ROLES OF THE ION CHANNEL NALCN IN NEURONAL EXCITABILITY CONTROL

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Abstract
The resting membrane potential (RMP) of a neuron is set by a complex balance between charged ions, ion channels and transporters. Many of the ion channels have been identified at the molecular level. Missing from the molecular identification has been the voltage-insensitive background sodium “leak” conductance that depolarizes the RMP from the equilibrium potential of potassium and provides a crucial contribution to neuronal excitability.

One candidate for the molecular identity of this conductance is the protein NALCN. NALCN is a previously uncharacterized orphan member in the sodium/calcium channel family. It is widely expressed in the nervous system. My thesis project was designed to uncover the properties of NALCN and to find its functional roles, especially its contribution to the neuronal excitability as an ion channel.

I found that NALCN formed a voltage-insensitive background sodium leak conductance. Such a conductance was detected in hippocampal neurons cultured from the wild-type mice but not the NALCN-/- mutant mice with targeted disruption in the NALCN gene. The conductance in the NALCN-/- neurons was restored when NALCN cDNA was transfected. These results suggest that NALCN provides the major contribution to the voltage-insensitive background sodium leak conductance.

We also discovered that the NALCN channel could be activated by the neuropeptides substance P (SP) and neurotensin (NT), and by lowering extracellular Ca2+ concentration ([Ca2+]e), both of which elicit excitatory effects on several types of neurons. In addition, we found that NALCN was activated by the two types of stimuli via distinct intracellular signaling pathways and the two had synergistic effects on each other. Finally, application of the neuropeptides or lowering [Ca2+]e did not excite hippocampal neurons cultured from the mutant, suggesting that the NALCN channel complex is a primary target for neuronal excitation control by the neuropeptides and extracellular Ca2+.

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ROLES OF THE ION CHANNEL NALCN IN NEURONAL EXCITABILITY CONTROL

Boxun Lu

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

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ABSTRACT

ROLES OF THE ION CHANNEL NALCN IN NEURONAL EXCITABILITY CONTROL

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Supervisor: Dr. Dejian Ren

The resting membrane potential (RMP) of a neuron is set by a complex balance between charged ions, ion channels and transporters. Many of the ion channels have been identified at the molecular level. Missing from the molecular identification has been the voltage-insensitive background sodium “leak” conductance that depolarizes the RMP from the equilibrium potential of potassium and provides a crucial contribution to neuronal excitability.

One candidate for the molecular identity of this conductance is the protein NALCN. NALCN is a previously uncharacterized orphan member in the sodium/calcium channel family. It is widely expressed in the nervous system. My thesis project was designed to uncover the properties of NALCN and to find its functional roles, especially its contribution to the neuronal excitability as an ion channel.

I found that NALCN formed a voltage-insensitive background sodium leak conductance. Such a conductance was detected in hippocampal neurons cultured from the wild-type mice but not the NALCN<sup>−/−</sup> mutant mice with targeted disruption in the NALCN gene. The conductance in the NALCN<sup>−/−</sup> neurons was restored when NALCN cDNA was transfected. These results suggest that NALCN provides the major contribution to the voltage-insensitive background sodium leak conductance.
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CHAPTER I
INTRODUCTION

Electrical Signals in Neuronal Communication

The most fundamental task of a neuron is to receive, process, and transmit signals. To perform these functions, neurons have evolved two properties: they are connected, and they conduct signals.

To connect with each other, neurons have developed a peculiar cellular structure: an axon to conduct signals away from the cell body toward distant targets, and several shorter branching dendrites extending from the cell body to receive signals. This structure allows connections to form between different neurons via synapses to establish neuronal circuits that control various functions of the body (Lopez-Munoz et al., 2006).

Electrical signals are conducted along the axon of a neuron by flow of currents. The membrane accumulates ionic charges on its inner and outer surfaces, functioning as a capacitor. The voltage established by the charges across the membrane is defined as the membrane potential, with extracellular space as the reference. An electrical signal applied at a given location can lead to local changes in the membrane potential, which could be used to conduct the signal. However, unless energy is utilized to amplify them, both the current flow and the local disturbance of membrane potential will decay as they travel with increasing distance, because of progressive loss of current due to leakage through plasma membrane.

For short distance communication, the signal attenuation may not be problematic.
For long-distance communication, however, a passive spread of signal is inadequate and an active signaling mechanism is employed. When the membrane potential change exceeds a threshold, an explosion of electrical activity (action potential) is triggered (discovered by Emil du Bois-Reymond in 1848). Action potentials propagate along the axon's plasma membrane and are sustained by amplification along the way. This traveling wave of membrane potential change (action potential) can carry a message at speeds as high as 100 meters per second or more without attenuation (Hodgkin, 1937a; Hodgkin, 1937b).

**Ion Channels and Transporters in Action Potential Generation**

Physiologists and chemists have long been studying the roles of ions in the excitation of nerve and muscle cells. In the 1880’s, Sidney Ringer showed that the solution perfusing a frog heart must contain salts of sodium, potassium and calcium mixed in a definite proportion for the heart to continue beating for a long period (Cordell, 1995; Moore, 1911). Later on, Walther Nernst’s work (1888) on electrical potentials arising from the diffusion of electrolytes in solution inspired speculations of ions as the determinants of bioelectric potentials (Edsall, 1974). Next, Julius Bernstein correctly proposed that excitable membranes are selectively permeable to K⁺ ions at rest and that during excitation the membrane permeability to other ions increases (Bernstein, 1902). During the twentieth century, major roles in excitation were discovered for each of the cations in Ringer’s solution (Na⁺, K⁺, Ca²⁺).

Excitable membranes have evolved elegant cellular devices to establish as well as utilize the concentration gradient of different ions across cell membrane. Because of its
hydrophobic interior, the lipid bilayer of neurons’ membranes serves as a barrier to the passage of ions. This barrier function is crucially important because it allows neurons to maintain the ionic concentrations of solutes in its cytosol different from those in the extracellular fluid and in the intracellular membrane-enclosed compartments. To make use of this barrier, however, neurons have had to evolve ways of transferring ions across their membranes in order to generate membrane potentials and to regulate intracellular ionic concentrations.

Cell membranes have an elaborate design in ion transport, with many and relatively selective transport devices handling different ions, often under separate physiological control. Earlier studies of the membrane transport mechanisms were largely based on physiological flux measurements. Largely based on kinetic criteria, these measurements revealed two separate transport mechanisms: transporters and channels.

The early literature viewed a transporter as a ferryboat diffusing back and forth across the membrane while carrying ions bound to stereospecific binding sites (Laprade et al., 1975). This view was modified because the numerous transporter devices that have been purified from membranes and cloned turned out to be too large to diffuse or to undergo dramatic structural changes at the rate needed to account for the fluxes they catalyze. Supported by the recently solved transporter structures (Gadsby, 2007), a more recent view is that subtle changes occur within the protein to expose the ion-binding sites alternatively to the intracellular and extracellular media.

The other class of ion transport devices, ion channels, has been firmly established to form aqueous pores. Ions passing through the channels are accessible from both sides
of the membrane simultaneously. The pore mechanism is supported by the fact that the rate of passage of ions through one single open channel—often more than $10^6$ per second, as resolved by recording from single molecules (channels)—is far too high for any transport mechanism other than a pore (Neher and Sakmann, 1976). The more direct evidence for the pore mechanism is the visualization of protein crystal structure of the expected continuous aqueous pathway with ions passing right through the channel molecule (Doyle et al., 1998).

Both transporters and channels are required for the proper functioning of excitable membranes. Transporters are used to establish the concentration gradient of the major ions. For example, Na$^+$, K$^+$ and Ca$^{2+}$ ions are actively pumped uphill by ATP-driven transporters to establish relatively high K$^+$ as well as low Na$^+$ and Ca$^{2+}$ concentrations inside the cell. These concentration gradients had been revealed since the 1940’s. The intracellular K$^+$ concentration inside neurons is usually at least 20 times the extracellular concentration (Hodgkin and Huxley, 1947), while the intracellular Na$^+$ concentration is ~10 times lower than the extracellular one (Hodgkin and Katz, 1949). These concentration gradients are mainly maintained by the Na$^+/K^+$ pump (Kawakami et al., 1985; Noguchi et al., 1986), which consumes ATP and transforms its energy into the chemical potential energy of the ions (Caldwell et al., 1960; Hodgkin and Keynes, 1955). This potential energy is then utilized in electric signal generation and propagation during excitation. Although the transporters establish the ion gradients needed for excitation, they themselves are not the major parts of the excitation process.

Ion channels mediate the movement of ions during excitation in the nervous system. Each channel may be viewed as an excitable molecule that is specifically
responsive to a certain stimulus: a membrane potential change (for voltage-gated channels), a neurotransmitter (for receptor-activated and ligand-activated channels), or a mechanical deformation (for mechanosensitive channels). The channel’s response is opening or closing of the ion permeable pore. For ion-selective ion channels, opening of the pore selectively allows certain ions to flow passively down their electrochemical gradient, while closing of the pore stops the ion fluxes. The opening and closing of the pore are random events of which the probability is affected by the intensity, timing and the nature of the stimulus (Hille, 2001; Neher and Sakmann, 1976).

The coordinated opening and closing of a certain set of ion channels can generate an action potential. In most neurons, for example, a fast and transient Na$^+$ influx occurs through voltage-gated Na$^+$ channels (Nav) when the neuronal membrane potential depolarize from its resting level to a threshold. The Na$^+$ influx results in a further depolarization and an overshoot of the membrane potential. However, this Na$^+$ influx quickly vanishes, due to the inactivation of Nav channels. The decrease of the Na$^+$ influx, together with a K$^+$ outflux through voltage-gated K$^+$ channels (Kv), repolarizes and hyperpolarizes the membrane potential to form an overall change called an action potential. The membrane is then able to fire the next action potential when the threshold is reached. The charge movement in this scenario was clearly illustrated in the historic work of Alan Lloyd Hodgkin and Andrew Fielding Huxley a half century ago (Hodgkin and Huxley, 1952), followed by the molecular identification of the ion channels involved. In summary, an action potential is generated by time- and voltage- dependent Na$^+$ and K$^+$ fluxes. However, in order to initiate an action potential, the membrane needs to be depolarized to the threshold from the resting membrane potential (RMP). The ease of
generating action potentials, referred to as the excitability of the membrane, is therefore affected by the RMP: the closer the RMP is to the threshold, the higher the probability is to generate action potentials (up to a certain point). The ionic basis that determines RMP will be discussed in the next section.

**Contribution of Leak Conductances to Resting Membrane Potential**

As mentioned above, one of the factors that control the excitability of the membrane is its resting potential, which is set by the ion fluxes through the membrane at its resting state. Quantitatively, each type of permeant ions tends to drive the membrane potential towards its own equilibrium potential, which is a point when the electric force and the chemical-gradient force for the particular ion are “balanced” such that there is no net flow of the ion (Nernst, 1893). The equilibrium potential, also called the “Nernst Potential”, can be calculated by the Nernst equation:

$$E = \frac{RT}{zF} \ln \frac{[\text{ion}]_{\text{out}}}{[\text{ion}]_{\text{in}}}$$

where

- $E$ is the Nernst potential in volts (inner – outer);
- $R$ is the ideal gas constant in joules per Kelvin-mole;
- $T$ is the temperature in Kelvin-degree;
- $F$ is Faraday's constant in coulombs/mol;
- $z$ is the valence of the ion;
- $[\text{ion}]_{\text{out}}$ is the extracellular concentration of the ion;
- $[\text{ion}]_{\text{in}}$ is the intracellular concentration of the ion.

Therefore, different types of ions drive the membrane potential towards different voltages.
The relative contribution of each ion in setting the membrane potential is weighed by its membrane conductance (in proportion to permeability), as determined by the Goldman-Hodgkin-Katz voltage equation (derived by David E. Goldman, Alan Lloyd Hodgkin and Bernard Katz). The GHK voltage equation for \( N \) positive and \( M \) negative monovalent ionic species is:

\[
E_m = \frac{RT}{F} \ln \left( \frac{\sum_{i=1}^{N} P_{C_i^+} [C_i^+]_{\text{out}} + \sum_{j=1}^{M} P_{A_j^-} [A_j^-]_{\text{in}}}{\sum_{i=1}^{N} P_{C_i^+} [C_i^+]_{\text{in}} + \sum_{j=1}^{M} P_{A_j^-} [A_j^-]_{\text{out}}} \right),
\]

where

- \( E_m \) is the membrane potential in volts (inner – outer);
- \( P_{\text{ion}} \) is the permeability of the ion;
- \( C_i \) refers to monovalent cations;
- \( A_j \) refers to monovalent anions.

In neurons, membrane potentials are usually largely determined by the Na\(^+\), K\(^+\), and Cl\(^-\) ions, due to these ions’ dominant concentrations and permeability through the membrane under physiological conditions. The GHK voltage equation could then be simplified as:

\[
E_m = \frac{RT}{F} \ln \left( \frac{P_{Na^+} [Na^+]_{\text{out}} + P_{K^+} [K^+]_{\text{out}} + P_{Cl^-} [Cl^-]_{\text{in}}}{P_{Na^+} [Na^+]_{\text{in}} + P_{K^+} [K^+]_{\text{in}} + P_{Cl^-} [Cl^-]_{\text{out}}} \right)
\]

When the neurons are at the resting state, most voltage- and time- dependent ion channels are not open; the voltage- and time- independent background “leak” conductances may provide the major contribution to the RMP (Hodgkin and Huxley, 1952). The K\(^+\) leak conductance is likely to be contributed by many ion channels, including the 15 K\(^+\)
channels in the K2P family (Goldstein et al., 2005). The K⁺ leak current drives RMP towards the equilibrium potential of K⁺ (E_K), near −90 mV in mammalian neurons under physiological conditions. However, the resting membrane potential of most mammalian neurons is in the range from −50 to −80 mV (Huettner and Baughman, 1988; Puil et al., 1986), considerably depolarized to E_K. This may be partially explained by the presence of a background Na⁺ leak conductance, observed in recordings from a variety of neurons (Hodgkin and Katz, 1949; Raman et al., 2000; Simasko, 1994; Tryba and Ramirez, 2004). Na⁺ has a ~10 times higher concentration outside than inside (established by the Na⁺/K⁺ ATPases); a Na⁺ leak therefore drives the RMP towards E_{Na} near +60 mV. The Na⁺ leak conductance is normally a fraction (~4% in the squid giant axon) of that of the K⁺ conductance at rest (Hodgkin and Katz, 1949), resulting an RMP much closer to E_K than to E_{Na}. On the other hand, since the E_{Na} is far from RMP (hence a large depolarizing driving force for Na⁺), a Na⁺ leak conductance is able to provide significant contribution to neuronal excitability control. Sources of the Na⁺ leak conductance have been speculated to be via Naᵥ channels (Chen and Lucero, 1999; Nicholls and Baylor, 1968), nonselective channels (Raman et al., 2000), Na⁺ leak through K⁺ channels (Heginbotham et al., 1994; Shrivastava et al., 2002), and transporters (Jacob et al., 1987), but experimental data showing the molecular identifications were largely absent before our studies (described in Chapter II).

**Signal Transmission between Neurons**

Neurons form synapses with each other so that the signals can be transmitted between them. When a nerve impulse arrives at the synapse, the signal could be
transmitted to the connected neuron in two ways: electrical synaptic transmission and chemical synaptic transmission.

Electrical synaptic transmission refers to the direct electrical coupling between the membrane sending out the signal (presynaptic membrane) and the one receiving the signal (postsynaptic membrane) (Figure 1.1A). When action potentials arrive at the presynaptic membrane electrically connected with the postsynaptic membrane, a direct intercellular current flow could be conducted to the postsynaptic membrane, which may lead to a depolarization sufficient to initiate action potentials in the postsynaptic membrane. This direct coupling mechanism was first demonstrated by intracellular microelectrode recordings from nerve fibers in the abdominal nerve cord of the crayfish (Furshpan and Potter, 1959; Watanabe and Grundfest, 1961). We now know that the presynaptic and postsynaptic membranes in electrical synapses are connected morphologically via gap junction channels formed by two connexon hemi-channels, each of which is an assembly of the connexin proteins (Figure 1.1A) (Goodenough, 1974). Gap junctions allow current to conduct from one cell to another. Electrical transmission has been observed at a wide variety of synapses. The resistance of the gap junction between neurons determines the degree of electrical coupling: the lower the resistance, the higher the percentage of the potential change to be transmitted. Because the electric current spreads directly from one cell to the next, there is little delay in the electrical synaptic transmission.

For chemical synaptic transmission, the presynaptic and postsynaptic membranes are separated by the synaptic cleft and signals are transmitted via chemical molecules called neurotransmitters (Figure 1.1B). When action potentials arrive at the presynaptic
membrane, they may trigger the release of neurotransmitters into the synaptic cleft. The released neurotransmitters may be sensed by the postsynaptic membrane to trigger membrane potential changes and action potentials. The chemical nature of the synaptic transmission was established by a direct and simple experiment done by Otto Loewi in the 1920s. He perfused the heart of a frog and stimulated the vagus nerve to slow the heartbeat. The fluid transferred from the slowed heart was able to slow down the heartbeating of another unstimulated heart, demonstrating that the stimulation of the vagus nerve of the first heart had caused release of a substance that diffused into the perfusate and was able to slow the heartbeating of the second heart. The substance was demonstrated in subsequent experiments by Loewi and his colleagues to be mimicked by acetylcholine (ACh), the first established neurotransmitter (Loewi, 1921; Raju, 1999).

Dozens of neurotransmitters have now been identified. They can be classified in many different ways. Chemically, the major neurotransmitters can be classified as small-molecule transmitters such as amino acids, monoamines and ACh, and the peptide neurotransmitters such as substance P and neurotensin. According to their effects, neurotransmitters are also classified as excitatory versus inhibitory. In addition, they can be classified by the transmission mechanism, as direct ones, which directly control the gating of ion channels to influence excitability, versus indirect ones, which influence ion channels via multi-component signaling pathways. Some neurotransmitters can be both excitatory and inhibitory, or both direct and indirect, because they may bind with different types of receptors on the postsynaptic membrane in various types of synapses.

In contrast to those of the small molecule neurotransmitters, the mechanisms by which some of the peptide neurotransmitters such as substance P and neurotensin excite
neurons are not well understood. In Chapter III, we found that they excite neurons by activating the cation ion channel NALCN, by a non-canonical mechanism: the activation is mediated by G-protein coupled receptors, but through a G-protein independent pathway, which requires both the Src-family kinases and the ion channel subunit UNC80.
Figure 1.1. Electrical and Chemical Synaptic Transmission.


(A) At electrical synapses, ionic currents flow directly from one cell to another through connexons, leading to changes in membrane potential of the postsynaptic cells.

(B) At chemical synapses, depolarization of the presynaptic nerve terminal triggers the release of neurotransmitters, which interact with receptors on the postsynaptic membrane, leading to excitation or inhibition.
The Role of Calcium in Neurotransmission and Neuronal Excitation

A key player in synaptic transmission is Ca\(^{2+}\). When placed in medium with low Ca\(^{2+}\) concentration, release of ACh at the neuromuscular junction was reduced and eventually disappeared (Del Castillo and Stark, 1952; Dodge and Rahamimoff, 1967). Focal application of Ca\(^{2+}\) by micropipette induced increase in release of acetylcholine by the nerve terminal, shown by a large increase in the number of quantal components of the end-plate potential (Katz and Miledi, 1965). Procedures that increase the intracellular calcium caused an increase in neurotransmitter release (Alnaes and Rahamimoff, 1975; Miledi, 1973). Calcium has later been established as an essential player in neurotransmitter release irrespective of the nature of the transmitter. The physiological concentration of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) of neurons at resting is within the range of 10 ~ 100 nM, thousands times lower than the ~mM physiological extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_e\)) (Clapham, 2007). When the presynaptic membrane is depolarized, voltage-gated Ca\(^{2+}\) (Ca\(_V\)) channels open, resulting in a Ca\(^{2+}\) entry into the cell and an increase in [Ca\(^{2+}\)]\(_i\), which triggers neurotransmitter release (Augustine et al., 1985).

While [Ca\(^{2+}\)]\(_i\) increases dramatically during synaptic transmission, [Ca\(^{2+}\)]\(_e\) may drop significantly as well due to Ca\(^{2+}\) moving into the cell from outside through Ca\(_V\) channels. Given that intracellular volume of any particular cell is usually negligibly small compared to the extracellular space and that the opening of Ca\(_V\) channels is transient, this [Ca\(^{2+}\)]\(_e\) drop usually may not be significant. However, repetitive stimulation in areas where extracellular space is limited can cause [Ca\(^{2+}\)]\(_e\) to decrease from approximately 1.3 to 0.1 mM (Benninger et al., 1980; Heinemann and Pumain, 1980;
Krnjevic et al., 1982; Nicholson et al., 1977; Pumain and Heinemann, 1985). In microdomains such as the synaptic cleft, single impulses may cause $[\text{Ca}^{2+}]_e$ to decrease as well (Borst and Sakmann, 1999; Rusakov and Fine, 2003; Stanley, 2000).

$[\text{Ca}^{2+}]_e$ has been found to negatively influence the neuronal excitability: a reduction in $[\text{Ca}^{2+}]_e$ normally increases excitability (Burgo et al., 2003; Chu et al., 2003; Frankenhaeuser and Hodgkin, 1957; Xiong et al., 1997; Yaari et al., 1983). This lies in contrast to the effect of $[\text{Na}^+]_e$ and $[\text{K}^+]_e$: a decrease in $[\text{Na}^+]_e$ or $[\text{K}^+]_e$ normally suppresses neuronal excitability, presumably through the action on leak currents. As a positively charged ion, $\text{Ca}^{2+}$ movement into the cell would depolarize the membrane, same as $\text{Na}^+$ and $\text{K}^+$. Therefore, a $[\text{Ca}^{2+}]_e$ drop decreases the chemical gradient of $\text{Ca}^{2+}$ and reduces $\text{Ca}^{2+}$ flux into the cell. The most “direct” effect of such a $[\text{Ca}^{2+}]_e$ drop would be a suppression of neuronal activity, in contrast to the observed excitatory effects.

The excitatory effect of $[\text{Ca}^{2+}]_e$ drop is then likely through mechanisms other than the change in the movement of $\text{Ca}^{2+}$ per se. One such mechanism is that $[\text{Ca}^{2+}]_e$ affects $\text{Na}_V$ and $\text{K}_V$ channels. In the 1950's, Bernhard Frankenhaeuser and Alan Lloyd Hodgkin examined the voltage dependence of the $\text{Na}_V$ channel current using voltage clamp experiments (Frankenhaeuser and Hodgkin, 1957). When $[\text{Ca}^{2+}]_e$ is reduced, the voltage dependence of the channel was shifted in the hyperpolarization direction. As a result, a lower threshold needs to be reached to activate the $\text{Na}_V$ channel and fire an action potential, resulting in an increase in neuronal excitability.

Frankenhaeuser and Hodgkin suggested that the effect might be due to the screening effect of $\text{Ca}^{2+}$: $\text{Ca}^{2+}$ screens/neutralizes the negative charges that, for example, arise from membrane proteins and attach to the outer surface. Upon removal of
extracellular Ca$^{2+}$, the unscreened negative charges would reduce the effective potential gradient across the membrane, while the electrical potential between intracellular and extracellular solutions remains the same (Figure 1.2). As a result, when [Ca$^{2+}$]$_e$ drops, less depolarization is needed to reduce the potential gradient to the threshold of Na$^+$ activation. One “complication” of the charge screening effect is that reducing the effective potential gradient across the membrane upon a removal of extracellular Ca$^{2+}$ should also shift the voltage dependence of Na$^+$ inactivation and K$^+$ activation in the same direction, which should lead to a decrease in excitability.

In addition to the reduction of surface charge screening, the excitation by lowering [Ca$^{2+}$]$_e$ is likely to be contributed by other effects as well. Indeed, it has been discovered that, in several types of neurons, a drop in [Ca$^{2+}$]$_e$ activates depolarizing, nonselective cation currents in cell bodies and nerve terminals (Formenti et al., 2001; Hablitz et al., 1986; Smith et al., 2004; Xiong et al., 1997). The molecular identities of the channels responsible for the currents and their contribution to the low [Ca$^{2+}$]$_e$-induced neuronal excitability has not been established. In Chapter IV, we discovered that low [Ca$^{2+}$]$_e$ activates the ion channel NALCN, and such an activation provides a major contribution to the excitatory effect of low [Ca$^{2+}$]$_e$ in the hippocampal neurons.
Figure 1.2. Effect of Surface Charge on Membrane Potential.


(A) The resting membrane potential, $V_R$, produced by ionic charge separation, is determined by the composition of the intracellular and extracellular fluids. The outer membrane surface includes negative charge, which is neutralized by divalent cations, so that the voltage gradient across the membrane is the same as $V_R$.

(B) When the divalent cations (e.g. Ca$^{2+}$) are removed from the extracellular solution, the shape of the potential profile (the blue line in A and B) is altered by the outer surface negativity, reducing the potential gradient across the membrane.
Overview of Thesis

In this thesis, I studied the multiple roles of the NALCN ion channel in neuronal excitability control. I also uncovered several mechanisms by which the channel can be regulated. In Chapter I, I briefly reviewed the ionic basis of neuronal excitation. In Chapter II, I presented experimental evidence showing that the then novel protein NALCN is an ion channel that controls the RMP by forming a background Na\(^+\) leak conductance. At the whole organism level, NALCN is required for the normal respiratory rhythm. This chapter was modified from a journal article originally published in Cell (Lu et al., 2007). In Chapter III, I described our experimental data showing that NALCN is activated by the peptide neurotransmitters substance P and neurotensin, through G-protein coupled receptors but via G-protein independent pathways. Instead, the activation is dependent on the Src family kinases (SFKs) and a NALCN-associate protein UNC80. The channel complex plays an important role in the excitatory effect of peptide neurotransmitters. This chapter was modified from a journal article originally published in Nature (Lu et al., 2009). In Chapter IV, I summarized experimental results revealing that NALCN is also activated by lowering \([\text{Ca}^{2+}]_e\), in a G-protein- and UNC80- dependent manner. This activation provides a major contribution to the excitation upon \([\text{Ca}^{2+}]_e\) drops. In Chapter V, I summarized the conclusions from our studies, and discussed future directions of research related to the NALCN channel complex.
CHAPTER II

THE NEURONAL CHANNEL NALCN CONTRIBUTES RESTING SODIUM PERMEABILITY AND IS REQUIRED FOR NORMAL RESPIRATORY RHYTHM

Summary

Sodium plays a key role in determining the basal excitability of the nervous systems through the resting “leak” Na⁺ permeabilities, but the molecular identities of the TTX- and Cs⁺-resistant Na⁺ leak conductance were previously unknown. Here we show that this conductance is formed by the protein NALCN, a substantially uncharacterized member of the sodium/calcium channel family. Unlike any of the other 20 family members, NALCN forms a voltage-independent, nonselective cation channel. NALCN mutant mice have a severely disrupted respiratory rhythm and die within 24 hours of birth. Brain stem-spinal cord recordings reveal reduced neuronal firing. The TTX- and Cs⁺-resistant background Na⁺ leak current is absent in the mutant hippocampal neurons. The resting membrane potentials of the mutant neurons are relatively insensitive to changes in extracellular Na⁺ concentration. Thus, NALCN, a nonselective cation channel, forms the background Na⁺ leak conductance and controls neuronal excitability.
Introduction

The resting membrane potential (RMP) of most mammalian neurons is in the range from −50 to −80 mV, considerably depolarized to the potassium equilibrium potential near −92 mV. This suggests that in addition to resting potassium conductances, there are also conductances due to other ions. In fact, recordings from a variety of neurons reveal a significant resting or background sodium permeability (Hodgkin and Katz, 1949; Raman et al., 2000; Simasko, 1994; Tryba and Ramirez, 2004). However, the molecular basis of resting sodium permeability remains unclear. In some neurons, there is resting permeability from hyperpolarization-activated cation channels (I_h, encoded by HCN genes; (Robinson and Siegelbaum, 2003)), and there can also be resting sodium permeability from TTX-sensitive sodium channels giving a subthreshold, persistent sodium current (Crill, 1996). However, both of these conductances are highly sensitive to voltage, and there is also evidence for a true voltage-insensitive background leak sodium permeability not blocked by Cs or TTX (Raman et al., 2000; Simasko, 1994). The leak Na^+ current (I_{L-Na}) is believed to play important roles in the regulation of neuronal excitability, but the in vivo function of such a leak conductance has not been tested because of a lack of specific blockers (Atherton and Bevan, 2005; Eggermann et al., 2003; Jackson et al., 2004; Jones, 1989; Pena and Ramirez, 2004; Raman et al., 2000; Robinson and Siegelbaum, 2003). Furthermore, the molecular basis for the voltage-independent sodium permeability was completely unknown previously.

In animals, voltage-gated Na^+ (Na_v) and Ca^{2+} (Ca_v)-selective channels have four homologous repeats (domains I–IV) of 6TM-spanning segments (S1–S6) (4x6TM) (Catterall, 2000; Goldin, 2002). The selectivity filter is formed by stretches of S5-S6 pore
(P) loops contributed by each 6TM domain (Figure 2.1A) (Catterall, 2000; Doyle et al., 1998; Sather and McCleskey, 2003). In Ca\textsubscript{v} channels, the calcium selectivity requires acidic amino acids that bind Ca (four glutamate [E] or aspartate [D] residues, one from each of the four homologous repeats; EEEE motif) in the mouth of the pore. Na\textsubscript{v} channel selectivity filters are generally DEKA in analogous positions (Heinemann et al., 1992; Kim et al., 1993; Sather and McCleskey, 2003; Tang et al., 1993; Yang et al., 1993) (Figure 2.1A). One member of the 21 genes encoding 4x6TM family NALCN (for sodium leak channel, nonselective; previous name, VGCNL1; accession number AAN10255) has not been characterized (Figure 2.1A) (Lee et al., 1999; Yu and Catterall, 2004). NALCN differs from Na\textsubscript{v} and Ca\textsubscript{v} channels in that it has fewer positively charged residues (arginine or lysine) in its voltage-sensing S4 segments. Additionally, NALCN has an EEKE motif, a mixture of the EEEE (Ca\textsubscript{v}) and DEKA (Na\textsubscript{v}) residues, in its predicted selectivity filter (Figure 2.1A).

NALCN is highly conserved in mammals (99% identity between human and rat). \textit{Drosophila melanogaster} has a NALCN homology named \textalpha\textsubscript{1}U (for unusual \alpha\textsubscript{1} subunit, accession number AY160083, 57% identity with the human homolog) (Littleton and Ganetzky, 2000). Hypomorphic alleles in the fruit flies with reduced NALCN expression, although viable and fertile, have altered locomotive behavioral circadian rhythms (Lear et al., 2005; Nash et al., 2002). Furthermore, the mutant flies display a narrow abdomen phenotype (\textit{na}) and have altered sensitivities to volatile anesthetics such as halothane (Krishnan and Nash, 1990; Mir et al., 1997; van Swinderen, 2006), but produce much smaller effect on the response to enflurane (Humphrey et al., 2007). In addition, two homologs also exist in \textit{Caenorhabditis elegans} (nca-1, accession number NP_741413;
The functional significance of NALCN in mammals and whether NALCN indeed encodes a plasma membrane ion channel were previously unknown (Lee et al., 1999).

Here we report that NALCN forms a voltage-independent, nonselective, noninactivating cation channel permeable to Na\(^+\), K\(^+\), and Ca\(^{2+}\). Mice with deletion of the NALCN gene have a severely disrupted respiratory rhythm characterized by a regular rhythm interrupted by periods of apnea. Mutant pups die within 24 hr of birth. Hippocampal neurons from the mutant lack I\(_{\text{L-NS}}\) and the current can be restored by NALCN cDNA transfection. We thus conclude that NALCN, a nonselective cation channel, encodes the neuronal background Na\(^+\) leak conductance.

**Materials and Methods**

**In Situ Staining**

Frozen mouse sections (10 \(\mu\)m thick) were used for in situ staining. A DNA fragment of 1811 bp, starting from nucleotide 4764, was amplified from mouse brain and subcloned into pBlueScript II SK(−) for single-strand, digoxigenin (DIG)-labeled RNA probe synthesis. This fragment has no significant sequence similarity with any other genes in the genome. Sense probe was used as negative control. Hybridizations were washed and signals were visualized using AP-conjugated anti-DIG antibody and BCIP/NBT substrate.

**Knockout Mice Generation**
Animal uses followed protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. The targeting vector was generated with pKO NTKV-1903 (Stratagene) as a backbone. The left and right arms have 3 kb and 1.5 kb, respectively. LoxP sites were engineered in the 5'UTR (20 bp from the translational start codon) and 360 bp into the intron following exon 1. A correctly targeted ES cell clone was used to generate chimeric mice. Heterozygous mice were mated to a line expressing CRE recombinase to delete exon 1 flanked by the LoxP sites. Mice used to generate the data were from mating between heterozygous that had been backcrossed to C57BL/6 for three or more generations. Due to a lack of specific antibodies, we have not determined the stability of protein encoded by mutant transcripts. Genomic PCR was used to identify the mutant. For RT-PCR, total RNAs were isolated from E18.5 embryos using the NucleoSpin RNA II Kit (Macherey-Nagel). cDNAs were synthesized using RETROscript Kit (Ambion). The primers amplifying 479 bp of NALCN mRNA span exons 1 and 4, a region containing introns of ~7 kb. The forward and reverse primers have the sequences (5'→3')

ATGCTCAAAAGAAAGCAGAGTTCCA

and

AATCGGAAATAATCCTGAAAGCCC, respectively. Primers amplifying a 261 bp fragment of the mouse HPRT gene were included in the same tube and served as an internal control. PCR conditions were the following: 28 cycles composed of 1 min denaturation at 95°C, 1 min primer annealing at 60°C, and 2 min extension at 72°C using 0.05 unit/μl Taq DNA Polymerase (Promega).

Plethysmograph
Plethysmography employed a differential pressure transducer (Omega Engineering, Model PX65, ±0.25% linearity). Data were collected through a digitizer and AxoScope software and band-pass filtered at 0.1 to 500 Hz (Axon). The experiments were performed at 32°C, close to the body temperature of newborn pups.

**C4 Nerve Root Recording**

C4 nerve recordings using the brain stem-spinal cord preparation followed previously described methods (Di Pasquale et al., 1996; Viemari et al., 2003). The brain stem and cervical spinal cords prepared from P0 pups were superfused with oxygenized (95% O2/5% CO2) artificial cerebrospinal fluid containing (in mM) 129 NaCl, 3.35 KCl, 1.26 CaCl2, 1.15 MgCl2, 21.0 NaHCO3, 0.58 NaH2PO4, and 30 glucose (pH 7.4). Burst activities recorded from the C4 ventral roots using suction electrodes (40–60 μm opening) were amplified (GeneClamp 500B, Axon), lowpass filtered at 1 kHz, and digitized at 2 kHz using a DigiData 1322A controlled by Clampex 9.2 software. Experiments were performed at ~28°C.

**Hippocampal Neuronal Culture**

Hippocampi were isolated from P0 pups following genotype identification. Neurons acutely dissociated with protease were used for patch-clamp recordings on the same day (Jackson et al., 2004). For long-term culture, hippocampi were dissected into cold Ca2+- and Mg2+-free Hank's or PBS buffer. Chopped small pieces were digested in solution containing 20 units/ml papain activated by 1 mM cysteine, for 20–30 min at 37°C. Neurons were dissociated by trituration with fire-polished glass pipettes and plated onto
polylysine-coated dishes at 4 × 10^5 cell/35 mm dish, or glia-preplated dishes at 2 × 10^5/35 mm dish. The growth medium was composed of 80% DMEM (with 4.5 g/L glucose, 25 mM HEPES, no glutamine), 10% Ham's F-12 (Cambrex), 10% bovine calf serum (iron supplemented, Hyclone) and 0.5× antibiotic-antimycotic (Invitrogen). Cultures were maintained in 37°C/5% CO₂ humidified incubator. When necessary, cytosine-arabinofuranoside (Sigma) was added at 6 μM to inhibit glial growth. Neurons were used between 7 days *in vitro* (DIV) and 18. At least 1 day before experiments, two-thirds of the medium was replaced with fresh medium without glia inhibitor and antibiotics.

**HEK293T Cell Culture and DNA Transfection**

HEK293T cells were grown in DMEM (GIBCO) medium supplemented with 10% FBS and 1% Glutamax at 37°C in a humidified atmosphere of 5% CO₂, 95% air. NALCN cDNA used for the transfection was constructed from a rat clone (Lee et al., 1999) in a pTracer-CMV based vector, with 44 bp from the Xenopus β-globin sequence inserted into the 5′UTR. Clones were sequenced to ensure that only the ones with correct sequence were used. A similar construct encoding the human NALCN (99% amino acid identity with the rat ortholog) was also used for some experiments. No functional difference was observed between the two orthologs. Site-directed mutagenesis on the rat form NALCN was used to generate the EEKA mutant (Figure 2.3). An EEEE mutant was also constructed, but this mutant did not generate measurable current. Lipofectamine 2000 (Invitrogen) was used as the transfection reagent for both the HEK293T cells and neurons. Neurons of ages of DIV 5–7 were used for transfection, and patch-clamp recordings were
performed 48–60 hr later. Transfected cells were identified by the green fluorescence protein encoded in the same vector.

**Patch-Clamp Analyses Using HEK293T Cells**

All experiments were carried out at room temperature (20–25°C). For transfected HEK293T cells, only cells with the highest expression level (~ top 5%) judged by intensity of the GFP marker were recorded. Whole-cell currents were recorded 48–72 hr after transfection. Unless otherwise stated, pipette solution contained the following (in mM): 150 Cs, 120 Mes, 10 NaCl, 10 EGTA, 2 Mg-ATP, and 10 HEPES (pH 7.4, Osm ~300 mOsm/L). Bath solutions contained 150 NaCl, 3.5 KCl, 2 MgCl₂, 1.2 CaCl₂, 10 HEPES, and 20 glucose (pH 7.4 with 5 mM NaOH, Osm ~320 mOsm/L). In the 0 Na⁺, 0 K⁺ bath, Na⁺ and K⁺ were replaced by Tris⁺ or NMDG⁺.

For selectivity measurement, the pipette solution contained 155 Cs, 120 Mes, 10 Cl, 10 EGTA, 2 Mg-ATP, and 20 HEPES (pH 7.4, Osm ~300 mOsm/L). Bath contained 155 Na, 150 Cl, 10 HEPES, and 20 glucose (pH 7.4, Osm = 320 mOsm/L). NaCl was isotonically replaced with KCl and CaCl₂ for the measurements of PNa/Pcs and PCa/Pcs, respectively. In the 10 Cl⁻-containing bath, Cl⁻ was replaced with Mes⁻. The following equation was used to calculate the relative permeabilities of Na⁺ and K⁺ to Cs⁺ (Goldman, 1943; Hille, 2001).

\[
P_{X} / P_{Cs} = [Cs]i \exp \left( \frac{E_{rev}F}{RT} \right) / [X]o,
\]

where Erev, F, R, and T are the reversal potential, Faraday constant, gas constant, and absolute temperature, respectively. The relative Ca²⁺ permeability was calculated as
$$P_{Ca}/P_{Cs} = \gamma_{Cs} [Cs]_i \exp\left(\frac{E_{rev} F}{RT}\right)[\exp\left(\frac{E_{rev} F}{RT}\right) + 1]/(4\gamma_{Ca}[Ca]_e)$$,

where $\gamma_{Cs} = 0.70$ and $\gamma_{Ca} = 0.331$ are the activity coefficients (Yue et al., 2002).

Because of the “leakiness” of NALCN, special attention was taken to ensure that the currents recorded were from NALCN channel instead of nonspecific leaks from cell damage or loose seals. In the end of recording, either cation ion replacement (with NMDG$^+$ or Tris$^+$) or blocker application was used to assure that only cells with a tight seal were used for analysis.

**Patch-Clamp Analysis Using Hippocampal Neurons**

All experiments were carried out at room temperature (20–25°C) with cultured hippocampal neurons, except that Ca$V$ currents were recorded using acutely dissociated neurons. Pyramidal neurons were morphologically identified. For Na$V$ current recording, pipette solution contained the following (in mM): 145 CsAsp, 5 NaCl, 5 KCl, 2 MgCl$_2$, 10 EGTA, and 10 HEPES (pH 7.3, 315 mOsm/L). Bath contained the following: 50 NaCl, 5 MgCl$_2$, 1 CaCl$_2$, 2 CoCl$_2$, 10 TEA-Cl, 20 glucose, 145 sucrose, and 10 HEPES (pH7.4, 330 mOsm/L). For Ca$V$ current recordings, pipette solution contained the following (in mM): 115 CsAsp, 5 MgCl$_2$, 10 EGTA, 20 HEPES, 4 Mg-ATP, 0.3 Tris-GTP, and 14 phosphocreatine (di-tris salt) (pH 7.4; 277 mOsm/L). Bath solution contained the following (in mM): 155 TEA-Cl, 10 BaCl$_2$, 3.5 KCl, 1 MgCl$_2$, 10 HEPES, 10 glucose, and 10 sucrose (pH 7.4, 311 mOsm/L).

For voltage clamp used in recording the background leak Na$^+$ currents (Raman et al., 2000; Simasko, 1994), pipette solution contained the following (in mM): 10 NaCl, 12 KCl,
59 K$_2$SO$_4$, 4 MgCl$_2$, 10 HEPES, 5 Tris-OH, 14 Tris-creatine phosphate, 4 Mg-ATP, and 0.3 Tris-GTP (pH 7.4, Osm ~300 mOsm/L). The 140 mM Na$^+$ bath contained the following (in mM): 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 6 glucose, 10 HEPES, 2 CsCl, and 1 μM TTX (pH 7.4 with Tris-OH, Osm ~315 mOsm/L). Tris-Cl was used to replace 126 mM NaCl in the 14 mM Na$^+$-containing bath. Because of the small sizes (~15 pA) of the leak Na$^+$ currents, special precaution was taken to ensure that the current was not a result of recording instability. After recording in a bath containing different [Na$^+$]$_e$, each recording was finished with the bath returning to the original Na$^+$ concentration. For non-transfected neurons, only those with a difference <5 pA between holding currents in the first and the last baths were used for further analysis. For the transfected ones, which had larger ΔI$_{L-Na}$, baseline fluctuation of 20% of ΔI$_{L-Na}$ was allowed in cell selection. Initial experiments comparing ΔI$_{L-Na}$ in the WT and mutant neurons were done in a blinded fashion, in which the experimentalist did not know the genotype of the cells during the experiments.

For current-clamp recordings, pipette solution contained the following (in mM): 135 K-Asp, 5 NaCl, 5 KCl, 2 MgCl$_2$, 1 EGTA, 10 HEPES, 4 Mg-ATP, 0.3 Tris-GTP, and 14 phosphocreatine (di-tris) (pH adjusted to 7.2 with KOH, total K$^+$ about 147 mM). Bath solution contained the following (in mM): 150 NaCl, 3.5 KCl, 2 MgCl$_2$, 1.2 CaCl$_2$, 10 HEPES, and 20 glucose (pH 7.4 with 5 NaOH, Osm ~320 mOsm/L). Neurons were isolated with APV (10 μM), bicuculline (20 μM), and CNQX (20 μM). Currents (WT, +1.6 ± 0.9 pA, n = 18; mutant, +9.2 ± 3.8 pA, n = 15) were injected to elicit firing (Figures 2.7C and 2.7D). Some of the neurons cultured from both the WT and mutant showed spontaneous firing at 0 holding current.
Patch-clamp recordings were performed with an Axopatch-200A amplifier controlled by pClamp 9.2 software (Axon). Signals were filtered at 5 KHz and digitized at 10–20 kHz with a Digidata 1322A digitizer. Liquid junction potentials ≥5 mV (calculated with pClamp 9.2) were corrected.

**Results**

**Expression of NALCN in Central Nervous System Neurons**

Northern blot analysis suggested that NALCN mRNA is expressed in all brain regions (Lee et al., 1999). To determine which types of cells express the gene, we carried out in situ hybridization of mouse brain slices with DIG-labeled RNA probes derived from its 1.8 kb cDNA (carboxyl terminus). In the cerebral cortex (Figure 2.1B) and hippocampus (Figure 2.1C), an antisense RNA probe detected NALCN mRNA expression in all layers and in essentially all the neurons. Sense RNA probe was minimally detected, demonstrating specificity of staining. Similarly, NALCN mRNA was detected in all neurons of the spinal cord dorsal and ventral horns (data not shown). Using northern blot and in situ hybridization, we did not detect NALCN expression in liver, muscle, lung, kidney, or testis (data not shown, see also (Lee et al., 1999)). These experiments suggest that NALCN is likely a widely expressed neuronal ion channel.
Figure 2.1

A

S4 region

I
MALCN LRIPFVMRIAFRIFTPF HSQFTTFQVLAVVRLIKIS
CaV1.1 VPKVAFPVMRLLEWGW ISVLRCETRLLAFRTW
NaV1.1 YSALATPVMALMTISVI LSVLRFSPFLAVFRKLS

II
MALCN AQLIMVLACLRPLRTFLY YKMGAVCPVIFTPFSSCPR
CaV1.1 VRKLYLVLVLRPLRAISA SAPPFLFMFRLLLKLSN
NaV1.1 IESLRTRLVPLRALFHF PRPLYAROLRLKGG

Pore region

I
MALCN SCRQGWVF TQAGWVD SLLGQVE TQDWNK
CaV1.1 TNNGWTD TQGWTTS TQQGWDF TQGWPK
CaV2.1 TNNGWTD TQGWTTS TQQGWDF TQGWPK
CaV3.1 TNNGWVD TQGWNK SLLGWVD TQDWNG
NaV1.1 TQQEWEN LQGWNIE TPQGWMD TSGWWD

B
RNA expression in cortex

antisense    sense control

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wm

C
RNA expression in hippocampus

antisense    sense control

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wm
Figure 2.1. NALCN Is Widely Expressed in the Central Nervous System

(A) Alignment of the S4 and pore regions between NALCN, representative CaV and NaV channels. Schematic drawing illustrates that the CaV and NaV pore-forming subunits (α1) are composed of four homologous repeats (I, II, III, IV). The EEEE (DEKA) motifs in Ca2+ (Na+) channel pore regions are highlighted. A key aromatic residue (F) in NaV1.1 important for NaV’s TTX sensitivity is underlined.

(B and C) In situ staining with slices from cerebral cortex (B) and hippocampus (C). The gray matter (I-VI) and white matter (wm) layers in the cortex are indicated (B). Scale bar, 100 μm in (B) and 250 μm in (C). Sense RNA probes (right panels) are used as negative control for signal specificity.
NALCN Is a Voltage-Independent Ion Channel

Attempts to functionally reconstitute NALCN as an ion channel in heterologous systems have been unsuccessful (Lear et al., 2005; Lee et al., 1999; Yu and Catterall, 2004). To determine if NALCN indeed encodes an ion channel, we transfected NALCN-encoding cDNA into HEK293T cells and recorded whole-cell current 48–72 hr after transfection (Figure 2.2A). In cells expressing the highest amount of NALCN (~top 5%; judged by the levels of green fluorescent protein (GFP) expression from the pTracer vector containing both GFP and NALCN under separate promoters), >80% (19 of 22) of NALCN-transfected cells appeared “leaky” immediately after break in, had input resistance below 1 GΩ, and displayed a current with a linear current-voltage (I-V) relationship (INALCN, −22 to −1691 pA; −573 ± 124 pA, at −80 mV; n = 22) that was largely absent in mock-transfected (−10 ± 3 pA, n = 15) and nontransfected control cells (Figure 2.2B). Since currents with a linear I-V curve (Figure 2.2B) could also be generated by nonspecific leaks from cell damage or through poor seals, we used ion replacement to ensure that the currents analyzed were from ion-selective conductances. When extracellular Na⁺ and K⁺ were replaced with larger ions Tris⁺ or NMDG⁺, the inward currents were largely abolished and the I-V curves became outwardly rectifying (Figure 2.2C). The input resistance (Rm) under this condition became >1 GΩ (at −80 mV). INALCN could also be suppressed by channel blockers (see below).
Figure 2.2. NALCN Forms a Voltage-Independent Ion Channel

(A) Expression of myc-tagged NALCN protein (detected using Western blot analysis with anti-myc antibody).

(B) Representative current traces elicited by step voltage protocols (upper left) from mock- (lower left) and NALCN- (middle) transfected cells. (Right) Averaged current-voltage (I-V) relationship showing voltage-independent activation of NALCN (n = 22).

(C) Currents elicited by a voltage ramp protocol (from -100 mV to +100 mV in one second, upper left) in bath with (155 Na, 3.5 K) or without (0 Na, 0 K) K⁺ and Na⁺. Representative recordings from one mock transfected (left two panels) and one NALCN transfected (right two panels) are shown.

(D) Lack of inactivation of INALCN. (Upper left) Representative current traces. (Lower left) Protocol, (right) normalized currents at -80 mV following prepulses of various voltages. Dotted lines indicate zero current level. Data are represented as mean ± SEM. All experiments were performed using HEK293T cells except that COS-7 cells were used for the protein work in (A).
Unlike the ten Ca\textsubscript{v} and nine Na\textsubscript{v} members in the 4x6TM channel family, NALCN's activation is voltage independent (Figure 2.2B). No significant voltage-dependent inactivation was observed (Figures 2.2C and 2.2D). The lack of voltage dependence of activation and inactivation is likely a result of the absence of some of the charged residues conserved in the S4 domains of voltage-gated channels (Figure 2.1A) (Bezanilla, 2005; Horn, 2002).

**NALCN Is Nonselective, Permeable to Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+}**

Replacement of >90\% of Cl\textsuperscript{−} in the bath by methanesulfonate (MES) did not result in a significant change in the size or reversal potential of the current (Figure 2.3A), indicating that NALCN was impermeable to anion and that the current was carried by cations. In contrast, changing [Na\textsuperscript{+}]\textsubscript{e} from 155 to 15.5 mM reduced the amplitude of inward current and shifted the reversal potential, suggesting Na\textsuperscript{+} as a permeant ion (Figure 2.3B). NALCN is also clearly permeable to K\textsuperscript{+} and Ca\textsuperscript{2+} (Figure 2.3B). We used reversal potentials of I\textsubscript{NALCN} under various bi-ionic conditions to estimate the ion selectivity of the channel. The selectivity sequence was P\textsubscript{Na} (1.3, n = 12) > P\textsubscript{K} (1.2, n = 5) > P\textsubscript{Cs} (1.0, n = 12) > P\textsubscript{Ca} (0.5, n = 7). Thus, NALCN is a nonselective cation channel, unlike any of the other 20 members in the 4x6TM channel family, which are highly selective for either Na\textsuperscript{+} (Na\textsubscript{v}1-9 and Na\textsubscript{x}) or Ca\textsuperscript{2+} (Ca\textsubscript{v}1.1-1.4, Ca\textsubscript{v}2.1-2.3, and Ca\textsubscript{v}3.1-3.3) (Yu et al., 2005). The channel's unique ion selectivity is likely the result of its unusual sequences of amino acids associated with the putative channel pore (Figure 2.1A), as suggested by the extensive mutagenesis studies carried out on the EEEE motif in Ca\textsubscript{v}s and DEKA in Na\textsubscript{v}s (Heinemann et al., 1992; Kim et al., 1993; Sather and McCleskey, 2003; Tang et al., 1993;
In support of the importance of the EEKE motif in NALCN's ion selectivity, a mutant NALCN with an EEKA motif had a largely decreased Ca$^{2+}$ permeability; \( \frac{P_{\text{Na}}}{P_{\text{K}}} = \frac{P_{\text{K}}}{P_{\text{Cs}}} > \frac{P_{\text{Cs}}}{P_{\text{Ca}}} \) (Figure 2.3C). The involvement of amino acids surrounding the EEKE locus (Figure 2.1A) in ion selectivity may explain why the EEKA mutant is not as Na$^+$ selective as NaV channels (DEKA motif).

\( I_{\text{NALCN}} \) was not blocked by the NaV channel blocker TTX (up to 10 \( \mu \)M) (Figures 2.4A and 2.4C) but could be partially blocked by several CaV blockers with low affinity (apparent \( IC_{50}s \), 0.15 mM for Cd$^{2+}$, 0.26 mM for Co$^{2+}$, and 0.38 mM for verapamil) (Figure 2.4B). The trivalent ion Gd$^{3+}$, which is often considered a high-affinity blocker for stretch-activated channels (Yang and Sachs, 1989), effectively blocked \( I_{\text{NALCN}} \) with an apparent \( IC_{50} \) of 1.4 \( \mu \)M (Figure 2.4). The overall properties of \( I_{\text{NALCN}} \) (including the I-V relationship, activation, inactivation, \( \frac{P_{\text{Ca}}}{P_{\text{Na}}} \), and pharmacology) are unlike those of the majority of TRP cation channels, making it unlikely that the currents we recorded were through nonspecific leaks or through other proteins such as TRPs that could have been upregulated by the NALCN cDNA transfection.
Figure 2.3

A

B

C

ELEK Mutant

159.9 Cl

10 Cl

0.1 s

500 pA

+80 mV

0 mV

-80 mV

155 Na

15.5 Na

155 K

15.5 K

100 Ca

10 Ca

155 Na

15.5 Na

155 K

15.5 K

100 Ca

10 Ca

0 pA

0 pA

0 pA

0 pA

1 s

200 pA

0 pA

0 pA

0 pA

0 pA

0 pA

0 pA
Figure 2.3. NALCN Is a Nonselective Cation Channel

(A) Representative currents recorded in bath with high (159.9 mM, left) and low (10 mM, right) [Cl\(^-\)] using a step protocol (-80 mV to 80 mV in steps of 20 mV; \(V_h = 0\) mV; upper panel).

(B) Currents from a representative NALCN-transfected cell in bi-ionic conditions with extracellular cation concentrations as indicated. NMDG was used to partially replace the cation in low-cation concentration baths (15.5 Na, 15.5 K or 10 Ca). A ramp protocol (\(V_h = 0\) mV, from -100 mV to 100 mV in 1 s; upper left) was used.

(C) Similar to (B) but from a cell transfected with an EEKA mutant NALCN channel with E1389 (in repeat IV, Figure 2.1A) mutated to A using site-directed mutagenesis.
Figure 2.4. Sensitivities of $I_{NALCN}$ to Divalent Ions, Trivalent Ions, and $Na_V$ and $Ca_V$ Blockers

(A) Representative sensitivities of $I_{NALCN}$ to blockade by TTX, verapamil, Cd$^{2+}$, and Gd$^{3+}$. A step protocol (-40 mV to 40 mV in step of 20 mV; 500 ms; $V_h = 0$ mV; upper) was used.

(B) Inhibition curves showing apparent IC$_{50}$s. The curves were reconstructed using the current sizes at -80 mV obtained with a ramp protocol (n = 5).

(C) Percentage of inhibitions of the current by various blockers of indicated concentrations. Numbers of cells tested are in parentheses. Data are represented as mean ± SEM.
Neonatal Lethality and Respiratory Rhythm Defects Caused by Targeted Disruption of the NALCN Gene

To uncover the in vivo function of NALCN, we generated mice with NALCN's exon 1 deleted (Figure 2.5A). Semiquantitative RT-PCR assays show that the homozygous mutant (E18.5 embryos) did not express the wild-type (WT) NALCN mRNA; heterozygotes expressed reduced amounts in brain (Figure 2.5A). Mice were born in a roughly Mendelian ratio (22% −/−, 27% +/+ and 51% +/−; from 1043 pups in 127 litters, tail clips used for genotyping collected within ~10 hr of birth), suggesting that the channel gene is not required for embryonic viability. We did not observe gross differences in embryonic development, righting responses, spontaneous limb movement, and toe/tail pinch responses between WT and mutant newborn pups. The homozygous mutants appeared normal up to 12 hr after birth (Figure 2.5A). No mutant, however, survived beyond 24 hr after birth (data from 52 mutant pups in 28 litters). Thus, NALCN is one of the few channels in the 4x6TM channel family required for neonatal survival.

A clear distinction between null and WT mouse breathing patterns was apparent. Null mice respiration was sporadic, with apnea for a few seconds before beginning a burst of deep breathing lasting 5–10 s (data from >20 pairs). We measured the respiratory patterns using a whole-body plethysmograph, which detected the pressure changes generated by the breathing activities of the mice. The WT and mutant pups were subjected to recording a few hours after they were born, when they appeared otherwise indistinguishable. WT mice breathed in a well-defined rhythm (Figure 2.5B). Null mice breathing was characterized by apnea for ~5 s followed by a burst of breathing of ~5 s, at a rate of ~5 apnea events/minute (Figures 2.5C and 2.5D). Intriguingly, this pattern is
reminiscent of the periodic breathing of Cheyne-Stokes respiration found in humans with
CNS damage (Strohl, 2003).

Respiratory rhythms are controlled by the central nervous system. Given the
expression of the NALCN channel in CNS neurons but absence in muscle, and the ability
of the mutant pups to breathe, we suspected that the disrupted respiratory patterns in the
mutant was due to a defect in neuronal control. We recorded the electrical activities from
the fourth cervical nerve root (C4) that innervates the diaphragm and discharges rhythmic
electrical signals that regulate breathing. The C4 nerves in isolated brain stem spinal cords
from newborn pups (P0) have robust rhythmic electrical activities in WT mice (Figures
2.5E and 2.5G). In null mice, however, such electrical activities were largely absent
(Figures 2.5F and 2.5G). These recordings support the hypothesis that severe defects in
the respiratory rhythm in the null mice were due to defects in electrical signaling in the
nervous system.
Figure 2.5

A

exon 1

1 kb

exon 2

NALCN

HPRT

B

Wilder-type

piethysmograph

C

Mutant

nerven recording

D

Wild-type

Mutant

(n = 7)

(n = 4)

E

wild-type

F

Mutant

(n = 9)

(n = 7)
Figure 2.5. NALCN Is Required for Normal Respiratory Rhythm and Neonatal Survival

(A) Targeted disruption of NALCN.  (Left) Partial genomic structure in the region containing exons 1 and 2.  Connected arrows mark the deletion in the mutant.  (Middle) Semiquantitive RT-PCR using brain RNAs from E18.5 embryos with various genotypes. NALCN primers amplify 479 bp.  A primer pair amplifying a housekeeping gene (HPRT) was also included as internal control.  RT reaction without reverse transcriptase was used as template in the PCR negative control (‘ctrl’ lane).  (Right) Representative appearances of newborn WT (+/+) and mutant (-/-) pups.

(B–G) Respiratory rhythm defects in the mutant, as measured by plethysmograph (B–D) and C4 nerve root recordings from brain stem spinal cord preparations (E–G).  Plethysmograph detected regular rhythmic respiratory activities in the WT P0 pups (B), but the rhythm was disrupted in the mutant (C).  The mutant pups displayed periodic apnea (indicated by the arrow in [C]; defined as the absence of inspiration for longer than 3 s) (D).  C4 nerve root recordings detected rhythmic electrical activities in the WT (E), but the burst activities were nearly absent in the mutant (F and G).  Scale bars, 5 ml for (B) and (C); 50 mV for (E) and (F).  Data are represented as the mean ± SEM.
NALCN Encodes the Cs⁺- and TTX-Resistant Background Na⁺ Conductance in Neurons

To determine the native channel that NALCN encodes, we measured currents in hippocampal neurons, which express NALCN mRNA and are among the best electrically characterized neurons (Figure 2.1C). Using a slow ramp protocol, the TTX-sensitive, persistent Na⁺ current was observed in both WT and mutant mice (data not shown) (Raman and Bean, 1997). In addition, we used step protocols to record the NaV and CaV currents. The NaV current density in the mutant was not significantly different from the WT (WT, −26.0 ± 7.1 pA/pF, n = 10; mutant, −25.3 ± 7.0 pA/pF, n = 10; at −17 mV). We next examined the CaV currents using Ba²⁺ as the charge carrier. Ba²⁺ currents elicited in the mutant and WT neurons were comparable (WT, −35.1 ± 6.0 pA/pF, n = 5; mutant, −25.7 ± 8.2 pA/pF, n = 5; at −10 mV). Consistent with this observation, depolarization with 40 mM [K⁺]ᵅ readily evoked [Ca²⁺]ᵅ increases in both WT and mutant neurons when assayed with Fura-2 Ca²⁺ imaging (data not shown). Thus, the disruption in NALCN likely did not lead to nonspecific effects on NaV or CaV channels.

NALCN expressed in HEK293T cells has a linear I-V relationship, is permeable to Na⁺, and is constitutively open (Figure 2.2 and Figure 2.3). These properties fit reasonably well with those of I_L-Na (Raman et al., 2000; Simasko, 1994). To compare the Na⁺ leak currents in hippocampal neurons cultured from WT and the mutant, we used extracellular baths containing TTX (1 μM) and Cs⁺ (2 mM) to block the NaV and Ih currents, respectively. The small I_L-Na leak current was further isolated by subtracting the currents recorded in low (14 mM) [Na⁺]ᵅ from those in high (140 mM) [Na⁺]ᵅ at holding potentials (ΔI_L-Na at −68 mV) (Raman et al., 2000; Simasko, 1994) (Figure 2.6A) (see the
Materials and Methods). In WT neurons, 97% (29 of 30) had $\Delta I_{\text{L-Na}}$ larger than 5 pA ($-13.2 \pm 0.8$ pA at $-68$ mV, $n = 30$). In contrast, none of the 15 mutant neurons measured had $\Delta I_{\text{L-Na}} > 5$ pA ($-0.2 \pm 0.7$ pA) within the resolution limit of our measurement (Figures 2.6A and 2.6B). Transfection of NALCN to the mutant neurons restored and enhanced the $\text{Na}^+$ current ($\Delta I_{\text{L-Na}}$, $-72.4 \pm 14.4$ pA, $n = 12$) (Figures 2.6A and 2.6B), while transfection of an empty vector failed to do so (Figure 2.6B). Taken together, these data suggest that the TTX- and $\text{Cs}^+$-insensitive background $\text{Na}^+$ leak current $I_{\text{L-Na}}$ is likely carried by the nonselective NALCN-containing channel.

Hypothetically, $I_{\text{L-Na}}$ would balance background $\text{K}^+$ currents to maintain the RMP and regulate neuronal excitability. Consistent with this notion, the mutant neurons were hyperpolarized (RMP, $-71.4 \pm 3.2$ mV, $n = 14$) compared with WT ($-61.3 \pm 1.2$ mV, $n = 24$) (Figure 2.6C). Furthermore, mutant neurons were markedly depolarized when transfected with NALCN cDNA ($-36.5 \pm 3.7$ mV, $n = 15$), but not with empty vector ($-67.9 \pm 3.0$ mV, $n = 5$) (Figure 2.6C).
Figure 2.6

A. Holding current

-+/+

-\( \Delta I_{Na} \)

-/-

-/-: NALCN

B. \( \Delta I_{Na} \) (pA)

\( \pm \)

(15)

(5)

(30)

(12)

C. Resting membrane potential

-/+/

-/-

-/-: NALCN

-/-: mock

D. \( V_m \) (mV)

\( \pm \)

(24)

(10)

(17)

(9)

(14)

(5)

(5)
Figure 2.6. NALCN Encodes the Cs⁺- and TTX-Insensitive Sodium Leak Current

**I\textsubscript{L-Na} in Hippocampal Neurons**

All recordings were done in the presence of TTX (1 μM) and Cs⁺ (2 mM).

(A) Representative holding currents (at -68 mV) in baths containing 140 mM (indicated by hatched bars) or 14 mM Na⁺ (open bars) from a WT (+/+) neuron, a mutant (-/-), and a mutant transfected with NALCN cDNA (-/-; NALCN). Δ\text{I\textsubscript{L-Na}} (indicated by double-headed arrow) was calculated as the difference recorded between the 140 mM and 14 mM Na⁺-containing baths. Note different scale for the transfected neuron. Recording of 0.25 s is shown for each condition.

(B) Summary of Δ\text{I\textsubscript{L-Na}}. Numbers of cells tested are given in parentheses.

(C) Averaged RMPs (measured with current clamp).

(D) Changes in membrane potential in the WT and mutant neurons in response to [Na⁺]\textsubscript{e} change from 140 mM to 14 mM. Each connected line represents an individual neuron. Several lines in the WT (+/+ ) overlap.

(E) Summary of membrane potential changes upon the [Na⁺]\textsubscript{e} drop. Data are represented as mean ± SEM.
To test if the NALCN-encoded channel contributes to the sensitivity of the neuron's membrane potential to extracellular \([\text{Na}^+]\), we monitored MPs while \([\text{Na}^+]_e\) was reduced. A drop of \([\text{Na}^+]_e\) from 140 mM to 14 mM led to a hyperpolarization (\(\Delta V_m, -13.7 \pm 1.6 \text{ mV}, n = 17\)) in the WT neurons. In contrast, such effect of a 10-fold \([\text{Na}^+]_e\) drop was largely diminished in the mutant (\(\Delta V_m, -3.1 \pm 1.5 \text{ mV}, n = 10\)) (Figures 2.6D and 2.6E). Transfection of NALCN cDNA to the mutant neurons restored and enhanced the sensitivity (\(\Delta V_m, -32.0 \pm 2.9 \text{ mV}, n = 9\)), while transfection with an empty vector did not have a similar effect (\(\Delta V_m, 0.0 \pm 0.8 \text{ mV}, n = 5\)) (Figures 2.6D and 2.6E).

**NALCN Regulates Neuronal Excitability**

In addition to the NALCN channel carrying the \(\text{Cs}^+\)- and TTX-insensitive \(\text{Na}^+\) leak (Figure 2.6B), \(\text{Na}_V\) (activated by depolarization, blocked by TTX) and \(I_h\) (activated by hyperpolarization, blocked by \(\text{Cs}^+\)) may be partially active and may serve as two additional paths for the total \(\text{Na}^+\) leak at RMPs in mammalian neurons (\textit{C. elegans} has two NALCN genes but no genes encoding \(\text{Na}_V\) or \(I_h\)). To determine the relative contribution of each of the three components, we measured \(\Delta I_{\text{L-Na}}\) in the same neuron in the absence of TTX and \(\text{Cs}^+\), in the presence of \(\text{Cs}^+\), and in the presence of both TTX and \(\text{Cs}^+\). The relative contributions of \(I_{\text{NALCN}}\), \(I_h\) and \(\text{Na}_V\) to the total background \(\text{Na}^+\) current were estimated to be 72.1\% \pm 4.1\%, 9.6\% \pm 11.5\%, and 18.3\% \pm 10.9\% \((n = 5)\), respectively (Figure 2.7A). These data suggest NALCN channel as the dominant path for the \(\text{Na}^+\) leak in the hippocampal neurons. Consistent with the major contribution of NALCN, mutant neurons' MPs are relatively insensitive to the change of \([\text{Na}^+]_e\), even in the absence of \(\text{Cs}^+\) and TTX (Figure 2.7B).
Finally, we tested if NALCN contributes to neuronal excitability. In WT neurons, the frequency of continuous firing could be significantly reduced by an application of 10 μM Gd$^{3+}$ (77.1% ± 5.3%, n = 7) (Figure 2.7C, upper panel; Figure 2.7D). Consistent with the idea that the frequency reduction was due to a blockade of the $I_{\text{NALCN}}$ leak current, injection of a depolarizing current restored the firing frequency. This blockade effect of 10 μM Gd$^{3+}$ was largely absent in the mutant neurons (10.6% ± 5.8%, n = 8) (Figure 2.7C, lower panel; Figure 2.7D). Similarly, application of another NALCN blocker, verapamil (0.5 mM), reduced the firing frequency in the WT neurons (84.4% ± 4.4%, n = 11) but had less effect in the mutant (22.2% ± 11.9%, n = 7) (Figure 2.7D). The residual effect of verapamil in the mutant was likely due to its action on other targets such as Ca$\text{v}$ channels.
Figure 2.7

A

Normalized current (%)

C

B

Vm (mV)

140 Na 14 Na 140 Na 14 Na

ΔVm (mV)

(13)

(18)

C

10 μM Gd³⁺

injection = 0 pA

injection = +50 pA

D

Inhibition (%)

Gd²⁺ Verapamil

(7) (11)

(8) (7)
Figure 2.7. NALCN Is a Major Determinant of the Total Background Na⁺ Currents and Controls Neuronal Excitability.

Recordings were performed in cultured hippocampal neurons.

(A) Background Na⁺ currents (ΔI_{L-Na} at -68 mV; WT neurons) in baths without Cs⁺ and TTX, with Cs⁺ alone (2 mM), or with both Cs⁺ and TTX (1 μM). The currents were normalized to that measured in baths without Cs⁺ and TTX. Note that the TTX- and Cs-insensitive component (72% of total) was abolished in the NALCN mutant (Figure 2.5).

(B) Changes in membrane potential in the WT and mutant neurons in response to [Na⁺]_{e} change, in the absence of Cs⁺ and TTX. Right panel gives the averaged changes.

(C) Representative effects on action potential firing frequency of 10 μM Gd³⁺ in a WT (upper, no current injection) and mutant (lower, continuous firing elicited by a +50 pA current injection) neuron. In the WT, the blockade could be ‘‘rescued’’ by injecting a depolarizing current (+5 pA). Note a hyperpolarization of baseline membrane potential by Gd³⁺ in the WT, but not in the mutant.

(D) Summary of percentage reductions of firing frequency in the WT and mutant by Gd³⁺ (10 μM) or verapamil (0.5 mM). Data are represented as mean ± SEM.
Conclusions and Discussion

Unlike any of the other 20 members in the 4x6TM channel family, NALCN forms a voltage-independent, nonselective, noninactivating cation channel. The Cs\(^+\) and TTX-insensitive background Na\(^+\) leak current is absent in NALCN mutant neurons and can be restored by transient expression of NALCN using cDNA transfection. The sensitivity of the RMP to [Na\(^+\)]\(_{e}\) changes is largely diminished in the mutant. Finally, blockers that suppress \(I_{\text{NALCN}}\) had little effect on the mutant neurons' excitability. The simplest interpretation of our work is that NALCN encodes a cation-nonselective, voltage-insensitive channel that is responsible for the background Na\(^+\) leak current (\(I_{\text{L-\text{Na}}}\)) in neurons and is indispensable for neonatal survival. The 21 mammalian members in the four-repeat (4x6TM) ion channel family can now be functionally divided into three subfamilies of Ca\(^{2+}\) channels (ten members), Na\(^+\) channels (ten members), and nonselective cation channel (one member).

In the NALCN-deficient mice, spinal cord nerves (C4) lack the rhythmic electrical discharge that affects respiratory behavior. Invertebrates do not have analogous spinal cord nerves, but the defects in locomotive behavioral circadian rhythms in the Drosophila mutant with reduced NALCN expression point to the possibility that a common function of the channel is in neuronal rhythm generation and/or coordination (Lear et al., 2005; Nash et al., 2002; Robinson and Siegelbaum, 2003). Alternatively, as the channel is widely expressed in the nervous systems, a global reduction of excitability in the mutant may lead to the disruption in respiration (Feldman et al., 2003). The exact causes of the neonatal fatality need to be further investigated. In addition to the defect in behavioral circadian rhythms, the Drosophila mutant also displays other phenotypes, including narrow
abdomen, locomotion deficiency and altered sensitivity to anesthetics (Humphrey et al., 2007; Jospin et al., 2007; Krishnan and Nash, 1990; Mir et al., 1997; van Swinderen, 2006; Yeh et al., 2008). The complexity of the phenotypes suggests additional physiological roles of the NALCN channel. A conditional knockout mouse model will be able to address the roles of NALCN in adults. In *C. elegans*, the action potential is clearly Na\(^+\) dependent (Franks et al., 2002). In contrast to mammals, the worm genome does not appear to contain genes encoding Na\(_V\) or I\(_h\) (Bargmann, 1998; Robinson and Siegelbaum, 2003), but has two NALCN homologs that could potentially serve as the background leak Na\(^+\) conductances.

Two major background cation leak conductances operate to oppose each other in establishing the RMP in neurons, with the Na\(^+\) leak conductance being normally a fraction (~4% in the squid giant axon) of that of the K\(^+\) conductance (Hodgkin and Katz, 1949). K\(^+\) leak current is likely contributed by many channels, including the 15 K\(^+\) channels in the K2P family (Goldstein et al., 2005). In support of this redundancy, none of the two K2P channel knockouts (KCNK2 and KCNK4) was shown to lead to defects in neuronal excitability or animal survival. Nor was a reduction in background K\(^+\) leak current reported, although the channels overexpressed in heterologous systems clearly showed properties of background K\(^+\) leak conductances (Heurteaux et al., 2004).

Sources of the Na\(^+\) leak conductance have been speculated to be via Na\(_V\) channels (Chen and Lucero, 1999; Nicholls and Baylor, 1968), nonselective channels (Raman et al., 2000), Na\(^+\) leak through K\(^+\) channels (Heginbotham et al., 1994; Shrivastava et al., 2002), and transporters (Jacob et al., 1987). Our data suggest that the molecular mechanism for the Na\(^+\) leak is surprisingly simple. In the NALCN mutant neurons, the Na\(^+\) leak is
undetectable in the presence of TTX and Cs+. These results suggest that the Na+ leak conductance consists of three components: a Cs+-sensitive (possibly from I_h), a TTX-sensitive (likely from Na_v), and a NALCN-dependent component, with NALCN providing the majority of leak current. In animals without voltage-activated I_h and Na_v, NALCN is likely the sole source for the major Na+ conductance. Other candidates for leak currents are some members of the 28 mammalian TRP channels that are involved in sensory transduction and activated by receptors and ligands (Ramsey et al., 2006). Most of the TRP currents are nonlinear and reverse at potentials above 0 mV. None of the ten reported TRP subtype knockouts has been reported to lead to defect in the Na+ leak or neuronal excitability (Nilius et al., 2005). Nevertheless, we cannot exclude the possibility that conductances beyond our detection limit and/or channels that are downregulated in the NALCN mutant but restored and enhanced upon NALCN transient expression also contribute to the leak current.

In summary, NALCN is widely expressed in the brain and spinal cord neurons and forms a major background Na+ conductance. NALCN channels are nonselective, voltage independent, and do not inactivate. As such, they are ideal for setting levels of neuronal excitability and thus controlling firing rates. Interesting questions for the future are whether their expression or activity can be altered by signal transduction events or neuronal plasticity or are targets for therapeutics.

**Others’ Contribution**

This chapter was modified from a research article originally published as “The neuronal channel NALCN contributes resting sodium permeability and is required
for normal respiratory rhythm” in *Cell* (Lu et al., 2007). Dr. Dejian Ren generated the mouse line, initiated the project, did the molecular biology work and characterized the phenotype of *NALCN*<sup>−/−</sup> mice (Figure 2.1 and 2.5A-D). Dr. Yanhua Su started the neuronal culture and did the C4 nerve root recording (Figure 2.5E - G).
CHAPTER III
PEPTIDE NEUROTRANSMITTERS ACTIVATE A CATION CHANNEL COMPLEX OF NALCN AND UNC80

Summary

Several neurotransmitters act through G-protein coupled receptors (GPCR) to evoke a “slow” excitation of neurons (Baskys, 1992; Iversen, 1984; Salio et al., 2006). These include peptides, such as substance P (SP) and neurotensin (NT), as well as acetylcholine and noradrenaline. Unlike the fast (approximately millisecond) ionotropic actions of small-molecule neurotransmitters, the slow excitation is poorly understood at the molecular level, but can be mainly attributed to suppressing K⁺ currents and/or activating a non-selective cation channel (Farkas et al., 1996; Jan et al., 1980; Kuba and Koketsu, 1978; Kuffler and Sejnowski, 1983; Shen and North, 1992a; Shen and Surprenant, 1993; Stanfield et al., 1985). The molecular identity of this cation channel has yet to be determined; similarly, how the channel is activated and its relative contribution to neuronal excitability induced by the neuropeptides are unknown. Here we show that, in the mouse hippocampal and ventral tegmental area neurons, SP and NT activate a channel complex containing NALCN and a large previously unknown protein UNC80. The activation by SP through TACR1 (a GPCR for SP) occurs by means of a unique mechanism: it does not require G-protein activation but is dependent on Src family kinases (SFKs). These findings identify NALCN as the cation channel activated by SP receptor, and suggest that UNC80 and SFKs, rather than a G-protein, are involved
in the coupling from receptor to channel.

**Introduction**

Neuropeptides form the largest and most diverse group of neuronal signaling molecules found in the nervous system (Wotjak et al., 2008). Many of these small peptides, such as substance P (SP), neurotensin (NT), orexin, dynorphin, and angiotensin, are important chemical messengers used by the nervous system for communication and to influence a wide spectrum of physiological functions ranging from respiration, pain detection, nutrient ingestion to cognition (Otsuka and Yoshioka, 1993). In contrast to more classical neurotransmitters, such as acetylcholine and glutamate, the molecular mechanisms underlying how a large number of neuropeptides influence neuronal function is largely unclear.

SP is an 11 amino acid peptide that was identified as the first peptide neurotransmitter ~ 80 years ago (Otsuka and Yoshioka, 1993). Like most neuropeptides, SP binds to G-protein coupled receptors (GPCRs). Both SP and its neurokinin receptors (particularly TACR1) are widely expressed in brain and spinal cord and have broad effects on nervous system function (Cao et al., 1998; DeFelipe et al., 1998; George et al., 2008; Murtra et al., 2000; Otsuka and Yoshioka, 1993; Pena and Ramirez, 2004). SP “slowly” enhances neuronal excitation and can do so by suppressing K⁺ currents, modulating voltage-gated Ca²⁺ (Caᵥ) channels, and activating a non-selective cation channel (Bley and Tsien, 1990; Jones, 1985; Murase et al., 1989; Shapiro and Hille, 1993; Stanfield et al., 1985). The molecular identity of this cation channel has yet to be determined; similarly how the channel is activated is unknown.
One likely candidate for the cation channel affected by neuropeptides is NALCN. NALCN is a unique member of the 24 transmembrane-spanning ion channel family that includes voltage-gated Na⁺ (Nav) and voltage-gated Ca²⁺ (Cav) channels (Lu et al., 2007). It differs from Cav and Nav channels in two major features. First, NALCN channels have fewer charged residues in the voltage-sensing S4 helices typically found in voltage-gated ion channels, suggesting that their activation may have a unique voltage-dependence or independence. Second, NALCN has an EEKE motif in its ion selective filter regions, a mixture of those of CavS (EEEE) and Navs (DEKA) (Lu et al., 2007). When overexpressed in HEK293T fibroblast cells, NALCN generates a Na⁺-permeable cation channel that is tetrodotoxin (TTX)-resistant, non-inactivating, Gd³⁺-blockable, and has a linear current (I)-voltage (V) relationship (Lu et al., 2007). NALCN knockout mice have severely disrupted respiratory rhythms and die within 24 hours of birth. The mutant neurons lack a TTX-resistant Na⁺-leak current. These biophysical and pharmacological properties of NALCN closely resemble those of SP-activated cation channel currents (I_sp) studied in several brain regions (Aosaki and Kawaguchi, 1996; Inoue et al., 1995; Lu et al., 2007; Otsuka and Yoshioka, 1993; Pena and Ramirez, 2004; Shen and North, 1992b), suggesting that I_sp might be carried by NALCN.

Materials and Methods

Cloning of mUNC80 and Antibody Generation

The mouse mUNC80 was cloned from single-stranded brain cDNA as four fragments using PCR with reverse transcription (RT–PCR) with primers designed from
partial sequences in NCBI databases. Multiple clones were sequenced from each fragment and clones without mutation were used to assemble the full-length sequence in vector pcDNA3.1(+). The start of the ORF was unambiguously identified by the presence of an in-frame stop codon in the 5' UTR. The anti-NALCN antibody was generated in rabbit with a glutathione S-transferase (GST)-fusion protein containing the last 80 amino acids of NALCN. The mUNC80 polyclonal (rabbit) antibody was generated against the carboxy terminus (Figure 3.8). Both antibodies were affinity-purified. Immunoprecipitation and western blotting followed previously described methods (Liu et al., 2007).

**Neuronal Culture and Transfection**

Hippocampal neurons were cultured from P0 pups on glia pre-plated 35-mm dishes and coverslips as described previously (Lu et al., 2007) and were used between 7 days *in vitro* (DIV) and DIV18. The protocol for VTA neuron culture was modified from one established in rat (Masuko et al., 1992). In brief, isolated brain was placed on ice cold dissection solution (DMEM with 2X Pen/strep). The ~1.5 mm thick brainstem slice containing the mesencephalon is then isolated and the VTA was then trimmed from adjacent areas according to its relative position (lower middle area in the slice) (Masuko et al., 1992). The VTA was then digested with papain (12 units per unit) for 30 min with occasional mixing; digestion was stopped with 10% serum. Tissue was then triturated and plated in culture medium (Neurobasal-A) supplemented with 2% B-27, 0.5x Pen/Strep and 1 mM Glutamax. Owing to the small size of P0 mice, the VTA neuron culture also probably contained neurons from adjacent brain areas. Putative dopaminergic neurons were confirmed by tyrosine hydroxylase staining and morphologically identified,
Typical DA neurons have several thick and relatively straight primary processes which extend from their cell bodies, as well as a single thin process with uniform diameter for hundreds of microns before it branched and exhibited varicosities (Grace and Onn, 1989; Masuko et al., 1992; Rayport et al., 1992). VTA neurons of ages DIV18 to DIV30 were used for patch-clamp analysis. For transfection with Lipofectamine 2000 (Invitrogen), younger neurons (hippocampal, DIV5-DIV7; VTA, DIV6-DIV7) were used because of their higher efficiency. Transfected neurons were used 48-60 hrs later.

**Patch-clamp Analysis**

All experiments were performed at room temperature (20–25 °C). For recording the basal leak current in HEK293T cells transfected with NALCN alone (Lu et al., 2007) (Figure 3.3), cells were transfected with 3 µg NALCN (in a pTracer vector expressing GFP under a separate promoter). Only the most fluorescent cells (top ~5% of all green ones) that presumably expressed the highest level of NALCN were selected. For recording SP-activated currents, NALCN (0.5 µg) was co-transfected with mUNC80 (0.5 µg, in pcDNA3.1(+) vector) and human TACR1 (2 µg, in pcDNA3 vector), unless otherwise stated. An empty vector was added to ensure that the same amount of DNA was transfected when one or more constructs were not included. Cells with an above average level of fluorescence (~40% of the total number of green cells) were selected for analysis. It is possible that some of the basal ‘leak’ currents in neurons and over-expressing HEK293T cells were a result of basal level of receptor activation and tyrosine kinase activity in the cells. Cells with non-specific leak (for example, due to cell damage) were identified by replacing Na⁺ and K⁺ with NMDG or by application of blockers. In
TACR1–mUNC80–NALCN transfected cells, ~70% (53 out of 77) of those analyzed had SP-activated currents >100 pA (at -100 mV). The absence of currents in the rest presumably reflects the efficiency of having all three proteins (two of which are very large) expressed in the same cell, or the varying levels of endogenous signaling molecules. For current amplitude averages, all cells (including the ones without current) were included. HEK293T cells cultured with several batches of sera did not yield robust currents unless they were serum-starved (with Opti-MEM medium) for 9–16 h before recording (not shown). Data from these batches of transfections were not included for analysis.

Standard pipette solutions used for HEK293T cells contained (in mM): 150 Cs, 120 methanesulphonate, 10 NaCl, 10 EGTA, 2 Mg-ATP, 4 CaCl₂, 0.3 Na₂GTP and 10 HEPES (pH 7.4, osmolality ~300 mOsm/L). Bath was a Tyrode's solution containing (in mM): 150 NaCl, 3.5 KCl, 1 MgCl₂, 1.2 CaCl₂, 10 HEPES and 20 glucose (pH 7.4 with NaOH; final Na⁺ 155 mM; ~320 mOsm/L). Some cells were patched with pipette solutions containing no GTP; no differences were observed, consistent with the independence of ISP from G-protein activation. GDPβS-containing pipette solutions contained 1 mM GDPβS and no GTP. As a control, intracellular dialysis with GDPβS-containing pipette solutions blocked the activation of TRPC6 current by carbachol, which is G-protein-dependent, in HEK293T cells co-transfected with TRPC6 and m3AChR (not shown). When pipette solutions containing anti-phospho-SFK (from Millipore) or recombinant active Src protein (from Stressgen) were used, heat-inactivated (100 °C for 30 min) proteins were used as a control. Storage buffer of the recombinant protein was exchanged for pipette solution with a dilution of ~30,000 times by spinning three times in a concentrator (Microcon-50, Millipore).
Pyramidal hippocampal neurons and presumably dopaminergic VTA neurons used in patch-clamp recordings were morphologically identified (Grace and Onn, 1989; Masuko et al., 1992; Rayport et al., 1992). Unless otherwise stated, pipette solutions used for neuronal ISP and INT recordings contained (in mM): 120 CsCl, 4 EGTA, 2 CaCl2, 2 MgCl2, 10 HEPES, 4 Mg-ATP, 0.3 Tris-GTP and 14 phosphocreatine (di-tris salt) (pH adjusted to 7.4 with CsOH; final [Cs+] ~143 mM; free [Ca2+] ~60 nM; 300 mOsm/L). When GTPγS (1.5 mM) or GDPβS (1 mM) was included in pipette solution, GTP was omitted and cells were dialysed for 6–9 min before stimulus application. In experiments with SFK-activator-containing pipette solution, 1 µM SFK activator (a tyrosine-phosphorylated peptide that binds to the SH2 domain of Src kinases, from Santa Cruz Biotechnology Inc., catalogue number sc-3052) was added. TTX (0.8 or 1 µM) was added in the bath of Tyrode's solution. For whole bath SP application, concentrated SP (5 mM) was diluted to 50 µM with bath solution and pipetted into the bath to generate a final concentration of ~1 µM. Currents were continuously recorded for 10–20 min on SP application and the peak currents were used to plot the I–V curves. In puffer applications, diluted SP (10 µM) was pressure-applied using a pneumatic picopump for 10 s with a glass pipette (~3–5 µm opening) placed ~20 µm away from the neuron.

For current clamp with the VTA neurons (Figure 3.7), Tyrode's solution was used as the bath; the pipette solution contained (in mM): 135 K-Asp, 5 NaCl, 5 KCl, 1 MgCl2, 1 EGTA, 10 HEPES, 4 Mg-ATP, 0.3 Tris-GTP and 14 phosphocreatine (di-tris) (pH adjusted to 7.4 with KOH; total [K+], ~147 mM). Neurons were isolated during current clamp with DL-2-amino-5-phosphonovaleric acid (APV, 10 µM), bicuculline (20 µM) and 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX, 20 µM). Some cultured
neurons from both the wild type and mutant showed spontaneous firing at 0 holding current. For others, small currents (wild-type, -2.2 \pm 4.6 \text{ pA}, n = 29; mutant, +10.2 \pm 4.4 \text{ pA}, n = 19) were injected to artificially elicit repetitive firing. Firing frequencies were calculated from time windows (5 min) before and 30 s after SP or NT application. Liquid junction potentials (estimated using the Clampex software) were corrected offline.

Statistical Analyses

Analyses were performed using Clampfit, Sigma Plot and Origin. Data are presented as mean \pm SEM.

Results

NALCN is a neuronal cation channel carrying a small background leak Na\(^+\) current at the resting membrane potential (Lu et al., 2007). When overexpressed in HEK293T fibroblast cells, it generates a Na\(^+\)-permeable cation channel that is voltage-independent, non-inactivating, tetrodotoxin (TTX)-resistant and Gd\(^{3+}\)-blockable (Lu et al., 2007). It is not known whether, like background K\(^+\) channels, NALCN is also regulated by neuromodulators, but the biophysical and pharmacological properties of the NALCN currents (I\(_{\text{NALCN}}\)) closely resemble those of the SP-activated cation channel currents (I\(_{\text{SP}}\)) studied in several brain regions (Aosaki and Kawaguchi, 1996; Inoue et al., 1995; Pena and Ramirez, 2004; Shen and North, 1992b).

To test the possibility that I\(_{\text{SP}}\) requires NALCN, we recorded I\(_{\text{SP}}\) in wild-type and NALCN knockout (NALCN\(^{-/-}\)) mouse neurons by means of patch-clamp with measures taken to minimize K\(^+\) channel effects and to block voltage-gated Na\(^+\) channel (Na\(_{\text{V}}\)) and
synaptic currents. In 16 of 34 wild-type hippocampal pyramidal neurons held at -67 mV, an inward current (> 50 pA) developed after a pulse of SP was delivered through a puffer pipette placed ~ 20 µm from the cell body (Figure 3.1A, E). I_{SP} was blocked by pre-incubating cells with a competitive peptide (n = 3, Figure 3.1B) or a TACR1 antagonist (L703606; n = 14; Figure 3.1A), suggesting involvement of TACR1 or another member of the tachykinin receptor family of GPCRs. Reducing the bath concentration of Na^+ from 155 mM to 5 mM largely abolished the I_{SP} (Figure 3.1A), suggesting that Na^+ was the main charge carrier of the current at this holding potential. I_{SP} has also been recorded in the rat ventral tegmental area (VTA) (Farkas et al., 1996). Of 43 putative dopaminergic (DA) neurons cultured from the VTA of wild-type mice (see Material and Methods), 32 had an I_{SP} > 50 pA (Figure 3.1D). In contrast to the wild-type, none of the 30 hippocampal (Figure 3.1C, E) and 29 VTA (Figure 3.1D) neurons from NALCN^{-/-} mutant mice showed a significant I_{SP} (Figure 3.1E).

Like I_{NALCN} (Lu et al., 2007), the SP-activated currents did not inactivate within 300 ms at any voltage (Figure 3.2A). The current-voltage (I-V) relationship was linear at negative membrane potentials, suggesting little voltage-dependence of conductance in this range (Figure 3.2D). The overall properties of the I_{SP} in cultured mouse hippocampal neurons were similar to those of the I_{SP} recorded from numerous other neuronal preparations, primarily from brain slices (Aosaki and Kawaguchi, 1996; Inoue et al., 1995; Otsuka and Yoshioka, 1993). Owing to low current amplitudes, potential space-clamping problems in neuronal recordings, and possible contamination by voltage-gated currents at positive potentials, a detailed biophysical characterization could not be performed in the neurons.
Figure 3.1

A  
+/+(Hippocampal)  
155 mM [Na⁺]₀  
5 mM [Na⁺]₀  
155 mM [Na⁺]₀  
SP  
0 pA  
50 pA  
1 min  
SP  
L703806

B  
+/+(Hippocampal)  
[0-Arg¹, D-Pro², D-Trp⁷, Leu¹¹]-SP  
SP  
0 pA  
100 pA  
1 min  
SP

C  
−/−(Hippocampal)  
SP  
0 pA

D  
+/+(VTA)  
−/−(VTA)  
SP  
0 pA  
200 pA  
1 min

E  
Hippocampal  
VTA  
\(\begin{array}{c|c|c|c}
(+/+) & (30) & (+/+) & (43) \\
\hline
(−/−) & (29) & (−/−) & \end{array}\)
Currents (at -67 mV) were recorded from wild-type (+/+) or NALCN knockout (-/-) hippocampal and VTA neurons. Horizontal and vertical arrows indicate zero current level and SP application (10 s of puffing), respectively.

(A) $I_{SP}$ developed in bath containing 155 mM Na$^+$, but not when Na$^+$ was lowered to 5 mM (replaced with NMDG). Incubation with L703606 (10 μM, 6 min) blocked $I_{SP}$.

(B) Blockade by a peptide TACR1 antagonist ([D-Arg$^1$, D-Pro$^2$, D-Trp$^7$,$^9$, Leu$^{11}$]-SP; 10 μM, 6 min incubation).

(C) $I_{SP}$ is missing NALCN$^{-/-}$ hippocampal neuron.

(D) $I_{SP}$ recording in wildtype (+/+) and NALCN$^{-/-}$ (-/-) VTA neurons.

(E) Summary of the $I_{SP}$ sizes. Numbers of cells are in parentheses. Error bars, mean and SEM.
Figure 3.2

A  
+/+
Before SP

After SP

Subtraction

500 pA
100 ms

B  
--/--

C  
--/-- NALCN

D  
Voltage (mV)

-80 -40 40

-100

-200

+/+ (33)

--/-- (18)

--/-- NALCN (19)

--/-- mock (8)

Protocol

+33 mV

-87 mV (Vh)

-87 mV

300 ms
Figure 3.2. Characterization of the $I_{SP}$ in Hippocampal Neurons.

(A - C) Net SP-activated currents at various voltages (right) were obtained by subtracting the currents before (left) from those after (middle) SP bath application (1 µM) in wild type (+/+, A), mutant (-/-, B) and mutant transfected with NALCN(-/-; NALCN, C). Dotted lines indicate zero current level.

(D) Averaged $I_{SP}$ amplitudes. The lines from mutant (-/-) and mutant transfected with empty vector (-/-; mock) overlap. Numbers of cells are in parentheses. The right panel shows the voltage step protocols used. $V_h$, holding voltage. Error bars, mean and SEM.
I_{SP} could be restored in the NALCN^{+/−} mutant neurons by transfecting a NALCN complementary DNA (Figure 3.2C, D). The restored I_{SP} (Figure 3.4B, D), like that from wild-type neurons (Figure 3.4A, D), was blocked by a trivalent NALCN blocker Gd^{3+} (10 μM). The current, however, became resistant to 10 μM Gd^{3+} (Figure 3.4C, D) when restored with a Gd^{3+}-resistant NALCN pore mutant (EEKA) that has a single amino acid change (E to A) in the ion filter region (Figure 3.3). Taken together, the similarity of I_{SP} and I_{NALCN}, the absence of I_{SP} in NALCN^{−/−} neurons and presence with NALCN cDNA transfection, and the alteration of I_{SP} pharmacology by the EEKA pore mutant strongly suggest that NALCN forms the pore conduit carrying the I_{SP}.

To determine the role of G-proteins (the immediate downstream effectors of GPCR TACR1) in the NALCN activation by SP, we “locked” G-proteins in active or inactive states with non-hydrolyzable analogues of GTP (GTPγS) or GDP (GDPβS), respectively, applied by means of patch pipettes. Surprisingly, SP still induced inward currents of comparable sizes (Figure 3.5A). Thus, the activation of NALCN by SP through GPCRs is probably by means of an unconventional mechanism that does not require G-protein stimulation.
Figure 3.3. A Gd\(^{3+}\)-resistant NALCN Pore Mutant (EEKA).

(A) A schematic showing a NALCN mutant with the EEKE motif sequence in the putative channel pore filter mutated to EEKA.

(B and C) Representative blockade by 10 µM Gd\(^{3+}\) of the wild-type NALCN (EEKE) (B) and the pore mutant (EEKA) (C) overexpressed in HEK293T cells. Step protocols (300 ms, \(V_h = 0\) mV, from -80 to +80 mV in step of 20 mV) were used. Both the wild-type and the EEKA mutant could be blocked by 1 mM verapamil (Lu et al., 2007).
Figure 3.4

A

B

C

D

$\text{I}_{\text{na}} \text{ inhibition by } 10 \, \mu\text{M Gd}^{3+}$
Figure 3.4. Alteration of $I_{SP}$ Pharmacology by a Pore Mutation in NALCN.

(A-C) Representative hippocampal neuron $I_{SP}$ currents induced by bath application of 1 µM SP (indicated by bars) from a wild-type (A), $NALCN^/-$ transfected with NALCN (B) or with the pore mutant (EEKA, C). Currents were recorded in a Tyrode’s solution containing 10 mM extracellular $K^+$ at holding potential of $E_K$ ($K^+$ Nernst potential, -67 mV) to minimize contribution from $K^+$ current. Pipette solution was the same as the K.As sp pipette used in current clamp. The current in (C) was insensitive to 10 µM Gd$^{3+}$ but was blocked by 1 mM verapamil.

(D) Summary of percentages of inhibition by 10 µM Gd$^{3+}$. Some neurons had >100% inhibition of $I_{SP}$ (amplitude calculated as the difference between inward current sizes before and after SP application) presumably because Gd$^{3+}$ also blocked the basal leak current that existed before SP application (Lu et al., 2007).
Some GPCRs may also activate the Src family of tyrosine kinases (SFKs) -- a more recently discovered GPCR signaling cascade that regulates downstream factors such as mitogen-activated protein kinase and gene expression (DeFea et al., 2000; Lefkowitz and Shenoy, 2005). Bath application of genistein (a phosphotyrosine kinase inhibitor) or PP1 (an SFK inhibitor) abolished I_sp (Figure 3.5B), suggesting that SFKs are required for the activation of NALCN by SP.

Similar to previous findings (Heuss et al., 1999), intracellular dialysis with an SFK activator (a tyrosine-phosphorylated peptide that binds to the SH2 domain of Src kinases, from Santa Cruz Biotechnology Inc., # sc-3052) by means of a patch pipette induced a gradual increase of inward current (defined as Isrc; Figure 3.5C, left panel). After the current plateaued, SP no longer activated an additional inward current (I_sp; Figure 3.5C, D), suggesting that SP and SFKs activate a common channel. Similar to I_sp, the Isrc was blocked by Gd³⁺ (Figure 3.5C, left). In contrast to wild-type neurons, NALCN⁻/⁻ neurons lacked Isrc (Figure 3.5C, D); this current was largely restored by transfection with NALCN cDNA (Figure 3.5D). These data suggest that SFK activation is both necessary and sufficient for I_sp. Given that many other ion channels can also be regulated by SFKs (Davis et al., 2001), the lack of Isrc in the NALCN⁻/⁻ neurons was surprising, but seems to suggest that NALCN is a major cation channel target for SFKs near the resting membrane potential and may also be modulated by the diverse array of stimuli (for example, neurotransmitters, growth factors, cytokines and cell adhesion molecules) that lead to SFK activation (Heuss and Gerber, 2000; Salters and Kalia, 2004).
Figure 3.5

A

B

C

D

Additional $I_{SP}$
Figure 3.5. $I_{SP}$ is G-protein-independent but Requires SFKs.

Hippocampal neurons were used.

(A) $I_{SP}$ (at -67 mV) from recordings with GTP-, GTPγS- and GDPβS- containing pipette solutions. Numbers of cells are in parentheses.

(B) Inhibition of $I_{SP}$ (at -67 mV) by genistein (30 μM; left) and PP1 (20 μM; middle). Right, summary.

(C) In wild-type neurons (left), a Gd^{3+}-blockable current developed after intracellular dialysis (start time indicated by arrow) with pipette solution containing an SFK activator (1 μM; a ramp from -67 to -47mV in 1.4 s was given every 10 s to monitor input resistance). After the current reached a plateau (size defined as $I_{Sec}$ for D), SP did not induce an additional current ($I_{SP}$ for D). Right, $NALCN^{-/-}$ neuron.

(D) Summary of $I_{Sec}$ (left) and additional currents activated by SP after the dialysis-induced currents plateaued (right). Error bars, mean and SEM.
Other neuropeptides such as NT also elicit slow depolarization of neurons and activate a cation current (I_{NT}) similar to I_{SP} (Farkas et al., 1996). In the wild-type VTA neurons, NT (10 µM) application via a puffer pipette elicited an inward current (-90.0 ± 25.3 pA at -67 mV; n = 29) that was blocked by an NT receptor antagonist SR48692 and the SFK inhibitor PP1 (not shown). In contrast, neurons from NALCN^{-/-} mice lacked an I_{NT} (Figure 3.6), suggesting that the I_{NT} is also through the NALCN channel.

To determine the role of the SP- and NT- activated NALCN currents in modulating neuronal excitability, we recorded action potentials in the VTA dopaminergic neurons. Firing frequencies were significantly increased by 1 µM SP (Figure 3.7A, C) or 1 µM NT (Figure 3.7B, C) in the wild-type, but not in the NALCN^{-/-} neurons (Figure 3.7A-C). The defect in the mutant was partially restored by transfecting with NALCN cDNA (Figure 3.7C). We conclude that the NALCN channel has a major role in the potentiation of neuronal excitability by the neuropeptides in these neurons. Under other conditions or in other cells, the peptides may also excite neurons by suppressing K^{+} currents (Farkas et al., 1996; Shen and Surprenant, 1993; Stanfield et al., 1985), or by activating some of the TRP family members (Clapham, 2003; Montell et al., 2002; Oh et al., 2003).
Figure 3.6. Requirement of NALCN in the Neurotensin-activated Cation Current ($I_{NT}$) in Ventral Tegmental Area Neurons.

$I_{NT}$ amplitudes were obtained at various voltages in the wild-type (+/+) and mutant (-/-) and mutant neurons transfected with NALCN (-/-; NALCN) or empty vector (-/-; mock). Data were obtained by subtracting currents before and after NT application. The lines for mutant (-/-) and mutant transfected with empty vector (-/-; mock) overlap and are not distinguished. Currents at potentials positive to -40 mV were not studied because of complications by voltage-activated currents in these neurons. Error bars, mean and SEM.
Figure 3.7: Potentiation of Neuronal Firing Frequency by SP and NT Requires NALCN.

(A and B) Bath application of SP (1 µM, A) or NT (1 µM, B) increased the firing frequency of VTA neurons from wild-type (left) but not the NALCN⁻/⁻ mutant (right). Recordings of 10 s are expanded below each panel.

(C) Average increases of firing frequency (in %) by SP and NT in wild-type (+/+), mutant (-/-), mutant neurons transfected with NALCN (-/-; NALCN) or with empty vector (-/-; mock). Error bars, mean and SEM.
Unlike in neurons, little robust $I_{SP}$ was observed from HEK293T fibroblast cells co-transfected with TACR1 and NALCN (see Figure 3.9E), possibly because of a lack of key components for the channel activation. In *Drosophila melanogaster* and *Caenorhabditis elegans*, NALCN homologs genetically interact with other genes such as *unc79* and *unc80* (Humphrey et al., 2007; Jospin et al., 2007; Yeh et al., 2008). To investigate whether UNC80 forms a physical complex with NALCN and influences channel function, we cloned a mammalian UNC80 homolog (hereafter called mUNC80) from mouse brain (Figure 3.8, see Material and Methods for antibody generation). The full-length mUNC80 is predicted to encode a 3326 amino acid cytosolic protein (molecular weight 371 kDa) (Figure 3.9A) with no obvious domains of known function. It has high (96%) and moderate (~30%) identities with its human and invertebrate homologs, respectively. NALCN and mUNC80 form a complex in mouse brain (Figure 3.9C), and in transfected HEK293T cells (Figure 3.9B). In addition, both NALCN and mUNC80 are tyrosine-phosphorylated and such phosphorylation was inhibited by PP1 (Figure 3.10).
Figure 3.8

**Figure 3.8.** Predicted Amino Acid Sequence of mUNC80.

The peptide sequence used for antibody generation is underlined; a putative PDZ domain-binding motif in the carboxyl-terminus is in bold.

80
Figure 3.9

A

HEK293T
mUNC-80
Mock
Mouse brain

IB: anti-mUNC-80
(kDa)
180
160

B

HEK293T
mUNC-80 + Flag-ctrl1
Flag-NALCN

IP: anti-mUNC-80; IB: anti-Flag
(kDa)
180
160

C

Mouse brain

IP: Anti-ctrl1
Anti-mUNC-80
Anti-ctrl2

IB: anti-NALCN

D

SP
NMDG

Current (nA)
0.5
0
-0.5
100 s

E

NALCN
mUNC-80 +
TACR1 +

(23)(16)

(77)

F

SP
Gd3+

Current (nA)
2
1
0
-1

100 s

G

EEKA
SP
Gd3+
PP1

H

SP
PP1
Figure 3.9. $I_{SP}$ Reconstituted in HEK293T Cells.

(A) Immunoblot (IB) with lysates from transfected HEK293T cells and brain.

(B and C) Immunoprecipitation (IP) showing the protein complex between mUNC80 and NALCN in HEK293T cells transfected as indicated (B) and in brain (C). Ctrl1 and ctrl2 are two unrelated proteins used as controls.

Recordings in (D) – (H) were performed using ramp protocols (holding voltage $V_h = -20$ mV; -100 to +100 mV in 1 s, every 20 s).

(D) Recordings from a cell transfected with TACR1, mUNC80 and NALCN. Currents at three time points are expanded in the right panel ((1) before SP; (2) after SP; and (3) after Na$^+$ and K$^+$ in the bath were replaced with NMDG).

(F and G) Recordings showing that $I_{SP}$ was blocked by Gd$^{3+}$ (F) but became resistant to Gd$^{3+}$ when NALCN was replaced by the EEKA pore mutant (G).

(H) Inhibition of $I_{SP}$ by PP1 (20 μM). Error bars, mean and SEM.
Figure 3.10

Lysates from HEK293T cells co-transfected with NALCN (FLAG-tagged), Src, with or without (lane 2) mUNC80 were immunoprecipitated with anti-FLAG (lanes 1, 2) or anti-UNC80 (lanes 3, 4), and probed with anti-phosphorylated tyrosine antibody. Cells used in lane 4 were treated with SFK inhibitor PP1 (10 µM for 2h) before being lysed. Lanes 1, 2 and lanes 3, 4 are from two separate gels.
In HEK293T cells with moderate levels of co-expression of TACR1, mUNC80 and NALCN (see Materials and Methods), SP activated a current (-550.6 ± 102.9 pA at -100 mV; 0 to -4904 pA; n = 77, Figure 3.9D, E). In contrast, minimal activation was observed in cells transfected with combinations of only two of the three components (Figure 3.9E). The activated currents had a linear I-V relationship (Figure 3.9D, right, curve 2). Like I_{SP} in neurons, the current was blocked by the NALCN blocker Gd^{3+} (Figure 3.9F), but became resistant to Gd^{3+} when NALCN was replaced with the EEKA pore mutant (Figure 3.9G). Inclusion of GDP\betaS in the pipette did not prevent current activation (-914.0 ± 317.1 pA, n = 10), suggesting independence of G-protein activation.

Consistent with a requirement of SFKs, the current was suppressed by SFK inhibitors PP1, PP2, and SU6656, but not by PP3, an inactive analog of PP1 and PP2 (Figure 3.9H and Figure 3.11). In addition, I_{SP} was completely abrogated when an anti-phospho-SFK antibody was included in the pipette, but not when the antibody was heat-inactivated (Figure 3.12).

Furthermore, when a constitutively active Src (with Y529 mutated to F) was co-transfected, a basal current was increased from -104.1 ± 22.1 pA (empty vector to replace Src Y529F in transfection; n = 22) to -434.3 ± 119.9 pA (n = 21), and application of SP no longer activated significant I_{SP} in the Src Y529F co-transfected cells (-64.6 ± 33.1 pA, n = 13; compared with -416.2 ± 89.6 pA in the empty vector co-transfection control, n = 13) (Figure 3.13). Finally, inclusion of a recombinant active Src kinase protein in the pipette induced a gradual increase of inward current (Figure 3.14), suggesting that, like in neurons (Figure 3.5C), SFKs are sufficient to activated the current.
Figure 3.11. Inhibition of $I_{SP}$ by SFK Inhibitors.

Recordings were done with HEK293T cells transfected with TACR1, NALCN and mUNC80. Phosphotyrosine kinase inhibitor genistein (with daidzein as a control) and SFK inhibitors PP1, PP2 (with an inactive analog, PP3, as a control), and SU6656 were bath-applied at concentrations as indicated. $I_{SP}$ amplitudes at -100 mV were used for analysis. Error bars, mean and SEM.
Figure 3.12: Inhibition of $I_{sp}$ by an Anti-phospho-SFK Antibody.

(A and B) Representative recordings of $I_{sp}$ in HEK293T cells transfected with NK1R, mUNC80, and NALCN with pipette solution containing anti-phospho-SFK antibody ((A) final 1 µg/ml, 1:1000 dilution) or heat-inactivated antibody (B).

(C) Summary of $I_{sp}$ sizes (at -100 mV) recorded with pipette solutions containing anti-phospho-SFK antibody, heat-inactivated anti-phospho-SFK antibody, antibody storage buffer, or control IgG. Recordings were done using ramp protocols ($V_h = -20$ mV; -100 to +100 mV in 1 s, every 20 s). Error bars, mean and SEM.
Figure 3.13. Activation of NALCN by a Constitutively Active Src.

Representative currents recorded from HEK293T Cells transfected with NK1R, mUNC80, NALCN, and a constitutively active Src (Y529F) (A) or empty vector (B). PP1, 20 µM. Open bars indicate perfusion with bath containing NMDG to replace Na⁺ and K⁺.
Figure 3.14

A

Recombinant Src

B

Heat-inactivated

C

Development

(13) (17)

(18)

(10)

Recombinant Src

Heat-inactivated
Figure 3.14: Activation of NALCN by a Recombinant Active Src Protein.

(A and B) Representative recordings from HEK293T cells transfected with NK1R, mUNC80, and NALCN with pipette solution containing recombinant active Src (A, ~1.6 U/ml) or heat-inativated protein (B). An inward current developed upon intracellular dialysis with pipette solution containing the recombinant active protein (A). After the current reached a steady state, application of SP (1 µM) did not induce an additional current (I_{SP} for C). This current development was not present in the recording with pipette solution containing the heat-inactivated protein (B).

(C) Summary of current development (as recorded in (A)) by cell dialysis with recombinant protein and additional currents activated by SP bath application after the cellular dialysis. PP1, 20 µM. Open bars indicate perfusion with bath containing NMDG to replace Na⁺ and K⁺. Recordings were done using ramp protocols (V_h = -20 mV; -100 to +100 mV in 1 s, every 20 s). Error bars, mean and SEM.
Conclusions and Discussion

The simplest interpretation of our work is that the SP- and NT- activated channel is a complex consisting of NALCN and mUNC80, both of which are widely expressed in brain. Although this activation occurs through a GPCR, it is G-protein independent and requires SFKs. In addition to its role as a background Na’-leak cation channel (Lu et al., 2007), the NALCN channel may also be activated by other neuromodulators. In support of this notion, previous recordings (largely from rat slices) have shown that some neuropeptides such as luteinizing hormone-releasing hormone, NT, orexin (hypocretin) and vasoactive intestinal polypeptide, as well as some other GPCR-activating neurotransmitters such as acetylcholine and glutamate, elicit “slow” depolarization in the nervous system and activate cation channels with properties similar to those of NALCN (Farkas et al., 1996; Jones, 1985; Kuffler and Sejnowski, 1983; Liu et al., 2002; Shen and Surprenant, 1993). Future work using slice recordings from conditional NALCN knockout mice will allow more detailed analysis of the contribution of the NALCN channel to these currents in neurons.

Hippocampal and VTA neurons deficient in NALCN completely lacked SP- and NT- activated cation currents, suggesting a dominant role of NALCN in neuropeptide-induced currents. Targets of other neuropeptides, or SP- and NT-activated channels in other neurons, may include some of non-selective channels in the transient receptor potential (TRP) channel family that can be triggered following GPCR activation when expressed in heterologous systems (Clapham, 2003; Montell et al., 2002; Oh et al., 2003). Given that many ion channels can be activated by SFKs (Davis et al., 2001), the finding that NALCN-deficient neurons completely lack the inward current induced by the Src-activating peptide was surprising. But this finding is consistent with the idea
that NALCN is an important channel carrying inward Na⁺ current near resting membrane potentials (Lu et al., 2007). In neurons, the SFK pathway can be activated by a diverse array of stimuli (for example, neurotransmitters, growth factors, cytokines and cell adhesion molecules) via tyrosine kinase receptors or other receptors coupled to non-receptor tyrosine kinases (Heuss and Gerber, 2000; Salters and Kalia, 2004). Future work will examine whether these stimuli regulate neuronal function through the NALCN channel. In addition, activation of some GPCRs has been shown to trigger a “second wave” of signaling involving molecules, such as β-arrestins and SFKs, a signaling cascade that has been linked to GPCR oncogenic and apoptotic effects (Lefkowitz and Shenoy, 2005; Luttrell and Luttrell, 2004). The functional expression system for the TACR1-mUNC80-NALCN complex in HEK293T cells and the NALCN⁻/⁻ mutant can be used to dissect the structure-function relationships and the signal transduction pathway coupling the “second wave” signaling to channel opening and neuronal excitability. How mUNC80 contributes to the coupling also needs further study. mUNC80 may function as a scaffolding protein for the signaling complex by binding to the pore-forming protein (NALCN), the receptor (TACR1) and signaling molecules, such as SFKs and perhaps phosphatases. Our preliminary studies also show that UNC79 is associated with mUNC80 and NALCN in brain; however, we did not observe an obvious influence of it on the Iₛᵣ current sizes recorded from the transfected HEK293T cells. Further studies may need to examine if UNC79 influences more subtle properties of the channel such as on and off rates of activation, which vary significantly between cells in both heterologous expression systems and neurons (not shown).
Our data show that genetic disruption of the NALCN channel alone is enough to largely abrogate SP and NT’s modulation of excitability in the hippocampal and VTA neurons. This dramatic effect points to a predominant role for activation of cation conductance in tachykinin modulation of neuronal excitability. Due to the large driving force of Na\(^+\) at resting membrane potential, \(I_{\text{NALCN}}\) should be a potent excitatory current in neurons. The currents are voltage-independent, and do not inactivate, and are thus ideal for modulating neuronal excitability by neuropeptides.

**Others’ Contribution**

This chapter was originally published as “**Peptide neurotransmitters activate a cation channel complex of NALCN and UNC80.**” in *Nature* (Lu et al., 2009). Dr. Yanhua Su contributed to neuronal recordings (Figure 3.1, 3.2, 3.6 and 3.7). Dr. Sudipto Das contributed to work in Figure 3.2. Drs. Haikun Wang, Yan Wang and Jin Liu did the protein work (Figure 3.9 and 3.10). Dr. Dejian Ren started the project, developed the cDNA constructs, and helped design experiments.
CHAPTER IV

EXTRACELLULAR CALCIUM CONTROLS BACKGROUND CURRENT AND NEURONAL EXCITABILITY VIA AN UNC79-UNC80-NALCN CATION CHANNEL COMPLEX

Summary

In contrast to its intracellular roles, the molecular mechanisms by which extracellular Ca\(^{2+}\) regulates neuronal excitability are unclear. Here we show that, in hippocampal neurons, extracellular Ca\(^{2+}\) controls the NALCN-dependent Na\(^+\)-leak current (I\(_{L-Na}\)). NALCN also indirectly associates with UNC79 via its interaction with UNC80. In neurons from \textit{UNC79} and \textit{NALCN} knockout mice, I\(_{L-Na}\) is insensitive to changes in extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_e\)), and reducing [Ca\(^{2+}\)]\(_e\) from 1.2 mM to 0.1 mM fails to elicit the excitatory effects seen in wild-type neurons. The coupling between [Ca\(^{2+}\)]\(_e\) and NALCN complex takes place through a G-protein-dependent mechanism by means of a Ca\(^{2+}\)-sensing G protein-coupled receptor (CaSR), and requires the last amino acid of NALCN and the intracellular carboxy-terminal tail of CaSR. Human CaSR mutations associated with epilepsy also disrupt the ability of CaSR to control the NALCN current. Therefore, extracellular Ca\(^{2+}\) can influence neuronal excitability through the UNC79-UNC80-NALCN complex.
**Introduction**

Na⁺, K⁺, and Ca²⁺ each regulate the excitability of neurons. The effect of extracellular Na⁺ and K⁺ can largely be explained by the cell’s permeability to these ions (P_{Na} and P_{K}). P_{Na} is normally a fraction of P_{K} at rest, at approximately 4% in the squid giant axon (Hodgkin and Katz, 1949), which results in a resting membrane potential closer to that of the equilibrium (Nernst) potential of K⁺ (E_K) than to E_{Na}. Many K⁺ channels, such as the two-pore K₂P leak channels, contribute to the resting P_{K} (Goldstein et al., 2005). At rest, Na⁺ is believed to leak into neurons through voltage-gated Na⁺ channels via the window conductance (Chen and Lucero, 1999; Nicholls and Baylor, 1968), hyperpolarization-activated channels (HCNs) (Robinson and Siegelbaum, 2003), Na⁺-coupled transporters (Jacob et al., 1987), and the recently characterized Na⁺-leak channel NALCN (Lu et al., 2007). However, the way in which extracellular Ca²⁺ influences the resting excitability is poorly understood at the molecular level. This lies in sharp contrast to the extensively studied roles of intracellular Ca²⁺ in physiological functions including muscle contraction, hormone secretion, synaptic transmission, and gene expression (Clapham, 2007).

Under both physiological and pathological conditions, [Ca^{2+}]_c can drop significantly in brain regions such as the hippocampus, neocortex, and cerebellum. For example, repetitive electrical or chemical stimulation in areas where extracellular space is limited can cause [Ca^{2+}]_c to decrease from approximately 1.3 to 0.1 mM, presumably as a result of the movement of extracellular Ca²⁺ into cells (Benninger et al., 1980; Heinemann and Pumain, 1980; Krnjevic et al., 1982; Nicholson et al., 1977; Pumain et al., 1985). Single stimuli are also believed to lead to Ca²⁺ depletion in microdomains such
as the synaptic cleft (Borst and Sakmann, 1999; Rusakov and Fine, 2003; Stanley, 2000). In the cerebral cortex of the cat during slow wave sleep, \([\text{Ca}^{2+}]_e\) levels have been reported to oscillate between 1.18 and 0.85 mM, in phase with membrane potential oscillation in this region, and \([\text{Ca}^{2+}]_e\) can drop further, below 0.5 mM, if such cortical oscillation evolves into a spike-wave seizure (Amzica et al., 2002). Large drops in \([\text{Ca}^{2+}]_e\) are also observed in a variety of other models of seizure, hypoxia, ischemia, and trauma (Heinemann et al., 1986; Morris and Trippenbach, 1993; Nilsson et al., 1993; Silver and Erecinska, 1990).

Unlike \(\text{Na}^+\) and \(\text{K}^+\), extracellular \(\text{Ca}^{2+}\) negatively influences neuronal excitability: a decrease in \([\text{Na}^+]_e\) or \([\text{K}^+]_e\) normally suppresses neuronal excitability, whereas a decrease in \([\text{Ca}^{2+}]_e\) usually excites neurons (Burgo et al., 2003; Chu et al., 2003; Frankenhaeuser and Hodgkin, 1957; Xiong et al., 1997; Yaari et al., 1983). Several mechanisms have been proposed to explain this negative regulation. First, \(\text{Ca}^{2+}\) neutralizes negative charges on the cell membrane. Such charge-screening effects can shift the voltage dependences of biophysical properties (activation and inactivation, for example) of many ion channels such as \(\text{Na}_V\) and \(\text{K}_V\) toward hyperpolarization (Frankenhaeuser and Hodgkin, 1957; Hille, 1968). In addition, \(\text{Ca}^{2+}\) can directly interact with channel gating machinery (Armstrong and Cota, 1991; Armstrong and Lopez-Barneo, 1987). A reduction in \([\text{Ca}^{2+}]_e\) also activates depolarizing, nonselective cation currents in cell bodies and nerve terminals (Formenti et al., 2001; Hablitz et al., 1986; Smith et al., 2004; Xiong et al., 1997) (Figure 4.1). The molecular identities of the channels responsible for the currents, the mechanisms by which \([\text{Ca}^{2+}]_e\) change is coupled to channel opening, and the role of these channels in the regulation of neuronal
excitability by $[\text{Ca}^{2+}]_e$ remain largely unknown.

NALCN (Na$^+$-leak channel, non-selective; (Lu et al., 2007)) is a member of the 24-transmembrane-spanning (24 TM) ion channel family, which also includes 10 voltage-gated Ca$^{2+}$ channels (CaVs) and 10 Na$^+$-selective channels (NaV1.1–1.9 and NaX) (Snutch and Monteil, 2007; Yu et al., 2005). The protein is unique in that its S4 transmembrane segments lack some of the charged residues (K and R) found at every third position in the S4s of the NaV, CaV, and KV channels. In addition, its pore filter regions have an EEKE motif, a mixture between the EEEE found in the CaVs and the DEKA of NaVs (Lee et al., 1999). Consistent with these unique structural features, NALCN is the only nonselective, non-inactivating, voltage-independent channel among the family's 21 members (Lu et al., 2007). Unlike some of the CaVs and NaVs, the subunit composition of NALCN has not been determined. NALCN is widely expressed in the nervous system. In cultured hippocampal neurons, it contributes the major TTX- and Cs-resistant Na$^+$ leak at voltages close to the resting membrane potential. Mice with a targeted disruption in NALCN have severely disrupted respiratory rhythms and die within 24 hours of birth (Lu et al., 2007). Mutations in the NALCN homolog genes in *Drosophila melanogaster* (*na*) and *Caenorhabditis elegans* (*nca*) lead to defects in locomotion, anesthetic sensitivity, rhythmic behaviors, and synaptic function (Humphrey et al., 2007; Jospin et al., 2007; Pierce-Shimomura et al., 2008; Yeh et al., 2008). In addition, mutant screening suggests that NALCN genetically interacts with other genes such as *unc-79* and *unc-80*, whose mammalian counterparts are UNC79 and UNC80, respectively (Humphrey et al., 2007; Jospin et al., 2007; Yeh et al., 2008). It is not known by what means NALCN, UNC79, and UNC80 might functionally interact in the
In this study, we identify NALCN as the cation channel that is activated by a reduction in $[\text{Ca}^{2+}]_e$. Its activation in neurons requires UNC79 and UNC80, where UNC79 indirectly associates with NALCN through its interaction with UNC80. The coupling between $[\text{Ca}^{2+}]_e$ and the channel occurs via a $[\text{Ca}^{2+}]_e$-sensing G protein-coupled receptor (GPCR) and requires both the intracellular carboxy termini of the channel and the GPCR.

**Materials and Methods**

**Animals**

Animal use was in accordance with protocols approved by the University of Pennsylvania IACUC. The generation of *NALCN* (Lu et al., 2007) and *UNC79* (other name KIAA1409) (Nakayama et al., 2006) knockout mice has been previously described. Mice were derived from heterozygous matings in lines that had been backcrossed to C57BL/6 for more than 10 generations.

**Cloning of Full-length UNC79**

The full-length mouse cDNA clone used in this study was assembled in a pcDNA3.1-based vector from three fragments, each of which had been PCR-amplified from mouse brain cDNA with primers designed from partial sequences predicted based on *Drosophila UNC79* sequences. All fragments used to assemble the full-length clones were sequenced to ensure that no sequence errors had been introduced during amplification. The human *UNC79* full-length clone was assembled from an EST clone
containing part of the ORF (GenBank access number AB037830, a gift from Kazusa DNA Research Institute) and a fragment obtained using 5’RACE from a human brain cDNA library. Sequences of the mouse UNC79 clone have been deposited in GenBank (#GQ334471). The mouse clone was used for the patch clamp experiments. In some protein chemistry experiments (Figure 4.8), the human UNC79 (94% identical with the mouse one) was used and the interaction was also confirmed with the mouse clone.

Cell Culture and DNA Transfection

Hippocampal neurons, dissociated from (postnatal day) P0 mouse brains, were digested with papain and plated on poly-L-lysine-coated glass coverslips (12- or 5-mm diameter) in 35-mm dishes at approximately 3–4 ×10⁵ cells/dish. The starting medium consisted of 80% DMEM (Lonza), 10% Ham’s F-12 (Lonza), 10% bovine calf serum (iron supplemented, Hyclone) and 0.5× penicillin-streptomycin (Invitrogen). Cells were changed the next day (DIV 1) to Neurobasal A medium (Gibco) supplemented with 2% B-27, 0.5× penicillin/streptomycin, and 1× Glutamax. Cultures were maintained in a 37°C humidified incubator at 5% CO₂. For some experiments (Figures 4.2, 4.7, and 4.11), the neurons (1.5–2 ×10⁵ cells/dish) were plated onto glia-preplated coverslips or dishes, and maintained in the starting medium. Neurons cultured under this condition are known to have a more robust SP-activated current ((Lu et al., 2009)). When necessary, cytosine-arabinofuranoside (Sigma) was added at 6 μM to suppress glial growth. Neurons were recorded between DIV 7 and 18. At least one day before the experiment, two-thirds of the medium was replaced with fresh medium without glial inhibitor and antibiotics.
The SH-SY5Y human neuroblastoma cell line was cultured in 1:1 DMEM /F-12 (Gibco) supplemented with 10% FBS and 1× penicillin-streptomycin. HEK293T fibroblasts were cultured in DMEM (Gibco) supplemented with 10% FBS, 1× Glutamax and 1× Penn/Strep. The cultures were kept in 37°C in a humidified 5% CO2 atmosphere.

For transfection experiments, Lipofectamine 2000 was used as the transfection reagent. Neurons between DIV 5 and 7 were used for transfection. The transfected SH-SY5Y and HEK293T cells were replated the day before (for SH-SY5Y) or on the day of (for HEK293T cells) recording. Recordings were done 48-72 hr (for HEK293T cells) or 48–60 hr (for the other) after transfection. Transfected cells were identified using the GFP and/or RFP marker.

**Immunoprecipitation and Western Blotting**

The anti-NALCN and anti-UNC80 antibodies used in this study have been previously described (Lu et al., 2009). The polyclonal anti-UNC79 antibody was generated in rabbit with a KLH-conjugated peptide (sequence, CQVEIQSSEAASQFYPL) derived from the carboxy-terminus, and was affinity-purified with the peptide.

For HEK293T cells, cells were lysed by incubation at 4°C for 1 hr in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, pH 7.4) supplemented with a protease inhibitor cocktail (PIC). After centrifuging for 30 min at 20,000 g, the supernatants were mixed with immunoprecipitating antibodies and incubated at 4°C for 2 hr. Samples were then mixed with buffer-equilibrated protein A-agarose at 4°C for 2–14 hr. After three 10-min washes with RIPA buffer, bound proteins were eluted with a lithium dodecyl sulfate (LDS) sample buffer.
For brain proteins, frozen adult (Figure 4.8A) or newborn (Figure 4.8C–E) brains were powdered in dry ice and homogenized in RIPA buffer with PIC. The homogenates were then solubilized at 4ºC for 30 min. After centrifuging at 20,000 g for 30 min, the supernatants were either used immediately for immunoprecipitation or stored at -80ºC for later use. One mg of total protein was precipitated with 1 μg of antibody.

Protein electrophoresis was performed with 10% SDS-PAGE gel in Tris-glycine-SDS running buffer (for CaSR, Figure 4.16D) or, for the other, with 4-12 % Bis-Tris gradient gels in MOPS-SDS running buffer (Invitrogen). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. After being blocked with 5% nonfat dry milk in PBS with 0.1% Tween-20 (PBST), membranes were incubated with primary antibodies at 4ºC overnight or, for anti-Flag, at room temperature for 2 hr. Following incubation with horseradish-peroxidase-labeled secondary antibodies for 1 hr at room temperature, membranes were developed with SuperSignal West Pico ECL or SuperSignal West Dura ECL.

**Patch Clamp Analysis Using Hippocampal Neurons and SH-SY5Y Cells**

All recordings were carried out at room temperature (20–25ºC). Pyramidal neurons morphologically identified were used for neuronal recordings. For voltage-clamp experiments, the pipette solution contained 120 mM CsCl, 4 mM EGTA, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 4 mM Mg-ATP, 0.3 mM Tris-GTP and 14 mM phosphocreatine (di-tris salt) (pH adjusted to 7.4 with CsOH; approximately 300 mOsm/L; intracellular free [Ca²⁺] of approximately 60 nM and [Mg²⁺] of approximately 2 mM, estimated with WEBMAXC). GTP was omitted in pipette solution containing
GTPγS (1.5 mM) or GDPβS (1 mM). The bath solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 10 mM HEPES, 2 mM CsCl, 6 mM glucose and CaCl$_2$ (2 mM unless otherwise indicated) (pH 7.4 with Tris-OH, approximately 315 mOsm/L). Tris-Cl was used to replace 126 mM NaCl in the bath containing 14 mM Na$^+$ bath. TTX (1 μM) and ABC mix (10 μM APV, 20 μM bicuculline, 20 μM CNQX) were applied in the bath to block Na$_v$ and synaptic currents. The $I_{L_{-}Na}$ leak current was measured by subtracting the currents recorded in low (14 mM) [Na$^+$]$_e$ from those in high (140 mM) [Na$^+$]$_e$ at holding potentials ($\Delta I_{L_{-}Na}$) (Lu et al., 2007). For $\Delta I_{L_{-}Na}$ measurement, special precaution was taken to ensure that the current was not a result of recording instability. After recording in a bath containing different [Na$^+$]$_e$, the bath was perfused back to the original [Na$^+$]$_e$. Only those with a fluctuation of the holding current change below 5 pA or 20% of $\Delta I_{L_{-}Na}$ between the first and the last baths, with the same [Na$^+$]$_e$, were used for further analysis. In some initial experiments used in Figures 4.2, a K$^+$-containing pipette solution was used for the $\Delta I_{L_{-}Na}$, which contained 10 mM NaCl, 12 mM KCl, 59 mM K$_2$SO$_4$, 4 mM MgCl$_2$, 10 mM HEPES, 14 mM phosphocreatine (di-tris salt), 4 mM Mg-ATP, 0.3 mM Tris-GTP (pH 7.4 with Tris-OH, approximately 300 mOsm/L). The low [Ca$^{2+}$]$_e$-activated current, $I_{LCA}$, was measured as the change in the size of $\Delta I_{L_{-}Na}$ or the change of the currents at fixed voltages when [Ca$^{2+}$]$_e$ was lowered as indicated. When $I_{LCA}$ was measured by the current change at fixed voltages, either blockers or [Na$^+$]$_e$ reduction was applied to ensure that the current was not due to non-specific leak.

For current-clamp recordings, the pipette solution contained 135 mM K-Asp, 5 mM NaCl, 5 mM KCl, 2 mM MgCl$_2$, 1 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, 0.3 mM Tris-GTP, 14 mM phosphocreatine (di-tris) (pH 7.4 with KOH). The bath solution
contained 150 mM NaCl, 3.5 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 20 mM glucose, and CaCl₂ at the indicated concentration (pH 7.4 with 5 mM NaOH, approximately 320 mOsm/L). Neurons were isolated with APV (10 µM), bicuculline (20 µM) and CNQX (20 µM).

**Patch Clamp Analyses Using HEK293T Cells**

The pipette solution contained 150 mM Cs, 120 mM Mes, 10 mM NaCl, 10 mM EGTA, 4 mM CaCl₂, 0.3 mM Na₂GTP, 2 mM Mg-ATP, 10 mM HEPES (pH 7.4, approximately 300 mOsm/L). Bath solutions contained 150 mM NaCl, 3.5 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 20 mM glucose, and CaCl₂ at the indicated concentrations (pH 7.4 with 5 mM NaOH, approximately 320 mOsm/L). In the NMDG bath, Na⁺ and K⁺ were replaced by NMDG⁺. In the I_{LCA} reconstitution experiments, 0.5 µg NALCN, 0.5 µg UNC80, 0.5 µg Src529, and 2 µg CaSR cDNA were cotransfected. The NALCN and CaSR constructs were made in vectors based on pTracer-CMV2 (Invitrogen) modified to also express eGFP (for NALCN) or mCherry RFP (for CaSR) under a separate promoter. The human CaSR insert was from an IMAGE EST clone (ID#8327704). CaSR mutants were constructed by introducing point mutations as follows: R185Q (nt554/G mutated to A; with the first nucleotide (nt) in the open reading frame numbered as 1), E354A (nt1065/A to C), R898Q (nt2693/G to A), A988V (nt2963/C to T), CaSR1-895 (nt2686-2688/CGC to TGA; amino acid 896 mutated to a stop codon). Only cells with moderate level of both GFP and RFP fluorescence signals were selected for recordings. In control experiments where one or more constructs were not included, an equal amount of empty vector DNA was used to ensure that all the
transfections contained the same amount of DNA.

Liquid junction potentials (estimated using Clampex software) were corrected offline. Patch clamp recordings were performed using an Axopatch-200A amplifier controlled with Clampex 9.2 or Clampex 10 software (Axon). Signals were digitized at 2–10 kHz with a Digidata 1322A or 1440 digitizer.

Results

NALCN Conducts the Low-[Ca$^{2+}$]$_e$-activated Cation Current (I$_{LCA}$)

In several types of neurons, a reduction in [Ca$^{2+}$]$_e$ induces a voltage-independent cation conductance that is Na$^+$-permeable but largely non-selective, TTX-resistant, and blockable by verapamil, Cd$^{2+}$, and Gd$^{3+}$ (Figure 4.1) (Formenti et al., 2001; Hablitz et al., 1986; Xiong et al., 1997). The selectivity, pharmacology, and expression of the conductance are in general agreement with those of the NALCN channel that carries the Cs$^+$- and TTX-insensitive Na$^+$ leak current (I$_{L\text{Na}}$) in hippocampal neurons (Lu et al., 2007). To test whether lowering [Ca$^{2+}$]$_e$ potentiates I$_{L\text{Na}}$, we measured the current in the presence of TTX and Cs$^+$, which we used to block the contribution from NaV$_S$ and HCNs, respectively. We further isolated the small Na$^+$ leak current by measuring the difference ($\Delta$I$_{\text{LNa}}$) between holding currents obtained in baths containing 140 mM and 14 mM Na$^+$ (Lu et al., 2007). When [Ca$^{2+}$]$_e$ was lowered from 2 mM to 0.1 mM, a large increase (6.5 ± 0.7 fold, $n = 5$) of $\Delta$I$_{\text{LNa}}$ was observed (Figures 4.2A and 4.2B). A further decrease of [Ca$^{2+}$]$_e$ to 0.01 mM led to a 10.7 ± 0.7 ($n = 6$) fold increase. Thus, the Na$^+$ leak current faithfully reflected [Ca$^{2+}$]$_e$ changes in a wide range between 0.01 to 2 mM (Figure 4.2B). Similar to currents generated by NALCN (I$_{\text{NALCN}}$) (Lu et al., 2007),
$I_{LCA}$ was blocked by 10 μM Gd$^{3+}$ (Figure 4.1, see also (Xiong et al., 1997)) and 1 mM verapamil (not shown).

In contrast to wild-type cells, mutant hippocampal neurons deficient in NALCN ($NALCN^{-/-}$) lacked an $I_{LCA}$ when $[Ca^{2+}]_e$ was lowered to 0.1 mM (Figures 4.2C and 4.2D). Transfection of NALCN cDNA, but not empty vector, into the $NALCN^{-/-}$ neurons restored the current (Figures 4.2E and 4.2F). These data suggest that $I_{LCA}$ is dependent on NALCN.

Like in the $NALCN^{-/-}$ neurons, transfection of NALCN cDNA into SH-SY5Y, a neuroblastoma cell line that lacks endogenous $I_{LCA}$, also reconstituted an $I_{LCA}$ (Figure 4.3). The I-V relationship (Figure 4.3D) of the current was similar to that of the native $I_{LCA}$ currents that have been recorded in neurons (Xiong et al., 1997).
Figure 4.1. Representative inward current activated by a decrease in $[\text{Ca}^{2+}]_e$ to 0.1 mM from 2 mM. Recorded from a wild-type neurons voltage-clamped at -68 mV; the current was blocked by a NALCN blocker (10 µM Gd$^{3+}$).
Figure 4.2

A

\[ (+/+) \]

\[ 2 \text{ Ca} \quad 0.1 \text{ Ca} \]

\[ (\text{pA}) \]

\[ 140 \text{ mM Na} \]

\[ 14 \text{ mM Na} \]

B

\[ \Delta I_{L_{Na}}/\Delta I_{L_{Na}}(2 \text{ mM } Ca^{2+}) \]

\[ [Ca^{2+}]_e \text{ (mM)} \]

C

\[ NALCN^{-/-} \]

\[ 2 \text{ Ca} \quad 0.1 \text{ Ca} \]

\[ (\text{pA}) \]

\[ 140 \text{ mM Na} \]

\[ 14 \text{ mM Na} \]

D

\[ [Ca^{2+}]_e \text{ (mM)} \]

\[ \Delta I_{L_{Na}} \text{ (pA)} \]

\[ +/+ \]

\[ NALCN^{-/-} \]

\[ 16 \quad 17 \quad 2 \quad 8 \quad 11 \]

E

\[ NALCN^{-/-}; NALCN \]

\[ 2 \text{ Ca} \quad 0.1 \text{ Ca} \]

\[ (\text{pA}) \]

\[ 140 \text{ mM Na} \]

\[ 14 \text{ mM Na} \]

F

\[ 2 \text{ Ca} \quad 0.1 \text{ Ca} \]

\[ NALCN^{-/-}; NALCN \]

\[ NALCN^{-/-}; \text{ mock} \]

(5)

(3)
Figure 4.2. Control of Resting Na⁺ Leak Current by Extracellular Ca²⁺ in Cultured Hippocampal Neurons.

(A) Representative holding currents at –68 mV in a wild-type (+/+) neuron. Na⁺-leak current is presented as ΔIₘₙ (indicated by the double arrow), defined as the difference between holding currents in 140 mM (solid bar) and 14 mM (open bar) Na⁺-containing baths. A 0.25 sec recording is shown for each condition. ΔIₘₙ increased when [Ca²⁺]ₑ was switched from 2 mM (indicated by the hatched bar labeled 2 Ca) to 0.1 mM (0.1 Ca).

(B) ΔIₘₙ in wild-type neurons measured at various [Ca²⁺]ₑ normalized to that measured with 2 mM [Ca²⁺]ₑ (n ≥ 5). The curve was fitted with the Sigmoidal dose-dependence equation:

\[ y = A_2 + (A_1-A_2)/(1 + (x/x_0)^p), \]

where y is ΔIₘₙ and x is [Ca²⁺]ₑ, with others being constant parameters.

(C) Similar to (A), but from a NALCN⁻/⁻ neuron.

(D) Comparison of ΔIₘₙ between wild-type (+/+) and NALCN⁻/⁻ neurons at a range of [Ca²⁺]ₑ, as indicated. The number of cells for each condition is indicated in parentheses.

(E) Representative recordings of ΔIₘₙ restored by NALCN cDNA transfection into the NALCN⁻/⁻ neurons.

(F) Summary of ΔIₘₙ generated by NALCN or mock (empty vector) transfection in 2 mM and 0.1 mM [Ca²⁺]ₑ (mean ± SEM).
Figure 4.3

SH-SY5Y cells

A

mock-transfected

NALCN-transfected

2 Ca

0.1 Ca

2 Ca

0.1 Ca

140 mM Na

14 mM Na

(pA)

-100

0

-100

A

B

[Ca^{2+}]_o (mM)

2

0.1

(5)

(5)

NALCN

mock

C

holding current

2 Ca

0.1 Ca

0

-100

-200

20 s

Verapamil

D

0.2 s

2 Ca

0.1 Ca

+80 mV

-80 mV

net current

-80 -40

40 80 (mV)

NALCN

mock

(n = 15)

(n = 5)
Figure 4.3. Heterologous Expression of NALCN as a $\left[\text{Ca}^{2+}\right]_e$-sensitive Channel in SH-SY5Y Neuroblastoma Cells.

(A) Representative $\Delta I_{L,Na}$ in mock- (left) and NALCN- (right) transfected cells in baths containing 2 mM or 0.1 mM $\text{Ca}^{2+}$.

(B) Average $\Delta I_{L,Na}$.

(C) Representative blockade, by 1 mM verapamil, of the low-$[\text{Ca}^{2+}]_e$-activated current from a NALCN-transfected cell.

(D) I-V relationship of the low-$[\text{Ca}^{2+}]_e$-activated currents. Currents were elicited with a voltage-step protocol (upper, -80 to +80 mV, 10 mV step, $V_h = -40$ mV). Net current activated by 0.1 mM $[\text{Ca}^{2+}]_e$ was obtained as the difference between currents measured in 2 mM and 0.1 mM $\text{Ca}^{2+}$-containing baths. Reduction of $[\text{Ca}^{2+}]_e$ activated a current with a linear I-V relationship (right) from NALCN-transfected cells, but minimal current from mock transfected ones.
I_{LCA} currents have also been postulated to arise via TRPM7 (MacDonald et al., 2006; Wei et al., 2007), a ubiquitously expressed TRP channel that normally opens when the intracellular [Mg^{2+}] is artificially low (Kozak et al., 2002; Kozak et al., 2005). Under our I_{LCA} recording conditions, with 2 mM free Mg^{2+} inside and 1 mM free Mg^{2+} outside the cells, TRPM7 transfected into SH-SY5Y generated essentially no I_{LCA} (0.8 ± 1.7 pA, n = 6; at -80 mV; compared to -235.6 ± 39.6 pA in the NALCN-transfected cells, n = 13; Figure 4.4B). When no Mg^{2+} was included in the pipette solution (0 [Mg^{2+}], Figure 4.4A), TRPM7 generated a current with an outwardly rectifying I-V relationship similar to that of the TRPM7 currents that have been recorded in many other cells (Jiang et al., 2005; Kozak and Cahalan, 2003; Monteilh-Zoller et al., 2003; Nadler et al., 2001), but distinct from those of the NALCN currents (Lu et al., 2007; Lu et al., 2009) and I_{LCA} currents (Xiong et al., 1997), both of which have a linear I-V relationship and are blocked by 10 μM Gd^{3+}. The TRPM7 current was essentially insensitive to a drop in [Ca^{2+}]_e from 2.0 mM to 0.1 mM (Figures 4.4A and 4.4B). These data suggest that, while TRPM7 may account for a small portion under artificially low [Mg^{2+}], and [Ca^{2+}]_e, I_{LCA} occurs largely through NALCN.
Figure 4.4

SH-SY5Y cells

A

B

TRPM7

0 [Mg]

+100 mV

-40 mV

2 mM [Ca^{2+}]_o

0.1 mM [Ca^{2+}]_o

net

400 pA

0.5 s

I_{Ca} (pA)

0

-300

(5) (6)

TRPM7

(13)

NALCN

0 [Mg]

2 mM [Mg]^{2+}
Figure 4.4. TRPM7 Currents Expressed in SH-SY5Y Cells.

(A) Representative currents recorded using a ramp protocol (upper) from a TRPM7-transfected cell, with pipette solution containing no Mg$^{2+}$ (0 [Mg$^{2+}$]; Mg-ATP was replaced with Tris-ATP and MgCl$_2$ was omitted in the pipette solution). Notice that the currents were steeply outwardly rectifying, in contrast to the low [Ca$^{2+}$]$_e$-activated currents in neurons. Lowering [Ca$^{2+}$]$_e$ from 2 mM (left) to 0.1 mM (middle) activated little inward current (right). Dotted line indicates zero current level.

(B) Statistics showing sizes of net currents activated by lowering [Ca$^{2+}$]$_e$ from 2.0 mM to 0.1 mM (at -80 mV) from TRPM7-transfected cells, compared with those from NALCN-transfected ones. Data for NALCN-transfected were replotted from Figure 4.3D for comparison. Further decreasing [Ca$^{2+}$]$_e$ to 10 µM in TRPM7-transfected cells recorded with 0 Mg$^{2+}$ pipettes generated low [Ca$^{2+}$]$_e$-activated currents of -11.9 ± 10.1 pA (at -80 mV, n = 4; not shown).
Sensitivity of the NALCN Current to $[\text{Ca}^{2+}]_e$ Is Dependent on the Carboxy-terminal Tail of NALCN Protein

To determine the structural requirements of NALCN protein for the channel current’s ($I_{\text{NALCN}}$) sensitivity to $[\text{Ca}^{2+}]_e$, we deleted residues in the NALCN carboxy terminus, one of the two large, presumably intracellular domains (the other one being the loop connecting repeats II and III, each of which contains approximately 280 amino acids [aa]). The carboxy terminus consists of two fragments highly conserved among vertebrates, separated by another that is less conserved (Figure 4.5A). A form of NALCN in which the last 202 aa were deleted was nonfunctional and failed to generate a basal Na$^+$ leak current (not shown). A mutant with a shorter deletion (101 aa, $\Delta$1638-1738) generated a basal Na$^+$ leak current ($\Delta$I$_{\text{L-Na}}$) when transfected into SH-SY5Y cells or $NALCN^{+/−}$ hippocampal neurons, but the current was not sensitive to $[\text{Ca}^{2+}]_e$ changes between 2 mM and 0.1 mM (Figures 4.5C and 4.6), suggesting that the last 101 amino acids are required for the sensitivity of $I_{\text{NALCN}}$ to $[\text{Ca}^{2+}]_e$. Results from additional deletions ($\Delta$1657-1699 and $\Delta$1623-1699) suggest that the non-conserved fragment is less important for the $[\text{Ca}^{2+}]_e$ sensitivity (Figures 4.5B and 4.5C). In contrast, the second highly conserved fragment is strictly required (see $\Delta$1733-1738 and $\Delta$1724-1732) (Figures 4.5B and 4.5C). In addition, deleting the last amino acid (I1738; $\Delta$1738) rendered the channel largely insensitive to changes in $[\text{Ca}^{2+}]_e$ (Figures 4.5B and 4.5C).
Figure 4.5. Dependence of Low $[\text{Ca}^{2+}]_e$-activated current ($I_{L\text{CA}}$) on the Carboxy-terminal Residues of NALCN.

(A) Schematic illustration of the location of the NALCN C-terminal mutants. The C-terminal sequences (from the rat isoform, accession # NP_705894) are shown (right). Non-conserved (red) and conserved (blue) amino acid substitutions in the chicken isoform (accession # XP_416967) are highlighted. Shadowed sequences indicate sequence non-essential to $I_{L\text{CA}}$ (deleted in Δ1623-1699).

(B) Representative $\Delta I_{L\text{Na}}$ recordings from SH-SY5Y cells transfected with NALCN deletion mutants Δ1623-1699 (with amino acids 1623-1699 deleted), Δ1724-1732 and Δ1738.

(C) Summary of potentiation of $\Delta I_{L\text{Na}}$ by lowering $[\text{Ca}^{2+}]_e$ from 2 mM to 0.1 mM, defined as percentage of increase ($\Delta I_{L\text{Na}}$ in 0.1 mM $\text{Ca}^{2+}$ - $\Delta I_{L\text{Na}}$ in 2 mM $\text{Ca}^{2+}$)/ $\Delta I_{L\text{Na}}$ in 2 mM $\text{Ca}^{2+}$). Data from transfected $NALCN^{-/-}$ and SH-SY5Y cells were pooled. Measurements from the 5 full-length NALCN- transfected neurons used in Figure 4.2F were also included for comparison. See figure 4.6 for averaged sizes of $\Delta I_{L\text{Na}}$ in 2 mM and 0.1 mM $\text{Ca}^{2+}$-containing baths.
Figure 4.6. Average ΔI_{L-Na} Sizes from the Full-length and Truncated NALCN-transfected Cells.

ΔI_{L-Na} values were pooled from SH-SY5Y and NALCN\textsuperscript{-/-} neurons transfected with cDNAs encoding the full-length and truncated NALCN in 2 mM and 0.1 mM Ca\textsuperscript{2+}-containing baths (see Figure 4.5). ΔI_{L-Na} from the 5 neurons used in Figure 4.2F was also included for the wild-type (full-length) NALCN bar for comparison.
Synergism between Low \([\text{Ca}^{2+}]_e\) and the Neuropeptide Substance P in NALCN Activation

NALCN is also activated by substance P (SP) in approximately 50% of the cultured hippocampal neurons (Lu et al., 2009). If lowered \([\text{Ca}^{2+}]_e\) and SP acted independently on the same target, the two would be expected to exert a synergistic effect. Consistent with this prediction, in wild-type neurons that had SP-activated current \(I_{SP}\), \(I_{LCA}\) was strongly potentiated by the SP application (7.6 ± 1.5 fold, \(n = 19\); Figures 4.7). In \(NALCN^{-/-}\) neurons, applying both stimuli simultaneously failed to activate significant current. Transfection of NALCN cDNA into the \(NALCN^{-/-}\) neurons restored the synergistic effect (Figures 4.7B). In contrast, a truncated NALCN that lacked the carboxy terminus (Δ1638-1738) restored the SP-activated current (-172 ± 89 pA, \(n = 12\)), but the current was largely insensitive to the \([\text{Ca}^{2+}]_e\) change (Figures 4.7B). The synergism between lowering \([\text{Ca}^{2+}]_e\) and SP application further supports the hypothesis that \(I_{LCA}\) is derived from NALCN.
Figure 4.7

A holding current

\( I_{Ca} \) without SP

\( I_{Ca} \) with SP

1 min

-400

-200

0

2 Ca

0.1 Ca

B

\( I_{Ca} \) without SP

\( I_{Ca} \) with SP

\( I_{SP} \)

1 min

-800

-400

0

2 Ca

0.1 Ca

Nalcn

Nalcn

Nalcn

Nalcn

1638-1728
Figure 4.7. Synergism between Low $[\text{Ca}^{2+}]_e$ and Substance P

(A) Representative recordings of $I_{\text{LCA}}$ in the presence and absence of substance P (1 µM), from a wild-type neuron cultured on pre-plated glial cells (left), or a $\text{NALCN}^{-/-}$ neuron cultured under the same conditions (right).

(B) Average $I_{\text{LCA}}$ of wild-type (+/+), $\text{NALCN}^{-/-}$, and full-length ($\text{NALCN}^{+/+}$; NALCN) or carboxy-terminal truncated ($\Delta1638-1738$) NALCN cDNA-transfected $\text{NALCN}^{-/-}$ neurons in the presence or absence of SP. For the wild-type or transfected neurons, only cells with greater than 20 pA SP-activated current ($I_{\text{SP}}$, measured under 2 mM $[\text{Ca}^{2+}]_e$ as illustrated by an arrow in panel A) were selected for analysis. $\text{NALCN}^{-/-}$ neurons had no detectable $I_{\text{SP}}$ (see (Lu et al., 2009)).
NALCN Associates with UNC79 via UNC80 in the Brain, and UNC79 Influences UNC80 Protein Levels

Our finding that the sensitivity of $I_{\text{NALCN}}$ to [Ca$^{2+}$]$_e$ requires the last amino acids in the intracellular tail of the NALCN protein suggests that the coupling between [Ca$^{2+}$]$_e$ changes and NALCN involves an intracellular mechanism. In addition, unlike in neurons and SH-SY5Y neuroblastoma cells, overexpression of NALCN alone in HEK293T fibroblasts generates a current that is insensitive to [Ca$^{2+}$]$_e$ changes (data not shown), suggesting that the sensitivity to [Ca$^{2+}$]$_e$ of NALCN in neurons may require interaction with others intracellular proteins. Recent genetic studies in the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* suggest that the *NALCN* gene interacts with other genes, including *UNC79* and *UNC80*, which encode two proteins that appear to be intracellular (Humphrey et al., 2007; Jospin et al., 2007; Pierce-Shimomura et al., 2008; Yeh et al., 2008). In the mouse brain, UNC80 associates with NALCN (Lu et al., 2009). To test whether UNC79 is also a part of the NALCN protein complex, we cloned mammalian UNC79 homologs from human and mouse brains, and developed a polyclonal antibody against them. The predicted mouse and human UNC79 proteins are 94% identical. They show 30–50% identity with their homologs from invertebrates such as fruit flies, soil worms, and sea urchins. Despite its large size (mouse, 2657 aa; human, 2654 aa), UNC79 has no similarity to domains with known function. Immunoprecipitating UNC79 from mouse brain also precipitated NALCN (Figure 4.8A, *lane 1*) and UNC80 (Figure 4.8A, *lane 4*), suggesting that the three proteins are physically associated in the brain.

In HEK293T cells cotransfected with an HA-tagged UNC79 (HA-UNC79) and a
GFP-tagged UNC80 (GFP-UNC80), immunoprecipitating GFP-UNC80 with an anti-GFP antibody also brought down HA-UNC79 (Figure 4.8B, lane 1), suggesting that the interaction between UNC79 and UNC80 does not require NALCN. Unlike UNC80 (Lu et al., 2009), UNC79 does not seem to interact with NALCN directly, as immunoprecipitating either of NALCN (Figure 4.8B, lane 3) or UNC79 (Figure 4.9A, lane 1) did not bring down the other when they were cotransfected in the absence of UNC80. When UNC80 was added to the transfection, however, immunoprecipitating NALCN also brought down UNC79 (Figure 4.8B, lane 4) and vice versa (Figure 4.9A, lane 2). These data suggest an UNC79-UNC80-NALCN complex model in which UNC79 interacts with UNC80, which in turn associates with the pore-forming subunit (NALCN) of the channel complex (Figure 4.9B).

Elimination of an ion channel subunit can lead to the instability of another component in the same complex (e.g. (Liu et al., 2007; Wang et al., 2009)). We examined the protein levels of UNC80 and NALCN in UNC79 (previous name KIAA1409, (Nakayama et al., 2006)) knockout mice, which have phenotypes similar to those of the NALCN mutant (Lu et al., 2007). The anti-UNC79 antibody recognized a specific protein band from wild-type brain (Figure 4.8C, lane WT), with a molecular weight close to that of the recombinant protein expressed in HEK293T cells, but this band was not detected in brain tissue from UNC79 knockout (KO) mice (Figure 4.8C, lane KO). In the UNC79 knockout, UNC80 protein was also undetectable (Figure 4.8D), but NALCN was present (Figure 4.8E, input). Consistent with the absence of both UNC79 and UNC80 in the UNC79 mutant, antibodies against UNC79 or UNC80 did not immunoprecipitate NALCN from the UNC79 KO brains, whereas they did in the...
wild-type (Figure 4.8E).

**UNC80 Is Essential for the NALCN’s $[\text{Ca}^{2+}]_c$ Sensitivity**

In the presence of 2 mM $\text{Ca}^{2+}$ in the bath, there was no obvious difference between $\Delta I_{\text{L-Na}}$ amplitudes in wild-type and $UNC79^{-/-}$ hippocampal neurons (Figures 4.10A and 4.10D). $\Delta I_{\text{L-Na}}$ was largely absent in the NALCN knockout (data not shown; see also Figure 4.2D and (Lu et al., 2007)). Together, these data suggest that NALCN can form a basal Na⁺-leak channel without an absolute requirement for UNC79, and perhaps also UNC80.

$\Delta I_{\text{L-Na}}$ in the absence of UNC79 and UNC80, as recorded from the $UNC79^{-/-}$ hippocampal neurons, however, was largely insensitive to changes in $[\text{Ca}^{2+}]_c$ (Figures 4.10A and 4.10D). Transfection of UNC79 cDNA into the $UNC79^{-/-}$ hippocampal neurons restored the sensitivity to $[\text{Ca}^{2+}]_c$ of $I_{\text{NALCN}}$ (Figures 4.10B and 4.10D). Furthermore, on the $UNC79^{-/-}$ background, transfection of UNC80 alone could bypass the requirement for UNC79 and rescue the $[\text{Ca}^{2+}]_c$ sensitivity (Figures 4.10C and 4.10D). Thus, the $[\text{Ca}^{2+}]_c$ sensitivity of $I_{\text{NALCN}}$ is dependent on UNC80, whereas UNC79 contributes to the sensitivity, perhaps indirectly, by affecting the UNC80 protein level.
Figure 4.8

A  WT brain

IP: α-UNC79  α-Ct171  α-UNC80  α-UNC79  α-Ct11  α-Chr2

IB: α-NALCN   α-UNC80

B  HEK293T

GFP  +  +  
FLAG-Ctrl3  +  
GFP-UNC80  +  +  
FLAG-NALCN  +  
HA-UNC79  +  +  +  

IP: α-GFP  
IB: α-HA

C  HEK293T  Brain

UNC79  Mock  WT  KO

IB: α-UNC79

D  Brain

Input  WT  KO

IB: α-UNC80

E  Brain

Input  WT  KO  WT  KO  WT  KO  WT  KO

IB: α-NALCN
Figure 4.8. UNC79 Forms a Complex with NALCN via Its Interaction with UNC80 in the Brain and Influences UNC80 Protein Level.

(A) Association of NALCN, UNC79, and UNC80 in the brain. Total mouse brain protein was immunoprecipitated (IP) with the indicated antibodies and blotted (IB) with anti-NALCN (left) or anti-UNC80 (right) antibodies. Anti-HA (α-Ctrl1) and anti-CATSPER1 (α-Ctrl2) were used as control antibodies for specificity.

(B) Association of UNC79 with the NALCN complex via its interaction with UNC80. Lysates from HEK293T cells transfected with the indicated combinations of plasmids were immunoprecipitated with anti-GFP (lanes 1–2) or anti-FLAG (lanes 3–5) and blotted with anti-HA (lanes 1–2 and lanes 3–5 are from two separate gels). A FLAG-tagged transmembrane protein, CATSPERβ (FLAG-Ctrl3), was used as a control.

(C) Western blot using total brain protein from wild-type (WT) and UNC79 knockout (KO), showing the recognition of native UNC79 protein by the anti-UNC79 antibody. Cell lysates from HEK293T cells transfected with UNC79 cDNA or empty vector (mock) were loaded for molecular weight comparison and assessment of antibody specificity.

(D) Western blot showing absence of detectable UNC80 protein in the UNC79 knockout brain.

(E) Western blot with anti-NALCN showing NALCN protein in the UNC79 KO (left two lanes). Immunoprecipitating with anti-UNC79 or anti-UNC80 failed to precipitate NALCN in the KO because of the absence of UNC79 and UNC80 in the mutant.
Figure 4.9  

**A**

<table>
<thead>
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<th></th>
<th>GFP</th>
<th>FLAG-Ctrl3</th>
<th>GFP-UNC80</th>
<th>FLAG-NALCN</th>
<th>HA-UNC79</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(KDa)

194

104

57

IP: α-HA IB: α-FLAG

**B**

An UNC79-UNC80-NALCN Complex Model

---

**Figure 4.9. NALCN Interacts with UNC79 Indirectly via UNC80.**

(A) Lysates from HEK293T cells transfected with the indicated combination of plasmids were incubated with anti-HA to immunoprecipitate HA-tagged UNC79, and then blotted with anti-FLAG to detect FLAG-tagged NALCN. A FLAG-tagged transmembrane protein CATSPERβ (FLAG-Ctrl3) was used as a control.

(B) An UNC79-UNC80-NALCN complex model illustrating the presumed sequence of interaction among the three proteins.
Figure 4.10

A

+/

2 Ca 0.1 Ca

\[ \Delta L_{\text{Na}} \]


\( \text{(pA)} \)


\(-200\)

\(-100\)

\(0\)

UNC79

2 Ca 0.1 Ca

\(140 \text{ mM Na}\)

\(14 \text{ mM Na}\)

5 s

B

UNC79\(^{-}\); UNC79

2 Ca 0.1 Ca

\[ \Delta L_{\text{Na}} (2 \text{ Ca}) \]

\[ \Delta L_{\text{Na}} (0.1 \text{ Ca}) \]

C

UNC79\(^{-}\); UNC80

2 Ca 0.1 Ca

\[ \Delta L_{\text{Na}} (2 \text{ Ca}) \]

\[ \Delta L_{\text{Na}} (0.1 \text{ Ca}) \]

\[ L_{\text{Ca}} = \Delta L_{\text{Na}} (0.1 \text{ Ca}) - \Delta L_{\text{Na}} (2 \text{ Ca}) \]

D

\[ \Delta L_{\text{Na}} (2 \text{ Ca}) \]

\[ \Delta L_{\text{Na}} (0.1 \text{ Ca}) \]

\[ L_{\text{Ca}} = \Delta L_{\text{Na}} (0.1 \text{ Ca}) - \Delta L_{\text{Na}} (2 \text{ Ca}) \]

+/

(20)

UNC79\(^{-}\) (19)

UNC79\(^{-}\); UNC79 (7)

UNC79\(^{-}\); UNC80 (8)
Figure 4.10. The Na⁺-leak Current Is Insensitive to [Ca²⁺]e in UNC79 Mutant Neurons, but the Sensitivity Can Be Rescued with UNC80.

(A–C) Representative ΔI_{LNa} in wild-type (A, +/+), UNC79 knockout (A, UNC79⁻/⁻), UNC79⁺/⁻ transfected with UNC79 cDNA (B), or UNC79⁺/⁻ transfected with UNC80 (C) neurons in baths containing 2 mM or 0.1 mM Ca²⁺.

(D) Summary of ΔI_{LNa} recorded with 2 mM [Ca²⁺]e (left group) and 0.1 mM [Ca²⁺]e (middle group), and the difference between the two (I_{LCA}, right group). Neurons cultured from littermates under identical conditions were used for comparison between the wild-type and mutant. The number of cells for each condition is indicated in parentheses.
The Control of \( I_{\text{NALCN}} \) by \([\text{Ca}^{2+}]_e\) Is through a G Protein-dependent Signaling Pathway

How does a decrease in \([\text{Ca}^{2+}]_e\) “activate” NALCN channel? The monovalent currents through \(\text{Ca}_V\) and several TRP channels are also \(\text{Ca}^{2+}\)-sensitive when \([\text{Ca}^{2+}]_e\) is artificially low, in the micromolar range (Almers and McCleskey, 1984; Hess et al., 1986; Owsianik et al., 2006). The major mechanism underlying this sensitivity is through a blockade of \(\text{Ca}^{2+}\) in the channel pore from outside of the cells; lowering \([\text{Ca}^{2+}]_e\) below 1 \(\mu\text{M}\) removes the blockade (Yang et al., 1993). However, it is unlikely that a similar pore-block mechanism accounts for the \([\text{Ca}^{2+}]_e\) sensitivity of NALCN at the more physiologically relevant, sub-mM \([\text{Ca}^{2+}]_e\) levels, since the channel expressed in HEK293T fibroblast cells is largely insensitive to \([\text{Ca}^{2+}]_e\) drops (see Figure 4.13B), as well as the fact that its \([\text{Ca}^{2+}]_e\) sensitivity requires NALCN’s intracellular C-terminal tail and the presumably intracellular protein UNC80.

We tested whether G proteins could act as an intracellular transducer to couple the \([\text{Ca}^{2+}]_e\) signal to the NALCN-UNC80 complex. Upon cell dialysis with a pipette solution containing GTP\(_\gamma\)S, a non-hydrolyzable GTP analog that constitutively activates G proteins, lowering \([\text{Ca}^{2+}]_e\) no longer increased the background leak \(\text{Na}^+\) current, suggesting that activation of G-protein by GTP\(_\gamma\)S suppressed the low-[\text{Ca}^{2+}]_e activation of NALCN (Figure 4.11A). As a further test, we recorded the current with pipette solutions containing GDP\(_\beta\)S, a non-hydrolyzable GDP analog that locks G-proteins in their inactive state. Upon break-in, an increase in holding current similar to that previously reported (Heuss et al., 1999) developed, even in the absence of \([\text{Ca}^{2+}]_e\) change. The current development was accompanied by an increase in input conductance, reflected
by the current magnitudes from a ramp protocol, suggesting an activation of one or more channels (Figure 4.11B). Once this current reached plateau, reduction of \([\text{Ca}^{2+}]_e\) no longer activated additional current, suggesting that GDP\(\beta\)S and low-\([\text{Ca}^{2+}]_e\) acted on the same channel. In support of NALCN as the channel target, the GDP\(\beta\)S-activated current was absent in \(NALCN^{+/−}\) neurons (Figures 4.11B and 4.11C), but could be restored by NALCN cDNA transfection (Figures 4.11D and 4.11E). Like the native current, the GDP\(\beta\)S-induced current was sensitive to Gd\(^{3+}\) when restored with a wild-type NALCN (Figure 4.11D, left), but became resistant when restored with a Gd\(^{3+}\)-insensitive NALCN pore mutant (Figure 4.11D, right; EEKA motif in the pore instead of the wild-type EEKE (Lu et al., 2009)). Thus, the GDP\(\beta\)S-induced current is through the NALCN channel.
Figure 4.11

A GTPγS

B GDPβS  

C Development

D GDPβS, NALCN−/−  

E Development

F Gd³⁺ inhibition
Figure 4.11. I\textsubscript{LCA} Is G Protein-dependent.

(A) Inclusion of GTP\textsubscript{γS} in the pipette solution blocked the low [Ca\textsuperscript{2+}]\textsubscript{e} potentiation of the Na\textsuperscript{+}-leak current, as shown in a representative recording (left, more than 6 min after break-in), and summarized at right.

(B) In wild-type neurons (left), an inward current developed upon dialysis with pipette solution containing GDP\textsubscript{βS} (V\textsubscript{h} = -68 mV; gap-free recording with a ramp from -68 mV to -48 mV in 1.4 s, every 10.3 s). After the current reached a plateau (defined as current development), reduction of [Ca\textsuperscript{2+}]\textsubscript{e} no longer activated additional current. GDP\textsubscript{βS} did not activate current in a NALCN\textsuperscript{-/-} neuron (right).

(C) Statistics of the GDP\textsubscript{βS}-activated current development, expressed as the size of the plateau current, and additional I\textsubscript{LCA} currents activated by lowering [Ca\textsuperscript{2+}]\textsubscript{e} to 0.01 mM in the presence of GDP\textsubscript{βS}, in the wild-type (+/+) and NALCN\textsuperscript{-/-} mutant.

(D) Representative inward current development upon GDP\textsubscript{βS} dialysis in NALCN\textsuperscript{-/-} neurons transfected with a wild-type NALCN (Gd\textsuperscript{3+} -sensitive; EEKE, left) or with a Gd\textsuperscript{3+}-resistant mutant (EEKA, right) NALCN. Note that lowering [Ca\textsuperscript{2+}]\textsubscript{e} did not activate further current in either cell. The EEKE-transfected neuron was blocked by 10 µM Gd\textsuperscript{3+}. The EEKA-transfected neuron was blocked by verapamil (ver, 1 mM, indicated by dashed arrow), but not by Gd\textsuperscript{3+} (10 µM, indicated by solid arrow).

(E) Summary of the peak currents.

(F) Sensitivity to Gd\textsuperscript{3+} (10 µM) blockade.
Co-expression of NALCN with UNC80 and a Ca^{2+}-sensing G-protein-coupled Receptor in HEK293T Cells Reconstitutes a [Ca^{2+}]_e-sensitive NALCN Channel

The involvement of G-proteins in I_{NALCN}'s [Ca^{2+}]_e sensitivity suggests that the signal that activates NALCN upon a decrease in [Ca^{2+}]_e is transmitted into cells through receptors coupled to the heterotrimeric G-proteins (G_\alpha & G_{\beta\gamma}) or small G-proteins. Consistent with the possibility that CaSR, a Ca^{2+}-sensing G-protein coupled receptor (GPCR), or its homologs, might be the putative receptor sensing the [Ca^{2+}]_e changes (Brown et al., 1995b; Pi et al., 2005), I_{LCA} could be inhibited by spermidine, a CaSR agonist (Figure 4.12).
Figure 4.12. Blockade of $I_{\text{LCA}}$ in Neurons by the CaSR Agonist Spermidine.

Lowering $[\text{Ca}^{2+}]_e$ from 2 mM to 0.1 mM activated an inward current that was blocked by 100 µM spermidine in the wild-type (+/+) hippocampal neurons; spermidine by itself did not elicit current in $\text{NALCN}^{-/-}$ neurons (right).
Together, these data suggest that, in neurons, $I_{\text{LCA}}$ requires at least a channel-pore-forming protein, NALCN, an intracellular protein, UNC80, and a GPCR capable of sensing $[\text{Ca}^{2+}]_e$ changes. We next tested whether these three proteins together were able to reconstitute $I_{\text{LCA}}$ in HEK293T fibroblast cells, which do not have significant endogenous $I_{\text{LCA}}$ (Figure 4.13A and 4.13D). In HEK293T cells, transfection of NALCN alone generates $I_{\text{NALCN}}$ in cells with the highest level of expression (approximately the top 5%, as estimated by the intensity of green fluorescent protein encoded in the same vector (Lu et al., 2009)). Addition of UNC80 and a constitutively active Src kinase (Src529, bearing a Y529F mutation) increases the percentage of cells with detectable $I_{\text{NALCN}}$ to approximately 40% (Lu et al., 2009). $I_{\text{NALCN}}$ from these cells was insensitive to a reduction in $[\text{Ca}^{2+}]_e$ from 1.2 mM to 0.1 mM (Figure 4.13B and 4.13D). However, cotransfection with CaSR rendered the current sensitive to $[\text{Ca}^{2+}]_e$. The current was largely suppressed in the presence of 1.2 mM $[\text{Ca}^{2+}]_e$, but this suppression was released in response to a decrease in $[\text{Ca}^{2+}]_e$ to 0.1 mM (Figure 4.13C, 4.13D and 4.15C). Similar to $I_{\text{LCA}}$ in neurons (Figure 4.12), the current reconstituted from CaSR, UNC80 and NALCN (Figure 4.14A), but not from the ones without CaSR (Figure 4.14B), was blocked by the CaSR agonist spermidine.

Like in neurons, $I_{\text{NALCN}}$ reconstituted in the HEK293T cells with CaSR and UNC80 became insensitive to $[\text{Ca}^{2+}]_e$ when the C-terminus of NALCN was deleted ($\Delta$1638-1738, Figures 4.15A and 4.15C). Similarly, a CaSR construct bearing a hyperparathyroidism- and hypercalcemia-associated point mutation in the receptor’s extracellular amino-terminus (R185Q), which renders the receptor unable to sense $[\text{Ca}^{2+}]_e$ (Bai et al., 1997), was also unable to reconstitute a $[\text{Ca}^{2+}]_e$-sensitive $I_{\text{NALCN}}$ in HEK293T.
CaSR Mutations Associated with Human Epilepsy Disrupt $I_{\text{NALCN}}$’s $[\text{Ca}^{2+}]_e$ Sensitivity

Although many human CaSR mutations are linked to epilepsy, the mechanisms underlying this phenotype are not well understood (Pidasheva et al., 2004). We tested a panel of CaSR mutations from epileptic patients (E354A, R898Q and A988V) (Kapoor et al., 2008). Each mutation reduced the inhibitory effect of CaSR on $I_{\text{NALCN}}$ (Figures 4.16B and 4.16C) even though the protein expression levels of the three mutants were comparable with that of the wild-type (Figure 4.16D). The R898Q mutation is of particular interest because it does not cause a change in whole body $\text{Ca}^{2+}$ or parathyroid hormone levels in patients (Kapoor et al., 2008). Although this suggests that R898Q retains the ability to sense $[\text{Ca}^{2+}]_e$, it nonetheless leads to the inheritable epilepsy phenotype (Kapoor et al., 2008). The R898 residue is located in the distal carboxy terminus of CaSR. This region has no obvious role in the receptor’s established function as a GPCR, since a truncated CaSR lacking the intracellular tail (CaSR 1-895, with the C-terminus residues beyond aa 895 deleted) is largely indistinguishable from the wild-type in stimulating IP$_3$ production and intracellular $\text{Ca}^{2+}$ release in response to extracellular stimuli (Chang et al., 2001; Gama and Breitwieser, 1998; Ray et al., 1997). However, this deletion largely abolished the receptor’s ability to reconstitute a $[\text{Ca}^{2+}]_e$-sensitive NALCN current (Figures 4.16A and 4.16C), suggesting a novel role for the conserved carboxy-terminus of CaSR.
Figure 4.13. Reconstitution of a [Ca$_{\text{e}}^{2+}$]-sensitive NALCN Current in HEK293T Fibroblasts with CaSR, NALCN, and UNC80.

(A–C) Representative currents obtained with a voltage-ramp protocol (-100 mV to +100 mV in 1 sec, $V_h = -20$ mV) from non-transfected cells (A) or cells transfected with various combination of NALCN, UNC80, and CaSR, as indicated (B, C) in baths containing 1.2 mM Ca$_{\text{e}}^{2+}$ (1.2 Ca) or 0.1 mM Ca$_{\text{e}}^{2+}$ (0.1 Ca). All baths contained 155 mM Na$^+$ except that NMDG$^+$ was used to replace Na$^+$ and K$^+$ in the (NMDG, 0.1 Ca) baths. All transfections also included a constitutively active Src (Src529) to increase the percentage of cells expressing detectable current (see Materials and Methods and (Lu et al., 2009)).

(D) Averaged size of the increase of inward current ($I_{\text{LCA}}$, at -100 mV) upon lowering [Ca$_{\text{e}}^{2+}$] from 1.2 mM to 0.1 mM ($I_{LCA}$). Cell number for each experiment is shown in parentheses.
Figure 4.14

A
CaSR + UNC80 + NALCN

B
UNC80 + NALCN

C
Inhibition by spermidine
Figure 4.14. Spermidine Blocks $I_{NALCN}$ in HEK293T Cells when CaSR Is Cotransfected with NALCN and UNC80, but Does Not Do So when CaSR Is Absent.

(A and B) Representative currents obtained with a voltage-ramp protocol (-100 mV to +100 mV in 1 s, $V_h = -20$ mV) from cells transfected with CaSR, UNC80, and NALCN (A), or without CaSR (B), in baths containing 1.2 mM Ca$^{2+}$ (1.2 Ca), 0.1 mM Ca$^{2+}$ (0.1 Ca), or 0.1 mM Ca$^{2+}$ with 100 µM spermidine (0.1 Ca + spermidine). All baths contained 155 mM Na$^+$ except that NMDG$^+$ was used to replace Na$^+$ and K$^+$ in the (NMDG, 0.1 Ca) baths. All transfections also included a constitutively active Src (Src529) to increase the percentage of cells expressing detectable current (see Experimental Procedures and (Lu et al., 2009)). $I_{NALCN}$ without CaSR (B) was not inhibited by spermidine but could be blocked by a NALCN blocker Gd$^{3+}$ (10 µM; (Lu et al., 2007)).

(C) Summary of the inhibition by spermidine at -100 mV. Cell number for each experiment is shown in parentheses.
Figure 4.15

A

CaSR + UNC80 + NALCN

1.2 Ca

0.5 nA

-100 (mV) - 100

0.5 nA

-100 (mV) - 100

0.5 nA

-100 (mV) - 100

NMDG, 0.1 Ca

B

CaSR R185Q + UNC80 + NALCN

1.2 Ca

0.5 nA

-100 (mV) - 100

0.5 nA

-100 (mV) - 100

0.5 nA

-100 (mV) - 100

0.1 Ca

NMDG, 0.1 Ca

C

\[
\frac{I_{1,2 \text{ Ca}}}{I_{0,1 \text{ Ca}}} \%
\]

(7) (10)

(37)
Figure 4.15. The \([\text{Ca}^2+]_e\) Sensitivity of \(I_{\text{NALCN}}\) Reconstituted in HEK293T Cells Requires both the Carboxy-terminus of NALCN and the Ability of CaSR to sense \([\text{Ca}^2+]_e\).

(A and B) Representative currents obtained with a voltage-ramp protocol, from -100 mV to +100 mV in 1 sec \((V_h = -20 \text{ mV})\), from cells transfected with CaSR, UNC80 and the truncated NALCN (Δ1638-1738) (A), or with UNC80, NALCN and a CaSR mutant (R185Q) that doesn’t sense the \([\text{Ca}^2+]_e\) changes (Bai et al., 1997) (B), in baths containing 1.2 mM Ca\(^{2+}\) (1.2 Ca) or 0.1 mM Ca\(^{2+}\) (0.1 Ca). NMDG\(^+\) was used to replace Na\(^+\) and K\(^+\) in the (NMDG, 0.1 Ca) baths. All transfections also contained a constitutively active Src (Src529) to increase the percentage of cells expressing detectable current (see Experimental Procedures and (Lu et al., 2009)).

(C) The sensitivity of \(I_{\text{NALCN}}\) to \([\text{Ca}^2+]_e\) from cells transfected with combinations of cDNA as indicated (expressed as the ratio of the current magnitude at -100 mV in a bath containing 1.2 mM Ca\(^{2+}\) to that in a bath containing 0.1 mM Ca\(^{2+}\)). Data for the “CaSR + UNC80 +NALCN” combination were from the cells used in Figure 4.13. Only the cells with a greater than 100 pA current (at -100 mV) in either 1.2 mM Ca\(^{2+}\)- or 0.1 mM Ca\(^{2+}\)-containing baths were selected for the ratio analysis.
Figure 4.16

A  CaSR 1-895 + UNC80 + NALCN

B  CaSR E354A + UNC80 + NALCN

C  $I_{\text{Ca}}$ normalized to WT CaSR (100%)

D  IB: anti-CaSR

IB: anti-β-actin
Figure 4.16. Epilepsy-associated CaSR Mutants Have Defects in Suppression of $I_{NALCN}$.

(A and B) Representative recordings of HEK293T cells cotransfected with NALCN, UNC80, together with the carboxy-terminal truncated CaSR (CaSR 1-895, A), or with the E354A CaSR mutant (B). See Figure 4.13 legend for detail.

(C) Sensitivity to $[Ca^{2+}]_e$ of the inward current at -100 mV (measured in the same way as Figure 4.15) in the wild-type (WT) or the various mutant CaSR -cotransfected HEK293T cells. Data for the “No CaSR” and “WT CaSR” combinations were from the cells used in Figure 4.13. Only the cells with a greater than 100 pA current (at -100 mV) in either 1.2 mM Ca$^{2+}$- or 0.1 mM Ca$^{2+}$-containing baths were selected for the analysis.

(D) Total cell lysates prepared from HEK293T cells transfected with empty vector (mock), wild-type or CaSR mutant as indicated, were blotted with anti-CaSR antibody (monoclonal, against the amino-terminus of CaSR, from Santa Cruz, #sc-47741; upper panel) or with anti-β-actin antibody as control (lower panel). The carboxy-terminal truncated CaSR mutant (CaSR 1-895) gave rise to apparently higher level of protein when the same amount of DNA (2 µg) as the wild-type was used for transfection. Recordings were also done with 1/3 amount of CaSR 1-895 (0.7 µg) as normally used; still, no obvious suppression of the NALCN current was observed ($I_{1.2Ca}/I_{0.1Ca} = 95.9 \pm 17.6\%$; $n = 8$).
NALCN Is Required for the Excitatory Effects of a Reduction in $[\text{Ca}^{2+}]_e$

Hypothetically, extracellular Ca$^{2+}$ can influence the basal excitability of neurons through its interaction with the cell membrane and with many of the proteins on the membrane (Hofer, 2005; Hofer and Brown, 2003). To determine whether the regulation of $I_{\text{NALCN}}$ by $[\text{Ca}^{2+}]_e$ plays a role in the excitatory effects of a moderate reduction in $[\text{Ca}^{2+}]_e$ within the range found in conditions such as hypocalcemia, which is often associate with seizure, and in brain regions during repetitive excitation, we used current-clamp recordings to compare the responses of wild-type and those of the UNC79$^{-/-}$ neurons, which have a $[\text{Ca}^{2+}]_e$-insensitive $I_{\text{NALCN}}$ (Figure 4.10). In both the wild-type (Figures 4.17A and 4.17C) and UNC79 mutant (Figures 4.17B and 4.17C) neurons, injection of depolarizing currents mimicking excitatory synaptic input elicited firing with frequencies proportional to the sizes of the current injected. In the wild-type, lowering $[\text{Ca}^{2+}]_e$ from 1.2 mM to 0.1 mM (with $[\text{Mg}^{2+}]_e$ fixed at 1 mM), increased the firing frequencies, sometimes converting a non-firing neuron to a firing one (Figures 4.17A and 4.17C). In contrast, the firing frequencies on average were not increased by the reduction of $[\text{Ca}^{2+}]_e$ in the UNC79$^{-/-}$ neurons (Figures 4.17B and 4.17C). Similar lack of the excitatory effect of lowering $[\text{Ca}^{2+}]_e$ was also seen in the NALCN$^{-/-}$ neurons (Figures 4.17C (to 0.1 mM $[\text{Ca}^{2+}]_e$) and 4.18 (to 0.5 mM $[\text{Ca}^{2+}]_e$)) and the effect could be partially restored by a NALCN cDNA transfection (Figures 4.18C (to 0.5 mM $[\text{Ca}^{2+}]_e$)).
Figure 4.17

A. Wild-type

B. UNC79

C. Averaged firing frequencies

1.2 mM [Ca\(^{2+}\)]

0.1 mM [Ca\(^{2+}\)]
Figure 4.17. Dependence of the Excitatory Action of Low \([\text{Ca}^{2+}]_e\) on UNC79 and on NALCN.

A hyperpolarizing holding current (\(I_{\text{Hold}}\), -60 pA in the neuron in panel A; -100 pA in panel B; -64.0 ± 18.8 pA (wild-type), -83.7 ± 19.2 pA (UNC79\(^{-/-}\)) and -32.7 ± 17.4 pA (NALCN\(^{-/-}\)) for panel C) was injected to bring each neuron’s steady membrane potential to -80 mV in 1.2 mM \(\text{Ca}^{2+}\)-containing bath. Pulses (10 s, as illustrated in lower right in panel B) of additional depolarizing currents with increasing amplitudes (+10 to +60 pA) were injected every 50 s.

(A, B) Examples of current-clamp recordings from a wild-type (A) and an UNC79 mutant neuron (B) in baths containing 1.2 mM (upper traces) or 0.1 mM (lower traces) \(\text{Ca}^{2+}\). Firing frequencies of the neurons during the 10 s depolarizing pulses are plotted in the right columns.

(C) Statistics of firing frequencies from wild-type (left) and UNC79\(^{-/-}\) neurons (right).
Figure 4.18

A  +/+  

1.2 mM [Ca²⁺]  0.5 mM [Ca²⁺]  (injection = 0 pA)  

B  NALCN⁻  

1.2 mM [Ca²⁺]  0.5 mM [Ca²⁺]  (injection = 0 pA)  

C  Firing frequency increase

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Figure 4.18. Defects in the Low [Ca^{2+}]_e Responses in NALCN^{−/−} and Rescue by cDNA Transfection.

(A-B) Examples of gap-free current-clamp recordings from a wild-type (A) and a NALCN^{−/−} (B) neuron while [Ca^{2+}]_e was lowered from 1.2 mM to 0.5 mM.

(C) Mean frequency increases caused by lowering [Ca^{2+}]_e in wild-type (+/+), NALCN mutant (NALCN^{−/−}), NALCN mutant transfected with NALCN cDNA (NALCN^{−/−}; NALCN) or with empty vector (NALCN^{−/−}; mock). Spike frequencies were calculated from greater-than-1-min time windows before and more than 45 seconds after the start of the solution change. Some cultured neurons from both the wild-type and mutant showed spontaneous firing at 0 holding current. For others, firing was elicited with current injection (wild-type, +1.7 ± 1.1 pA, n = 15; NALCN^{−/−} mutant, +3.5 ± 1.8 pA, n = 17; NALCN-transfected mutant, -7.2 ± 2.0 pA, n = 5; mock-transfected mutant: +9.0 ± 5.5 pA, n = 4) were injected to elicit firing.
Discussion

We have shown that $[\text{Ca}^{2+}]_e$ controls the size of NALCN channel currents. This control appears to be a major mechanism by which a change in $[\text{Ca}^{2+}]_e$ around the physiological concentrations influences the resting excitability of neurons. Thus, while extracellular K$^+$ may regulate neuronal excitability through many channels including the 15 two-pore domain K$^+$ leak channels (Goldstein et al., 2005), both Na$^+$ and Ca$^{2+}$ can exert their influence through the NALCN Na$^+$-leak channel, where Ca$^{2+}$ indirectly regulates the size of the NALCN current through a G-protein-coupled receptor, which senses $[\text{Ca}^{2+}]_e$, and a G protein-dependent intracellular mechanism that couples the signal to the UNC79-UNC80-NALCN channel complex.

The ability of Ca$^{2+}$ to act as an intracellular messenger has been extensively studied at the molecular level, but the ion’s potential role as an extracellular messenger is poorly understood. $[\text{Ca}^{2+}]_e$ has long been known to influence neuronal excitability (Frankenhaeuser and Hodgkin, 1955; Frankenhaeuser and Hodgkin, 1957). Although $[\text{Ca}^{2+}]_e$ is considered relatively stable, it has been shown to fluctuate under conditions such as prolonged stimulation, or in microdomains, where extracellular space is limited, during physiological processes such as synaptic transmission and sleep, or pathophysiological conditions such as seizure and hypocalcemia. Artificially lowering $[\text{Ca}^{2+}]_e$ can induce seizure in intact animals and seizure-like activities in brain slices and single neurons (Feng and Durand, 2003; Kaczmarek and Adey, 1975). Neurons cultured from NALCN$^{-/-}$ and UNC79$^{-/-}$ hippocampi are insensitive to the $[\text{Ca}^{2+}]_e$ drop from 1.2 mM to 0.1 mM, suggesting a major role of the NALCN complex in the low $[\text{Ca}^{2+}]_e$–induced neuronal excitability. Other mechanisms, such as the charge screening effects, may play
roles during more dramatic reduction of total extracellular divalent ions. Future experiments, with a tissue-specific conditional knockout or a knockin to engineer animals with a $[\text{Ca}^{2+}]_e$-insensitive NALCN, will further define the \textit{in vivo} roles of the regulation of NALCN by $[\text{Ca}^{2+}]_e$ in physiological functions, such as synaptic plasticity, and pathophysiological conditions such as seizure.

We have also uncovered several major components that appear to couple a drop in $[\text{Ca}^{2+}]_e$ to an opening of the channel: a $[\text{Ca}^{2+}]_e$-sensitive GPCR (CaSR), an UNC79-UNC80 complex, and the carboxy terminus of NALCN. These findings are consistent with a model in which there is a tonic inhibition of NALCN current by a $[\text{Ca}^{2+}]_e$-sensing GPCR and where, in turn, lowering $[\text{Ca}^{2+}]_e$ releases the inhibition and thereby activates the channel (Figure 4.19). However, the precise mechanism by which a $[\text{Ca}^{2+}]_e$ signal is transmitted to the NALCN complex remains to be established. CaSR, other members in the Class C GPCR family such as GPRC6A (Pi et al., 2005), mGluR1 (Kubo et al., 1998) and GABAB (Wise et al., 1999), as well as the heterodimers among them (Gama et al., 2001) can all sense changes in both $[\text{Ca}^{2+}]_e$ and other stimuli such as amino acids (Hofer and Brown, 2003). The major known function of CaSR is to detect $[\text{Ca}^{2+}]_e$ in organs such as the parathyroid gland, which secretes parathyroid hormone to regulate systemic $\text{Ca}^{2+}$ levels (Hofer and Brown, 2003). Like NALCN, CaSR is also widely expressed in the brain, where it is found at particularly high levels in regions such as the hippocampus and cerebellum (Ruat et al., 1995). The neuronal function of CaSR is beginning to emerge: activated CaSR stimulates the dendritic growth of neurons (Vizard et al., 2008) and suppresses synaptic transmission (Phillips et al., 2008), although the mechanisms are largely unknown. CaSR is coupled to $\text{G}_{\alpha}$ and leads to an
intracellular IP₃ and Ca²⁺ level increase upon stimulation (Hofer and Brown, 2003). This well established function of CaSR appears to be insufficient for the receptor’s ability to suppress NALCN current. Although the 183 amino acids of the distal carboxy terminus of CaSR are well-conserved among vertebrates, suggesting that they have physiological function, they are not required for the receptor’s ability to sense [Ca²⁺]ₑ changes and trigger IP₃ production ((Chang et al., 2001; Gama and Breitwieser, 1998; Ray et al., 1997), and data not shown). In contrast, this segment is essential for the ability of CaSR to suppress NALCN (Figure 4.16A), suggesting a novel function for this intracellular tail. Consistent with this proposed function of the carboxy terminus of CaSR in NALCN regulation, but not in the [Ca²⁺]ₑ detection operating in the parathyroid gland, the R898Q mutation in the region found in human patients did not lead to abnormality in whole body Ca²⁺ homeostasis; it did, however, lead to a clear deficiency in I_LCA (Figure 4.16) and is associated with heritable epilepsy (Kapoor et al., 2008). Future studies will examine whether this domain may be involved in the interaction between UNC80 and the intracellular carboxy tail of NALCN, both of which are required for I_LCA.

We found that low [Ca²⁺]ₑ can act synergistically with the neuropeptide substance P to potentiate I_NALCN in the hippocampal neurons (Figure 4.7). Similar synergism was also found between low [Ca²⁺]ₑ and the neuropeptide neurotensin in activating a cation current similar to I_NALCN in midbrain dopaminergic neurons (Farkas et al., 1996). Although both the action of [Ca²⁺]ₑ and that of SP require UNC80, the signal transduction pathways underlying them are distinct. The [Ca²⁺]ₑ action through CaSR is dependent on G proteins and the last amino acids of the NALCN protein, whereas the
effect of SP through TACR1 (the GPCR for SP) is independent of G proteins and the carboxy terminus of NALCN protein but requires the Src family of tyrosine-protein kinases (SFKs) (Lu et al., 2009). The last amino acids of NALCN contain a potential class II PDZ-domain binding motif (-LDI-COOH, Figure 4.5A) that may interact with PDZ-domain containing proteins essential for the channel’s [Ca\(^{2+}\)]\(_e\) sensitivity. Single channel recording and protein trafficking studies of NALCN will be required to determine whether the two modes of action have distinct channel parameters, such as the number of channels (\(N\)) and the opening probability (\(P_o\)). [Ca\(^{2+}\)]\(_e\) drop in brain regions occurs during seizure, where lowering [Ca\(^{2+}\)]\(_e\) is enough to trigger seizure. Similarly, an increase of SP expression in the hippocampus has been observed in a model of status epilepticus, where it was proposed to be critical for the maintenance of the epilepticus state (Liu et al., 1999). A synergetic effect between [Ca\(^{2+}\)]\(_e\) drop and Src kinases has also been observed in the “paradoxical” excitation of neurons and the increase of [Ca\(^{2+}\)]\(_i\), by low [Ca\(^{2+}\)]\(_e\) (Burgo et al., 2003). A simultaneous decrease in [Ca\(^{2+}\)]\(_e\) and an activation of the kinases by neuropeptides and other stimuli would be expected to provide a powerful excitatory signal to the neurons through the synergetic activation of NALCN (Figure 4.19).

Our findings indicate that the NALCN complex contains at least three proteins: NALCN, UNC79, and UNC80, with a predicted total molecular weight of approximately 800 kDa (assuming a monomeric stoichiometry), a size larger than those of some of the Na\(_V\)s and Ca\(_V\)s (Arikkath and Campbell, 2003). Several lines of evidence support the possibility that UNC79 and UNC80 represent auxiliary subunits of the NALCN channel. First, the three proteins are physically associated. Second, UNC79 affects the UNC80
protein level (Figure 4.8). Third, UNC80 is required for the control of the NALCN channel by GPCRs (Figure 4.10 and (Lu et al., 2009)). Fourth, mutations in \textit{UNC79}, \textit{UNC80} and \textit{NALCN} have similar phenotypes, which are in turn similar to the double-mutant phenotype (Humphrey et al., 2007; Jospin et al., 2007; Nakayama et al., 2006; Yeh et al., 2008), suggesting that the major roles of UNC79/UNC80 are NALCN-related.

The sizes of the basal leak currents ($\Delta I_{L-Na}$) in WT and \textit{UNC79} KO neurons were comparable (Figure 4.10D). Overexpression of NALCN in the \textit{UNC79} mutant background actually generated a leak current larger than that in the wild-type (not shown). Although, because the antibodies we developed do not optimally recognize native proteins in immunocytochemical preparations, we cannot exclude the possibility that NALCN has a different localization in the \textit{UNC79} mutant, these data are consistent with our previous findings that NALCN can form an ion channel without an apparent requirement for UNC79 (Lu et al., 2007; Lu et al., 2009). Our preliminary studies did not observe an obvious influence of UNC79 on properties of NALCN expressed in HEK293T cells. Further studies may need to examine if UNC79 influences the more subtle characteristics of the channel. Both \textit{UNC79} and \textit{NALCN} knockout mice have disrupted breathing rhythms, fail to nurse, and die as neonates ((Lu et al., 2007; Nakayama et al., 2006), and unpublished observations). Some \textit{UNC79} KO mice survive beyond the first day, whereas \textit{NALCN} KO mice die within 24 hr of birth. The slightly weaker phenotype of \textit{UNC79} KOs apparently reflects the function of the basal leak current through NALCN without UNC79/UNC80. However, it is clear that this current is not sufficient to support the animal’s viability. One possible reason for the residual
current’s inability to support life is that its localization may be defective in the \textit{UNC79} mutant. Another possibility is that the ability of NALCN to be activated or suppressed by GPCRs, which is dependent on the presence of UNC80 missing in the \textit{UNC79} mutant, is critical to survival. It has been proposed that activation of background currents similar to \(I_{\text{NALCN}}\) by neurotransmitters may be critical to the generation of respiratory rhythms in the brainstem, such that modulation of the \(\text{Na}^+\)-leak may play a fundamental role (Ptak et al., 2009).

In summary, we have uncovered a novel molecular mechanism by which extracellular \(\text{Ca}^{2+}\) ion influences the resting excitability of neurons. The signaling cascade includes a GPCR that senses \([\text{Ca}^{2+}]_e\) change and transmits the signal into the cell, as well as an \textit{UNC79}/\textit{UNC80} complex that may couple the signal to the NALCN carboxy terminus. This regulatory pathway also interacts with a G protein-independent mode of control by neuropeptides. NALCN does not inactivate and is \(\text{Na}^+\)-permeable. Control of the \textit{UNC79}-\textit{UNC80}-NALCN channel complex represents a powerful mechanism to influence neuronal excitability.
There is a tonic suppression of $I_{\text{NALCN}}$ by activated CaSR or its homologs at high $[\text{Ca}^{2+}]_e$ level (~1.2 mM), which involves UNC80, the last amino acids of NALCN and the carboxy-terminus of CaSR. Lowering $[\text{Ca}^{2+}]_e$ releases the suppression, increases $I_{\text{NALCN}}$, generates a low $[\text{Ca}^{2+}]_e$-activated inward current ($I_{\text{LCA}}$) and excites neurons. The R185Q CaSR mutant does not support $I_{\text{LCA}}$ because it can not sense $[\text{Ca}^{2+}]_e$ changes (Bai et al., 1997); the CaSR 1-895 mutant can sense $[\text{Ca}^{2+}]_e$ changes (Chang et al., 2001; Gama and Breitwieser, 1998; Ray et al., 1997) but lacks the essential function (unidentified) carried out by its carboxy-terminus. NALCN can also be activated by substance P (SP) receptor TACR1 in a G protein-independent manner that requires the Src family of kinases (SFKs) and UNC80 (Lu et al., 2009). The interplay between the positive regulation of NALCN through SFKs and the negative control via G proteins may determine the basal levels of Na$^+$ leak in neurons. A simultaneous decrease of $[\text{Ca}^{2+}]_e$ and increase of Src kinase activity generates a synergetic excitatory action.
Others’ Contribution

Drs. Qi Zhang and Haikun Wang did the protein work (Figure 4.8, 4.9 and 4.16D).

Dr. Manabu Nakayama provided the \textit{UNC79} knockout mouse line. Dr. Dejian Ren started the project, developed the cDNA constructs and helped design experiments.
CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

More than a hundred years ago, Sydney Ringer invented the Ringer’s solution which has been used for countless *in vitro* physiological studies. Over the past one hundred years, the major cation components, Na\(^+\), K\(^+\) and Ca\(^{2+}\), have been found to control the membrane excitability, especially via ion channels. The molecular identity of some of the ion channels involved has just begun to be uncovered. In this thesis, my colleagues and I discovered that, in mammalian hippocampal neurons, the ion channel NALCN provides a major contribution to the neuronal excitability control by both extracellular Na\(^+\) and Ca\(^{2+}\). Considering that extracellular K\(^+\) regulates neuronal excitability through many channels including the 15 two-pore domain K\(^+\) leak channels (Goldstein et al., 2005), our discovery that NALCN is responsible for the major action of both Na\(^+\) and Ca\(^{2+}\) is surprising. In addition, while NALCN forms a background “leak” conductance, it is a regulated leak that can be activated by peptide neurotransmitters and influenced by extracellular Ca\(^{2+}\). In summary, this thesis project revealed multiple functions of NALCN in neuronal excitation and provided insights into how neuronal excitability is controlled at the basal level and under certain stimuli.

In *Chapter II*, our data established NALCN as a voltage-independent, non-selective cation channel. We further identified the channel as a major contributor to the background Na\(^+\) leak conductance that is required for the basal level of neuronal excitability.

In *Chapter III* and *Chapter IV*, we found that NALCN forms a complex with
two novel proteins UNC79 and UNC80, and the channel complex could be activated by two types of stimuli: peptide neurotransmitters and [Ca\(^{2+}\)]\(_e\) drops. Interestingly, both stimuli are likely to function via receptors, yet through different signaling pathways. As a result, the two stimuli have synergistic effects when applied simultaneously.

Our results have revealed several relatively well-defined roles of NALCN in neuronal excitability control; they also raise many interesting questions for further studies. In this chapter, I will briefly discuss several future directions of NALCN-related research, including potential disease-related studies.

The Structure-function Relationship of NALCN

On the molecular level, the biophysical property of the NALCN current and its relationship with the NALCN protein structure need further investigation. The S4 segments of NALCN, while missing many conserved positively charged residues, still maintain most of them (Figure 2.1A). Why the NALCN current is voltage-independent in the presence of these positively charged residues remains an interesting question. One possible way to address this question is to record the currents from channel chimeras between Na\(_V\)s and NALCN or between K\(_V\)s and NALCN. A similar study of chimera proteins of Na\(_V\)s and K\(_V\)s revealed important information about Na\(_V\)s’ voltage-sensor functions (Bosmans et al., 2008). The experimental design would be to replace the voltage sensors of K\(_V\)s and/or Na\(_V\)s with the corresponding segments of NALCN, and to test how the voltage-dependence of the current is altered, if the channels are still functional. These studies may reveal which residue(s) in NALCN change the voltage sensitivity, and in which way. On the other hand, our data showed that NALCN is
voltage-independent within the range of -100 mV to +100 mV, but it is yet to be
determined whether the voltage-independence persists in wider voltage ranges.
Voltage-clamping at extreme voltages >100 mV might be difficult in HEK293T cells. In
addition, these cells have endogenous TRPM7 current which contaminates the recording
especially at membrane potentials >+50 mV. If NALCN could be functionally
expressed in the Xenopus oocyte expression system, it would be easier to test the
voltage-independence in a much wider voltage range.

Another important biophysical feature of the NALCN channel is its selectivity, or
the lack of. The putative selectivity filter of NALCN (EEKE) is a mixture of those of
NaVs (DEKA) and CaVs (EEEE, Figure 2.1), and the NALCN channel turned out to be
relatively nonselective among the major monovalent cations K⁺, Na⁺ and Cs⁺. The
channel is largely impermeable to Tris⁺ and NMDG⁺, probably because of the large sizes
of these ions. A NALCN mutant with a single point mutation in the EEKE signature
motif (to EEKA) has a decreased sensitivity to Gd³⁺ and a lowered permeability to Ca²⁺
(Lu et al., 2007). The EEKA mutant confirmed the importance of the EEKE motif in
the ion filter of NALCN. However, the relative complete loss of selectivity in wild-type
NALCN is somewhat unexpected, since a Naᵥ mutant with a similar selectivity filter
signature (DEKE) still maintains much of its selectivity, with \( P_{Na} >> P_K \) and no detectable
\( P_{Ca} \) (Heinemann et al., 1992). Moreover, even when the selectivity filter motif of
NALCN is mutated into EEKA, which closely resembles those of Naᵥs (DEKA), the
channel is still relatively non-selective. It is likely that NALCN’s selectivity is
determined not only by the EEKE motif but also the residues adjacent to it. Further
mutagenesis studies of these adjacent residues are needed for a better understanding of
the selectivity determination of the 24TM family channels.

**Intracellular Signaling Pathways of NALCN Activation**

Our results uncovered at least two pathways that can lead to NALCN activation. Both of the pathways are through G-protein coupled receptors (GPCRs) and require the NALCN-associated protein UNC80, yet they operate through different intracellular signaling mechanisms.

The neuropeptide substance P (SP) activates NALCN through a G-protein independent, but Src family kinases (SFKs) dependent pathway. However, how the SFKs couple to the SP receptor TACR1 and to NALCN activation via UNC80 is not clear. TACR1 may undergo endocytosis upon activation, which leads to a recruitment of β-arrestins that bind to and activate SFKs (DeFea et al., 2000). It would be interesting to test whether the same is true in the NALCN activation pathway. As for the coupling between SFKs and the NALCN-UNC80 complex, our lab has shown that UNC80 functions as a scaffold for Src kinases in NALCN channel function: UNC80 physically interacts with Src and can be phosphorylated by Src. UNC80 also enhances the phosphorylation of NALCN by Src (Wang and Ren, 2009). Whether the NALCN activation is mediated by direct phosphorylation of NALCN or UNC80 would be important to determine, and this can be tested by mutagenesis of potential phosphorylation sites. The coupling between SFKs and NALCN might also be independent of UNC80 upon activation by other receptors. A recent study showed that NALCN could be activated by muscarinic acetylcholine receptor type 3 (M3AchR) through a similar G-protein-independent and SFK-dependent pathway (Gilon and
However, the activation did not seem to require UNC80. NALCN can also be activated by lowering extracellular \([\text{Ca}^{2+}]\) in the hippocampal neurons. Our data suggested that extracellular \(\text{Ca}^{2+}\) inhibits NALCN, likely through the \(\text{Ca}^{2+}\)-sensing receptor GPCR, and that lowering extracellular \(\text{Ca}^{2+}\) releases this inhibition. Several other GPCRs are also able to sense \([\text{Ca}^{2+}]_e\). Further studies are needed to establish the identities of the \(\text{Ca}^{2+}\)-sensing receptors coupled to the NALCN complex \textit{in vivo}. In addition, the mechanisms by which the receptor(s) are coupled to the channel complex need to be probed.

Interestingly, the activation of NALCN both by SP and by low \([\text{Ca}^{2+}]_e\) requires UNC80. The two stimuli also have synergistic effects on the channel, suggesting that they influence distinct domains in UNC80, or distinct domains in NALCN through UNC80. Consistent with the second prediction, the low \(\text{Ca}^{2+}\) activation is dependent on the carboxyl-termini tail of NALCN, while the neuropeptide activation does not require this region. Furthermore, UNC80 interacts with two distinct regions of NALCN, one in the carboxyl-terminal tail, and the other in the loop between the second and third repeats (the II-III loop). Interestingly, overexpression of the carboxyl-terminal tail alone is able to disrupt the interaction between the NALCN II-III loop and UNC80 (unpublished data from Dr. Qi Zhang in our lab). This suggests a possibility that, by regulating the II-III loop interaction with UNC80, the NALCN carboxyl-terminal tail may function as a molecular switch to control channel activation by receptors. Further detailed studies on the interaction domains followed by mutagenesis may give new insight into the mechanism of NALCN activation.
Potential Roles of NALCN in Pathophysiology

NALCN is involved in many physiological functions. Earlier studies of the *Drosophila* NALCN homolog (*na*) revealed its role in circadian rhythm control. The hypomorphic alleles in the fruit flies with reduced expression of the NALCN homolog, although viable and fertile, have altered locomotive behavioral circadian rhythms (Lear et al., 2005; Nash et al., 2002). Later studies with *Drosophila melanogaster* and *C. elegans* knockout models of NALCN homologs suggested that NALCN also plays essential roles in the animal’s sensitivity to anesthetics and the generation of locomotion patterns (Humphrey et al., 2007; Jospin et al., 2007; Pierce-Shimomura et al., 2008; Yeh et al., 2008). Unlike the invertebrate NALCN knockout models, the NALCN knockout mice are not viable 24 hours after birth, probably due to a disruption of the normal respiratory rhythm (Lu et al., 2007). Further functional characterization of the mammalian NALCN would be greatly facilitated if a conditional NALCN knockout mouse line could be established and used to study the adult mutant.

The multiple physiological functions of NALCN raise the possibility of targeting NALCN for future therapeutic treatment and disease research. A few possible directions of NALCN related disease studies are discussed as follows.

**Epilepsy.** Epilepsy is a common chronic neurological disorder characterized by repeated episodes of seizures, which are symptoms of excessive or synchronous neuronal activities in the brain (Fisher et al., 2005). Epileptiform activities can be induced in several brain slice preparations by lowering $[\text{Ca}^{2+}]_e$ in the absence of chemical synaptic transmission (Albrecht and Heinemann, 1989; Albrecht et al., 1989; Jefferys, 1998; Jefferys and Haas, 1982; Richardson and O'Reilly, 1995). Our discovery that NALCN
provides a major contribution to the excitatory effect of low $[\text{Ca}^{2+}]_e$ implies a possible role of NALCN in epilepsy. Consistent with this possibility, our results showed that several idiopathic epilepsy syndrome-associated $\text{Ca}^{2+}$-sensing receptor mutants are poorly coupled to NALCN, compared to the wild-type receptor. To further test this possibility, hippocampal slices from $\text{NALCN}$ or $\text{UNC79}$ knockout mice, together with those from wild-type, could be used for low $\text{Ca}^{2+}$ perfusion, or other epileptiform inducing treatments, to test whether the mutant is more resistant to the induction of epilepsy. Similar studies could be carried out at the animal level as well. Finally, clinical studies could be performed for possible linkage between NALCN mutations and inherited epilepsy. Given that NALCN may broadly regulate the basal neuronal activities by providing the Na$^+$ leak conductance, even if NALCN were not directly linked to epilepsy, it may still be a target for epilepsy treatment by reducing basal neuronal excitability.

**Hypocalcaemia/Hypercalcaemia.** Serum $\text{Ca}^{2+}$ levels in the blood are controlled within the physiological range (2.12 mM to 2.55 mM) (Zieve, 2007). Levels that are too low or too high are referred to as hypocalcaemia or hypercalcaemia, respectively. Abnormal levels of plasma $\text{Ca}^{2+}$ may be asymptomatic, but under severe conditions, may also lead to life-threatening disorders including numerous neuropsychiatric and cardiovascular symptoms. The $\text{Ca}^{2+}$ levels can be detected (“sensed”) by the parathyroid cells via $\text{Ca}^{2+}$-sensing receptor (CaSR), a G-protein-coupled-receptor activated by extracellular $\text{Ca}^{2+}$ (Brown et al., 1995a). CaSR activation by extracellular $\text{Ca}^{2+}$ leads to an activation of the phospholipase C pathway, and an inhibition of the release of the parathyroid hormone (PTH), which acts to increase $\text{Ca}^{2+}$ concentration in the blood (Brown, 2007). PTH release could also be stimulated
by membrane potential depolarization induced by high $[K^+]_e$ (Dempster et al., 1982; Morrissey and Klahr, 1983). Our results showed that CaSR is coupled to the NALCN current, and, as a consequence, to the membrane potential. This functional interaction between CaSR and membrane potential via NALCN may provide a mechanism of coupling between CaSR and PTH release. However, little is known about NALCN in the parathyroid cells. Biochemical and electrophysiology approaches are needed to test the expression and function of NALCN in these cells. If NALCN is functional in those cells, its coupling to the CaSR could then be tested. Measurement of serum Ca$^{2+}$ and PTH release in NALCN knock-down or knockout mouse models could then be utilized to test whether NALCN plays a role in these physiological functions.

How neuronal excitability is controlled is one of the most fundamental questions in neuroscience. It is also pertinent to important physiological functions and diseases. Our discovery of NALCN’s roles in neuronal excitability control is therefore of scientific importance as well as potential pharmaceutical and therapeutic values. Future studies will need to solve the many mysteries related to the properties and functions of the NALCN-UNC79-UNC80 complex, its regulation, and its roles in human health and diseases.
BIBIOGRAPHY


Physiol 58, 349-362.


chemosensitivity. Annu Rev Neurosci 26, 239-266.


channel characteristics conferred on the sodium channel by single mutations. Nature 356, 441-443.


channels coupled with tachykinin receptors in rat sensory neurons. J Neurophysiol 73, 736-742.


and mechanism of changes in extracellular K+ and Ca2+ concentrations in the hippocampus. Can J Physiol Pharmacol 60, 1658-1671.


Brain Res Bull 70, 391-405.


tachykinins. Physiol Rev 73, 229-308.


receptor: molecular cloning in rat and localization to nerve terminals. Proc Natl Acad Sci USA 92, 3161-3165.


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Calcium sensing properties of the GABA(B) receptor. Neuropharmacology 38, 1647-1656.


