveform; \( I_{KNa} \): \( \tau = 14.55 \pm 4.29 \) s; measured as mean current during repolarizing phase of action potential waveform). In the cell shown if Figure 6, \( I_{KNa} \) (red trace) is much larger than \( I_{KV} \) (grey trace, revealed by subtracting currents in TTX, TEA and 4AP from currents in TTX) during the repolarizing phase of the action potential.
DISCUSSION

The results presented here demonstrate that the decrease in $I_{\text{KNa}}$ observed after blocking Na$^+$-influx takes place at a much slower rate than the blockade of Na$^+$-influx itself (Figure 1). This is consistent with our previous studies suggesting that TTX removes a large, sustained outward current as a secondary consequence of its action on Na$\text{V}$ channels. It is also consistent with our previous interpretations that K$_{\text{Na}}$ channel activity evoked by a voltage pulse is not dependent on Na$^+$-influx during the pulse itself. Instead K$_{\text{Na}}$ channel is dependent on Na$^+$-influx across a broad range of voltages, including the resting potential, which primes K$_{\text{Na}}$ channels to be active upon a depolarizing stimulus. We have taken advantage of the slow decrease in K$_{\text{Na}}$ channel activity to isolate $I_{\text{KNa}}$ evoked by various voltage pulses and reveal prominent activity of $I_{\text{KNa}}$ during action potential waveforms. The lack of a specific blocker for K$_{\text{Na}}$ channels has not allowed this to be achieved before.

*Mechanism of slow decline of K$_{\text{Na}}$ channel activity*

The observation of a slow decay in K$_{\text{Na}}$ channel activity following either Na$\text{V}$ channel blockade or removal of external Na$^+$ may be indicative of the mechanism by which the activity of K$_{\text{Na}}$ channels is coupled to Na$^+$ influx. It is possible that the rate at which Na$^+$ diffuses away from the membrane is slow and responsible for the slow decay in K$_{\text{Na}}$ channel activity. Alternatively, Na$^+$ may slowly unbind from K$_{\text{Na}}$ channels, resulting in the observed slow decay in $I_{\text{KNa}}$. No significant difference in the rate of decay
or recovery of $K_{Na}$ following application of TTX or replacement of external $Na^+$ with $Li^+$ was observed using internal sodium concentrations of 10 mM (control), 0 mM or 60 mM. Other experimental manipulations thought to change the gating of $K_{Na}$ channels such as intracellular $[Cl^-]$ (Yuan et al., 2003; Bhattacharjee et al., 2003), intracellular pH (Ruffin et al., 2008), or intracellular NAD$^+$ (Tamsett et al., 2009), only altered the proportion of current sensitive to $Na^+$ influx, with no detectable change to the rate of current decay (data not shown). This could suggest that the mechanism of coupling is not dependent on $K_{Na}$ channel gating behavior (slow dissociation of $Na^+$ from the channel) and favor the hypothesis of a subcellular diffusion barrier. However, it is possible that while the experimental manipulations tested in this study alter the gating of $K_{Na}$ channels, they do not change the rate at which $Na^+$ dissociates from the channel and therefore, the slow dissociation of $Na^+$ from the channel cannot be ruled out.

Increases in $[Na^+]_i$ evoked by synaptic or voltage-clamp stimulation have been measured using the fluorescent $Na^+$-sensitive dye SBFI (Rose & Ransom, 1997; Mittman et al., 1997; Rose & Konnerth, 2001; Fleidervish et al., 2010). These studies report that $Na^+$-transients within soma and dendrites display a slow decay time constant of up to 10 seconds. This supports the hypothesis that the slow decay in $K_{Na}$ channel activity is a result of slow intracellular diffusion of $Na^+$ rather than slow dissociation of $Na^+$ from $K_{Na}$ channels. In future studies, it could be revealing to measure the decay in $I_{KNa}$ while simultaneously imaging $[Na^+]_i$ dynamics. While this study further demonstrates that $K_{Na}$ channel activity is sensitive to $Na^+$-influx, the mechanism of coupling remains unclear.
No transient $I_{KNa}$ activity is observed

Early studies of $K^+$ currents activated by $Na^+$-influx, described $I_{KNa}$ as transient and suggested the activation of $K_{Na}$ channels could take place as a result of $Na^+$ influx during a single action potential (Bader et al., 1985; Hartung et al., 1985; Dryer et al., 1989). Unlike these previous studies (which have been suggested to be distorted by artifacts of inadequate voltage clamp), we find that the peak amplitudes of $I_{Na}$ and $I_{KNa}$ do not correlate (see Chapter 1 Figure 1c for example from Bader et al., 1985). That is, the peak $I_{Na}$ is completely blocked before any decay in $I_{KNa}$ is found (Figure 5). Previous studies have demonstrated the importance of $I_{NaP}$ in activation of $I_{KNa}$ (Budelli et al., 2009; Hage & Salkoff, 2012). While we still cannot rule out some contribution of transient $I_{Na}$ to activation of $I_{KNa}$, we have yet to find any conclusive evidence of transient activity of $I_{KNa}$. Most of our experiments have been conducted in olfactory bulb tufted/mitral cells to take advantage of the abundance of $K_{Na}$ channels. It is possible that greater coupling of transient $I_{Na}$ to $K_{Na}$ channels is present within different cell types.

Contribution of $K_{Na}$ channels to neuronal excitability

The data presented in Figure 6 suggests one physiological role of $K_{Na}$ channels is to repolarize the action potential. Other functions of $K_{Na}$ channels have been suggested such as generation of slow afterhyperpolarizations (Schwindt et al., 1989; Foehring et al., 1989; Safronov & Vogel, 1996; Franceschetti et al., 2003; Zhang et al., 2010). $K_{Na}$ channels have been suggested to lower the excitability of dorsal root ganglia neurons (Nuwer et al., 2010), but also permit the high frequency firing of neurons from the medial
nucleus of the trapezoid body by lowering the membrane resistance and membrane time constant of the cell (Yang et al., 2007). Such diverse proposed roles are not as contradictory as they may at first appear. It is important to remember that $K_{Na}$ channels are a family of channels and the extent of their molecular and physiological diversity remains unknown. In field of neurophysiology as a whole, there is an increased appreciation that some channels contribute to multiple aspects of neuronal firing (review by Bean, 2007). For example, voltage-gated sodium channels have a suprathreshold role, mediated by $I_{NaT}$, in which they are responsible for initiation and depolarization of the action potential as well as a subthreshold role, mediated by $I_{NaP}$, in controlling spike frequency and patterning. Furthermore, the behavior of all ion channels during neuronal physiology will depend on the context of other channels expressed by the neuron. For example, the large width of action potentials recorded in tufted/mitral cells (Figure 6) could result in $K_{Na}$ channels contributing to the repolarization of action potentials in such neurons, while in neurons with shorter duration action potentials, $K_{Na}$ channels will instead contribute to slow afterhyperpolarizations (Schwindt et al., 1989; Foehring et al., 1989; Safronov & Vogel, 1996; Franceschetti et al., 2003; Zhang et al., 2010).

This strategy of using action potential clamp to reveal the activity of $K_{Na}$ channels during an action potential is amenable to young dissociated cultures as recordings can be made from neurons with minimally extended processes. It would be exciting to extend this strategy to other neurons and preparations such as acute slices in which the behavior neuronal membrane is thought to more closely resemble that of the intact animal. Unfortunately, use of this technique in preparations in which ionic conductances may
originated far from the recording site would likely be prone to the pitfalls of inadequate
voltage-clamp that has tempered acceptance of early studies of \(K_{Na}\) channel activity in
neurons. Indeed, when we attempted to perform action potential clamp experiments with
neurons that had been cultured for several days, there were some indications that transient
\(I_{Na}\) was not well controlled.

\(K_{Na}\) channel activity across the brain

\(K_{Na}\) channel activity has been reported in excised patches from many different
neuronal preparations (review by Dale 1994; see also Chapter 1). Abundant expression
of Slack and Slick has been reported in many different brain regions (Bhattacharjee et al.,
2002; 2005; Allen Brain Atlas, see Chapter 1 Figure 3). Consistent with this, we have
now observed coupling between \(Na^+\)-influx and \(I_{KNa}\) in neurons cultured from
cerebellum, cortex, hippocampus, olfactory bulb and striatum (Figure 4). It is possible
that the young age of neurons used to make these cultures, or the culture conditions
themselves promote expression of \(K_{Na}\) channels to a greater extent than neurons in other
experimental preparations. The slow decay of \(K_{Na}\) channel activity and the application of
action potential clamp that we have described here could be powerful tools to reveal role
\(K_{Na}\) channels in normal and pathological neuronal excitability.
Figure 1: Differential rates of decline of \( I_{Na} \) and \( I_{KNa} \) following application of TTX

a) Currents evoked from a holding potential of -90 mV by a 500 ms voltage ramp to 0 mV (bottom). Voltage stimulus was repeated every 3 seconds. Control currents (black trace) represent net current through both \( Na^+ \) and \( K^+ \) channels. Within 6 seconds of TTX (1 µM) application, inward \( Na^+ \) currents are removed (green trace labeled “TTX immediate”). After blockade of inward \( Na^+ \) current, outward \( K^+ \) currents decay at a slower rate (see Figure 1b). Approximately 30 seconds after initial TTX application, outward currents reached minimal amplitude (blue trace labeled “TTX subsequent”). b) \( I_{Na} \) amplitudes (■) were measured as the mean current amplitude between -65 and -45 mV and \( I_{K} \) amplitudes (○) were measured as the mean current amplitude between -20 and 0 mV for each voltage sweep. Current amplitudes were then normalized to the maxima and minima over the course of the experiment. Plotted are the mean normalized current amplitudes before and after application of TTX (1 µM) for 7 neurons from dissociated olfactory bulb cultures. Error bars represent standard error of the mean. TTX was applied at time point 0, indicated by black arrow and perfused for the remainder of the experiment. Decreases in current amplitudes were fit by single exponential decay function, shown in red. Inward \( I_{Na} \) was relatively rapidly blocked by TTX, declining with a time constant of \( 2.72 \pm 0.67 \) seconds. Outward \( I_{K} \) declined with a much greater time constant of \( 12.43 \pm 1.52 \) seconds.
a

- black: control
- green: TTX immediate
- blue: TTX subsequent

100 pA
60 ms
-90 mV
0 mV
-90 mV

b

$I_{\text{Na}} \tau = 2.72 \pm 0.67 \text{ s}$

$I_{\text{K}} \tau = 12.43 \pm 1.52 \text{ s}$

Normalized current amplitude

Time (seconds)
Figure 2: Direct blockade of $I_K$ with TEA is rapid and leaves $I_{Na}$ in tact.

a) Currents were evoked using the same depolarizing voltage ramp as in Figure 1. Control current shown in black. Outward $I_K$ was rapidly blocked by application of 30 mM TEA (green trace) while inward $I_{Na}$ was grossly unchanged. In the continued presence of TEA, $I_{Na}$ was rapidly blocked by TTX (1 µM) (blue trace). No reduction in $I_K$ was observed following application of TTX in the presence of TEA. b) $I_{Na}$ (■) and $I_K$ (○) were measured and plotted as in Figure 1.
Figure 3: Replacement of external Na\(^+\) with Li\(^+\) results in slow \(I_K\) decline. Recovery of \(I_K\) following restoration of external Na\(^+\) is rapid.

Plot of mean normalized \(I_K\) amplitude for 10 olfactory bulb neurons. Currents were evoked with same depolarizing ramp protocol as Figures 1 & 2. External Na\(^+\) was replaced by Li\(^+\) at time point 0 for 48 seconds (black bar along x-axis). Decline and recovery were both well fit with mono-exponential decay and growth functions, respectively (fit indicated by red lines; \(\tau = 13.37 \pm 1.14\) and \(1.92 \pm 0.23\) seconds, respectively).
Figure 4: Rapid blockade of $I_{Na}$ followed by slow decay of $I_K$ is observed in neurons cultured from several different brain regions. Exemplary experiments from specified brain regions are presented here. All experiments used a 500 ms voltage ramp from -90 to 0 mV every 3 seconds, as described in Figure 1.

a) currents evoked from a medium spiny neuron of the striatum
b) currents evoked from an olfactory bulb tufted/mitral neuron
c) & d) currents evoked from cortical pyramidal neurons
e) currents evoked from hippocampal bipolar neuron
f) currents evoked from hippocampal pyramidal neuron
g) currents evoked from cerebellar bipolar neuron
h) currents evoked from cerebellar pyramidal neuron.
Figure 5: Difference in rate of block of $I_{Na}$ and subsequent decline in $I_K$ can be used to reveal isolated currents

a) control currents (black trace) evoked by a voltage step from -90 to -20 mV contains both $I_{Na}$ and $I_K$. Within 6 seconds of TTX application, $I_{Na}$ is completely blocked (green trace) followed by a slow decline in the outward currents that reach a minimum after approximately 30 seconds (blue trace). b) using data in a one can reveal $I_{Na}$ and $I_{KNa}$ evoked during the repeated voltage step. The black trace represents the total current blocked by TTX ($I_{Na}$ and $I_{KNa}$) and was generated by subtracting trace labeled “TTX subsequent” from “control”. The green trace represents the current rapidly blocked by TTX ($I_{Na}$) and was generated by subtracting “TTX immediate” from control. The blue trace represents the current slowly removed following application of TTX ($I_{KNa}$) and was generated by subtracting “TTX subsequent” from “TTX immediate”. Dashed line indicates 0 current level.
Figure a: Current traces showing different conditions: control, TTX immediate, and TTX subsequent.

Figure b: Traces illustrating total current blocked by TTX ($I_{Na} \& I_{KNa}$), current rapidly blocked TTX ($I_{Na}$), and current slowly blocked TTX ($I_{KNa}$).
**Figure 6: Action potential clamp reveals $K_{Na}$ channel activity during action potential waveform.**

Isolated currents evoked during an action potential command waveform in tufted/mitral cell of olfactory bulb. Blue trace shows kinetics of the voltage clamp command waveform previously recorded from the cell. Total (net) current, in black determined by subtracting currents evoked in the presence of TTX (1 µM), TEA (150 mM) and 4-AP (5 mM) from currents evoked in control conditions. $I_{Na}$ in green, determined by subtracting currents ~6 seconds after application of TTX from currents in control conditions. $I_{KNa}$, in red, determined by subtracting currents ~30 seconds after application of TTX from currents recorded ~6 seconds after application of TTX. $I_{KV}$, in grey, determined by subtracting currents in TTX, TEA and 4-AP from currents recorded ~30 seconds after applying TTX.


Chapter 5

Conclusions
In the preceding chapters I have demonstrated that Slo2/KNa channels are active participants in normal physiology. The activation of these channels by Na\(^+\)-influx has been demonstrated using whole cell recordings from multiple cell types and excised patch recordings containing a small number of KNa channels. Furthermore, I have demonstrated the immediate and secondary effects of preventing Na\(^+\)-influx take place with distinct time scales. I have exploited this to reveal the activity of KNa channels during the action potential waveform. There is much to be learned about KNa channels regarding their contribution to neuronal physiology, the biophysics of their gating and the mechanism of their functional coupling to Na\(^+\)-influx. As discussion of the experimental results has taken place within Chapters 2-4, I will focus here on potential avenues for future studies of KNa/Slo2 channels.

*What are the physiological roles of KNa/Slo2 channels?*

An active area of research is directed towards a better understanding of physiological roles of KNa/Slo2 channels. As described throughout the thesis, there is evidence suggesting a contribution of KNa/Slo2 channels to the afterhyperpolarizations following individual or bursts of action potentials, and evidence from my own data illustrating a contribution of KNa channels to the repolarization of the action potential. Both are likely to be true, but future studies would benefit from specific pharmacological blocker(s) of KNa/Slo2. As described in Chapter 1, no such compound is currently available.
For all ion channels, most compounds used as selective-blockers are selective in terms of physiologically-defined currents, but block many different molecularly-defined ion channels. For example, TTX is selective for the voltage-gated sodium current, but blocks 7 different $\alpha$-subunits ($Na_v1.1$-$7$). Furthermore, the contribution of accessory subunits to the gating of ion channels can be rather dramatic and the ability to target channels composed of specific accessory subunits is nearly non-existent with pharmacology. An interesting exception may be found with carbamazapine, which has long been used clinically as a preferential inhibitor of $I_{\text{NaP}}$, and has been suggested to differentially affect $I_{\text{NaP}}$ dependent on the presence of the $\beta_1$-subunit (Uebachs et al., 2010). Therefore, while a specific blocker of Slo2 channels would be of great use, future studies will also benefit from molecular dissection of the Slo2 family.

Generation of animals in which Slo2.1, Slo2.2 or both genes have been genetically removed could be useful in determining the contribution of the channels to action potential firing or even behavior of the animal. With knockouts of any member(s) of a gene family, there is potential for compensatory changes in expression of other genes. Cortical pyramidal neurons from $K_{V}4.2$ knockout mice closely resemble wild type mice in the generation of action potentials in response to current injection, but dramatically differ in ionic conductances underlying their similar neuronal excitability (Nerbonne et al., 2008). It is difficult to predict whether there may be similar compensatory changes in expression of other ion channels in response to genetic deletion of Slick and/or Slack, but expression of Slack is reported to change as a result of deletion of a voltage-gated K$^+$ channel. Slack is reported to be up-regulated in $K_{V}1.3$ knockout mice (Lu et al., 2010).
This line of mice is sometimes described as “super-smeller” mice as they display lower odorant thresholds and improved odorant discrimination compared to wild type mice (Fadool et al., 2004). The contribution of increased Slack expression to this phenotype is unclear (Lu et al., 2010). The generation of conditional knockouts, constitutive or conditional knockdowns using siRNA, or expression of dominant negative constructs could also be of potential use to investigate the physiological role of Slo2 channels and determine if there are unique roles of channels encoded by Slick versus Slack. While compensatory changes may be more limited in these strategies, alternative concerns arise such as efficacy of knockdown and off-target effects.

**Structural basis of sensitivity to Na\(^+\)-influx**

There are several lines of experiments that could provide more information on the mechanism by which the activity of K\(_{Na}/\)Slo2 channels is dependent on Na\(^+\)-influx. What is the distance between K\(_{Na}/\)Slo2 channels and the source of Na\(^+\)-influx? Use of fluorescence resonance energy transfer (FRET) could be used to estimate the distance between Slo2 channels and voltage-gated Na\(^+\) channels. Such experiments would depend on highly-selective antibodies for both channels for which epitopes of both channels were on the same side of the plasma membrane.

Is there a direct interaction between Slo2 channels and Na\(_V\) channels? This could be tested by attempting co-immunoprecipitation of the two channel types. Again, such experiments would be aided by improved antibodies for Slo2 channels. It is possible that Slo2 channels and Na\(_V\) channels do not directly bind to each other but are kept in close proximity by other mechanisms.
proximity by one or more intermediary regulatory or scaffolding proteins. In this case, the conditions in which the proteins were isolated prior to immunoprecipitation could greatly affect the ability to detect any interaction by this line of experiments.

One proposed mechanism of the activation of $K_{Na}$ channels by $Na^+$-influx is the presence of a submembrane domain or fuzzy space. Such an interpretation could imply limits to the diffusion of $Na^+$ by organelles or other subcellular structures. While such structures can be seen in electron micrographs of neurons, it is not known if $K_{Na}$/Slo2 channels are localized to such regions. Immunogold labeling of Slo2 channels could allow identification of potential barriers to diffusion near the channels. Furthermore, co-labeling of $NaV$ channels with gold particles of a different diameter could also provide a measure of the proximity of $K_{Na}$ and $NaV$ channels.

Heterologous systems could be used to determine if Slo2 channels and $NaV$ channels are capable of forming the appropriate structures in the absence of specialized scaffolds or accessory proteins. Challenges that currently limit undertaking such studies include uncertainty of whether or not a specific $NaV$ channel interacts with Slo2 channels in neurons. Furthermore, many $NaV$ channels and Slick channels are “poor expressers” even when studied in isolation. Finally, a precise stoichiometry could be required for coupling of $NaV$ and Slo2 channels.

**Mechanism of Slo2 $Na^+$-gating**

What is the biophysical mechanism of Slo2 channel sensitivity to $Na^+$? A site in Slo2.2 that resembles a $Na^+$-coordination site present in some inward-rectifier $K^+$-
channels is reported to confer \( \text{Na}^+ \)-sensitivity to Slo2.2 channels (residues D818 and H823) (Zhang et al., 2010). There is not a homologous sequence in Slo2.1, but channels encoded by Slo2.1 and Slo2.2 display similar sensitivity to \( \text{Na}^+ \). It is intriguing that in an investigation of the activation of Slo2.2 channels by Fragile-X Mental Retardation Protein (FMRP) (Brown et al., 2010), the Kaczmarek laboratory generated a Slo2.2 construct in which the channel was truncated at amino acid 804 - upstream of the reported \( \text{Na}^+ \)-binding site – but no change in \( \text{Na}^+ \)-sensitivity of the truncated channel was reported. It will be interesting to see if other laboratories confirm this motif as a \( \text{Na}^+ \)-binding site in Slo2.2 channels and if a distinct mechanism of \( \text{Na}^+ \)-gating is really present in Slo2.1.

The fundamental property of neurons is electrical excitability. An essential question in neuroscience is how the repertoire of ion channels gives rise to the electrical behavior of neurons. This question is not just a matter of what proteins are there, but also how each element interacts with all the others. These interactions can be direct interactions between proteins as with pore-forming channel subunits and accessory subunits. Furthermore, many ion channels are controlled by membrane voltage which in turn is dictated by the activity of ion channels. Similarly, the chemical signaling that takes place between and within neurons can govern the gating of ion channels while ion channels can also be the source of the signal. The work presented in this thesis demonstrates the unexpected coupling of two widespread elements of neuronal excitability. \( \text{Na}^+ \)-influx through \( I_{\text{NaP}} \) allows the participation of \( K_{\text{Na}} \) channels in normal
physiology. While potential avenues for further elucidating the interactions between these two elements are described above, this system should ultimately be considered as part of the complex tapestry of elements governing neuronal excitability.
WORKS CITED


