Potassium and Sodium Currents Regulating Pacemaking and Burst Firing in Substantia Nigra Dopamine Neurons

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Potassium and Sodium Currents Regulating Pacemaking and Burst Firing in Substantia Nigra Dopamine Neurons

Abstract

Dopamine-releasing neurons with cell bodies in the substantia nigra pars compacta (SNc) are a primary source of dopamine in the mammalian brain. Dysfunction of dopaminergic signaling is associated with numerous psychiatric disorders, and degeneration of the SNc is one of the hallmarks of Parkinson’s disease. These neurons are autonomous pacemakers. Their spontaneous action potentials supply target areas with baseline dopaminergic tone, while synaptically-triggered bursts signal salient events. My goal was to understand the ionic currents that regulate spontaneous and burst firing in these neurons, using acutely dissociated somata from mouse SNc.

Little is known about the potassium channels that participate in action potential repolarization in SNc neurons. Chapter 2 describes major complementary contributions of large-conductance calcium-activated potassium (BK) channels and voltage-gated Kv2 channels. Inhibiting either type of channel individually had little effect on pacemaking because the resulting small spike broadening recruited more current through the other type and because there is a functional “reserve” of both types. In contrast, these channels regulate evoked burst firing in distinct ways: the frequency of evoked firing was increased by inhibition of Kv2 channels but decreased by inhibition of BK channels. The opposing effects on burst firing can be understood through the different channel kinetics, with BK channels activating and deactivating much faster than Kv2 channels.

Sodium channels are critical components of action potential generation. Current models of SNc firing rely on sodium channel data obtained at reduced temperatures using non-physiological
solutions. Chapter 3 describes characteristics of voltage-gated sodium channels in SNc neurons at 37°C using physiological ionic conditions. Based on these results, we constructed a computational model of voltage-gated sodium channels and explored how their gating helps regulate both pacemaking and burst-like firing.

A variety of peptide toxins have been critical for separating currents carried by particular ion channels in central neurons. Chapter 4 describes the surprising observation that SNX-482, commonly used as a specific inhibitor of R-type Cav2.3 channels, is actually even more potent as an inhibitor of A-type potassium current in SNc neurons. Further experiments using transfected HEK-293 cells revealed that SNX-482 inhibits both Kv4.3 and Kv4.2 channels.
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Chapter 1
Introduction

Electrical excitability in the nervous system is produced by the finely-coordinated activity of ion channels (Hille, 2001). Ion channels integrate synaptic events, initiate (or fail to initiate) and propagate action potentials, and enable release of neurotransmitters onto downstream cells, thereby allowing rapid communication between neurons even over long distances. While voltage-gated sodium channels and delayed rectifier potassium channels are alone sufficient to generate action potentials (Hodgkin and Huxley, 1952a-d), in the mammalian nervous system, interactions among an extensive repertoire of voltage- and ligand-gated channels produce a multitude of spike shapes and firing patterns (Bean, 2007).

Neuronal activity is finely regulated, and aberrations in ion channel function can lead to dramatic physiological deficiencies. A wide range of disorders termed “channelopathies” results from ion channel mutations (Cain and Snutch, 2011; Savio-Galimberti et al., 2012; Waxman and Zamponi, 2014). These include several forms of epilepsy (Fletcher et al., 1996; Vacher and Trimmer, 2012; Catterall, 2014), pain disorders (Yang et al., 2004; Cox et al., 2006; Fertleman et al., 2006; Faber et al., 2012a, b), ataxia (Fletcher et al., 1996; Ophoff et al., 1996; Mori et al., 2000), and migraine (Ophoff et al., 1996; Pietrobon, 2013). Some drugs restore normal function or rescue proper physiological activity through their actions on ion channels. For example, many anticonvulsant drugs dampen neuronal excitability by altering channel gating, typically by promoting sodium channel inactivation or by enhancing activation of potassium channels (Kuo, 1988; Gunthorpe et al., 2012).
Neuronal membrane potentials reflect the coordinated activity of numerous types of channels. The complexity of simultaneous interactions among multiple conductances, including many which themselves depend on transmembrane voltage, makes it difficult to predict \textit{a priori} how a particular type of channel contributes to overall excitability (e.g. Jackson and Bean, 2007; Kispersky et al., 2012). Disparate sets of ion channels can produce extremely similar electrical outputs (Goaillard et al., 2009; O’Leary et al., 2013). In some contexts, drastic alterations in channel properties have little effect on spiking behavior, while in others, small modulations produce dramatic changes (Goldman et al., 2001; Burdakov and Ashcroft, 2002; Swensen and Bean, 2005). For this reason, understanding properties of individual ionic currents in isolation has been a critical step towards understanding how they jointly contribute to electrical excitability. Voltage clamp experiments, which allow currents to be recorded in response to well-defined transmembrane voltages, have been extremely useful in unraveling the separate components of neuronal excitability, and models informed by these types of experiments have been very successful in predicting spiking behavior (Hodgkin and Huxley, 1952d; Connor and Stevens, 1971). The primary focus of this thesis was improving our understanding of the ion channels that regulate action potentials in dopaminergic neurons of the \textit{substantia nigra pars compacta} (SNC).

The SNC is a nucleus of mostly dopaminergic neurons located in the midbrain, situated ventral to the midbrain reticular nucleus and along the dorsal surface of the \textit{substantia nigra pars reticulata} (SNr). Along with the nearby but more medially located ventral tegmental area (VTA), it is one of the primary sources of dopamine in the mammalian brain. Dopaminergic signaling has many behaviorally significant functions and is involved in motor control, salience encoding, and reward-based learning (Schultz, 2002; Schultz, 2007). Its dysregulation has been implicated in numerous psychiatric disorders, such as schizophrenia, ADHD, and addiction (Grace, 1991; Oades, 1987; Dalley and Roiser, 2012; Koob and Le Moal, 1997); the degeneration of dopamine cells of the SNC in particular is the hallmark of Parkinson’s disease and accounts for many of its symptoms.
(Ehringer and Hornykiewicz, 1960; Schober, 2004). Many neuroleptic drugs act by inhibiting dopamine receptors and produce side effects that mimic Parkinson’s, including dyskinesia, bradykinesia, rigidity, and tremor (Mackay, 1981). The disease states brought about by abnormal dopaminergic signaling point to important physiological roles for dopamine neurons and the ion channels governing their excitability.

**Connections and functions of SNc**

The primary and best-studied connection of the SNc is to the striatum, where it modulates cortical and thalamic inputs to the basal ganglia (Surmeier et al., 2011a) and from which it receives substantial inhibition (Somogyi et al., 1981; Watabe-Uchida et al., 2012). SNc neurons innervate GABAergic medium spiny neurons (MSNs), which are the principal cells of the striatum, as well as several types of striatal interneurons. MSNs are segregated into two classes based on their connectivity and receptor expression patterns: direct pathway MSNs express D1 dopamine receptors and project to the SNr; indirect pathway MSNs express D2 dopamine receptors and project to the external part of the globus pallidus. According to a classic model, the direct and indirect pathways of the basal ganglia operate as two parallel systems with opposing effects: the direct pathway promotes movement, while the indirect pathway suppresses it (Albin et al., 1989). Activating the direct pathway increases inhibition of the SNr, which sends an inhibitory projection to motor regions of the thalamus, resulting in disinhibition of motor signals. Activating the indirect pathway inhibits the external part of the globus pallidus, causing disinhibition of the subthalamic nucleus (STN). The STN is glutamatergic and tonically excites both the SNr and medial pallidal regions, which inhibit the thalamus, so the overall effect of indirect pathway activation is inhibition of motor signals. On striatal MSNs, background levels of dopamine supplied by the SNc act at high-affinity D2 receptors, providing inhibition of the indirect pathway. Transient increases in dopamine release are thought to activate lower-affinity D1 receptors on direct pathway neurons, increasing
their excitability and facilitating movement. The wiring of the basal ganglia is an area of ongoing research, and although this model fails to take into account the effects of dopamine on striatal interneurons and their downstream targets, it nonetheless predicts several aspects of basal ganglia function and dysfunction, including the paucity of movement seen in Parkinson's patients.

The symptomology of Parkinson's established a role for the SNc in control of voluntary movements, but the SNc is also involved in signaling salient and reward-related sensory stimuli. Excitatory inputs arise from areas related to sensory information or movement, including the superior colliculus (Comoli et al., 2003; Coizet et al., 2006); somatosensory, motor, and prefrontal cortices (Tong et al., 1996; Naito and Kita, 1994; Watabe-Uchida et al., 2012); the parabrachial and subthalamic nuclei (Coizet et al., 2010; Iribe et al., 1999); and the pedunculopontine tegmentum (Clarke et al., 1987; Futami et al., 1995; Floresco et al., 2003; Watabe-Uchida et al., 2012).

Numerous inhibitory inputs to SNc arise from elsewhere in the basal ganglia. A prominent source of inhibition is the SNr, an adjacent nucleus of GABAergic projection neurons into which SNc cells extend their dendrites (Lee et al., 2004; Grace and Bunney, 1979). It appears that several basal ganglia nuclei send GABAergic projections to both SNc and SNr, and that monosynaptic inhibition of SNc is typically dominated by disynaptic disinhibition through effects on SNr (Lee et al., 2004; Grace and Bunney, 1985). In addition to these GABAergic inputs, SNc neurons also release dopamine somatodendritically within the SNc itself, where it acts on autoreceptors to dampen subsequent excitability and dopamine release (Bernardini et al., 1991; Benoit-Marand et al., 2001).

**Cellular effects of dopamine**

Dopamine receptors are G protein-coupled and are grouped into two families: D1-like, comprising D1 and D5 receptors, and D2-like, comprising D2, D3, and D4 receptors. As is typical for transmitters acting at GPCRs, the effects of dopamine are complex and depend on the identity and state of the downstream neuron (reviewed in Neve et al., 2004; Beaulieu and Gainetdinov, 2011;
and Tritsch and Sabatini, 2012). D1-like receptors tend to be coupled to $\text{Ga}_{s/o}$ and are positively coupled to adenylyl cyclase (AC) activity, which enhances production of cyclic adenosine monophosphate and protein kinase A. Activation of D1 receptors on direct pathway MSNs is thought to increase excitability by enhancing surface expression of NMDA receptors (Hallett et al., 2006), and possibly also by more direct interactions (Liu et al., 2004). In other cell types, D1 receptor activation can also lead to decreases in sodium channel and N-type calcium channel availability (Cantrell and Catterall, 2001; Kisilevsky et al., 2008); it is unknown whether these processes also take place in striatal neurons.

D2-like receptors are generally coupled to $\text{Ga}_{i/o}$, and their activation conversely inhibits AC. Following dissociation of the $\text{Ga}$ subunit, the freed $\text{G}b\gamma$ complex may also diffuse within the membrane to exert additional effects, including activating a hyperpolarizing current through G-protein activated inwardly rectifying potassium (GIRK) channels of the $K_{\text{ir}}3$ family and inhibiting voltage-gated calcium channels. For indirect pathway MSNs, activation of D2 receptors decreases AMPA receptor and L-type calcium currents, dampening their excitability (Hernández-Echeagaray et al., 2004; Hernández-Lopez et al., 2000). Dopamine neurons themselves express D2 and D3 autoreceptors; while the effects of D3 receptor activation are not clear, D2 receptors on dopamine neurons are coupled to GIRK current activation (Davila et al., 2003; Gantz et al., 2013).

Heteromultimers of D1 and D2 receptors may couple to $\text{Ga}_q$, activating phospholipase C, increasing production of inositol triphosphate and diacylglycerol, and facilitating calcium release from intracellular stores. Dopamine receptors may additionally modulate the function of ligand-gated ion channels via direct interactions. Because the targets of dopamine neurons are likely to receive modulatory inputs from multiple sources simultaneously, it is difficult to make general statements about specific effects of dopamine on neuronal circuits.
Electrophysiology of dopaminergic neurons

In vivo, two distinct modes of firing have been observed in SNc dopamine cells: regular (and sometimes irregular) single-spike firing, and burst firing (Grace and Bunney, 1984a, b). Single-spike pacemaking is thought to be important for establishing baseline dopaminergic tone in target brain regions, while burst firing is associated with transient increases in dopamine release (Gonon, 1988). Pacemaking occurs cell-autonomously; firing rate and pattern are both modulated by synaptic inputs (Kitai et al., 1999). A significant body of work describes some of the ion channels important for spontaneous firing and for the transition between tonic pacemaking and burst firing, but a complete picture is still lacking. Multiple different sets of ionic conductances can produce similar outputs in terms of firing pattern, but understanding the details of the currents involved is necessary to accurately predict how modulation either by endogenous peptides or by therapeutics will affect spike rate, firing pattern, and responses to additional perturbation.

Depolarizing currents

In many types of pacemaking neurons, spontaneous depolarization results mainly from subthreshold or persistent sodium current (Taddese and Bean, 2002; Do and Bean, 2003; Khaliq and Bean, 2010; Yamada-Hanff and Bean, 2013). In SNc dopamine cells, both sodium current and low voltage-activated L-type calcium current through Cav1.3 channels play important roles in the depolarization leading up to action potential threshold (Durante et al., 2004; Chan et al., 2007; Puopolo et al., 2007; Guzman et al., 2009; Putzier et al., 2009). One proposed explanation for the deterioration of SNc neurons in Parkinson’s disease is that the large calcium influx during pacemaking and burst firing makes SNc dopamine cells especially vulnerable to oxidative stress and mitochondrial dysfunction (Chan et al., 2010; Surmeier et al., 2011b).

In addition to L-type channels, SNc dopamine neurons also contain P/Q-, N-, T-, and R-type calcium channels (Cardozo and Bean, 1995; Durante et al., 2004). Inhibiting P/Q-type channels can
slow or stop spontaneous firing, indicating a potential role for these channels in pacemaking as well (Puopolo et al., 2007). Based on effects of TRP channel blockers and ionic substitution, it appears that SNc neurons also have substantial resting cation and sodium conductances (Puopolo et al., 2007; Kim et al., 2007; Mrejeru et al., 2011).

The hyperpolarization-activated cation current $I_h$, mediated in dopaminergic SNc neurons primarily by HCN2 and HCN4 channels (Franz et al., 2000; Neuhoff et al., 2002; Dufour et al., 2014), has often been used to identify SNc dopamine neurons electrophysiologically. $I_h$ is an inward current activated at hyperpolarized potentials, so it has been an attractive candidate for the pacemaking generator. However, its role is unclear, as inhibiting $I_h$ with ZD-7288 usually does not stop pacemaking (Puopolo et al., 2007; Guzman et al., 2009). While it may contribute to the depolarization in some SNc neurons, other currents are involved as well; other functions of $I_h$ remain to be understood. Some neurons within SNc cells lack $I_h$ but express tyrosine hydroxylase, a key enzyme required for dopamine synthesis, suggesting that the lack of $I_h$ is not sufficient to rule out dopaminergic identity (Neuhoff et al., 2002).

Currents implicated in burst firing

In vivo, application of glutamate or glutamatergic agonists to dopaminergic SNc neurons induces burst firing (Grace and Bunney, 1984b). Stimulation of the pedunculopontine nucleus, which supplies glutamatergic and cholinergic inputs to SNc, can also evoke bursting, as can relief of GABAergic inhibition (Hong and Hikosaka, 2014; Lokwan et al., 1999; Tepper and Lee, 2007). It is unclear and somewhat controversial how well burst firing can be reproduced in slice preparations, but burst-like activity can be evoked by a variety of experimental protocols. These include stimulating glutamatergic afferents or applying AMPA and NMDA receptor agonists (Blythe et al., 2007), blocking the small conductance calcium-activated potassium (SK) current while injecting
depolarizing current (Johnson and Wu, 2004), simultaneously activating $K_{\text{ATP}}$ channels and NMDA receptors (Schiemann et al., 2012), and injecting depolarizing current alone (Blythe et al., 2009).

In addition to transmitter receptors, several channels have been identified that influence the transition between tonic and burst firing. In the absence of functional $K_{\text{ATP}}$ channels, burst firing is eliminated from a subpopulation of SNc neurons (Schiemann et al., 2012). Blockade of SK channels in vitro promotes bursting (Shepard and Bunney, 1988), and positive or negative modulation of these channels in vivo increases or decreases firing regularity (Herrik et al., 2010). Ether-a-go-go related gene (ERG) potassium channels (Ji et al, 2012), M-type channels (Drion et al., 2010), and transient receptor potential (TRP) channels (Mrejeru et al., 2011) have also been identified as potentially being involved in burst firing.

Hyperpolarizing currents

Most of what is known about the potassium channels in SNc neurons revolves around potential involvement in the control of burst firing; less is known about the hyperpolarizing currents tuning action potentials. In addition to their potential role opposing burst initiation, SK channels, particularly SK3, appear to influence regularity of firing (Wolfart et al., 2001). In dopamine neurons of the VTA, A-type potassium current ($I_{A}$) counteracts the pacemaker depolarization and stabilizes slow, regular firing (Khaliq and Bean, 2008). SNc dopamine neurons also have $I_{A}$, mediated by Kv4.3 channels; expression of these channels is inversely correlated with firing frequency, suggesting that they play a similar role in SNc neurons (Liss et al., 2001; Hahn et al., 2003). Additional potassium channels have been identified in SNc dopamine neurons, including Kv1 and Kv3 (Dufour et al., 2014), but their precise functions are unknown.
Summary of research

I began this research with the goal of understanding more about the ionic mechanisms contributing to pacemaking and burst firing in dopamine neurons. I pursued this question using a preparation of acutely dissociated cell bodies, which lack cellular processes and surrounding brain tissue, but continue to generate spontaneous action potentials. This preparation offers several advantages for investigating the ionic currents important for spontaneous pacemaking. Isolated somata are electrotonically compact, making voltage clamp experiments more tractable than in neurons in more intact settings. And because there is no tissue surrounding the cell bodies after dissociation, it is possible to quickly and completely exchange the solutions bathing the cell, thus facilitating pharmacological experiments. Meanwhile, the persistence of pacemaking even following dissociation suggests that the currents essential for generating spontaneous action potentials are present within the cell body. This makes it possible to investigate the relevant channels in a system where currents can be evoked in response to well-defined voltages, and where they can be pharmacologically isolated using well-defined drug concentrations.
Chapter 2

Kv2 and BK channels in substantia nigra dopamine neurons differentially regulate pacemaking and burst-like firing

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Abstract

Dopamine neurons of the substantia nigra pars compacta (SNc) have two major modes of activity: slow tonic pacemaking and higher-frequency bursts evoked by synaptic input. Substantial work has explored the ionic mechanisms underlying the spontaneous depolarization of these cells and the commencement and cessation of burst firing, but less is known about the voltage-dependent potassium currents contributing to spike repolarization and to the timing of action potentials within a burst. Using action potential clamp experiments with acutely-dissociated mouse SNc dopamine neurons at 37°C, we found that the outward current flowing during spike repolarization included major contributions from BK calcium-activated potassium channels and Kv2 channels. Given the large BK current during action potentials, we were surprised that in current clamp experiments, blocking BK current with paxilline had no clear effect on firing frequency and only small effects on spike shape. Similarly, inhibiting Kv2 channels using Guangxitoxin-1E (GxTx) also had no apparent effect on spontaneous firing frequency. However, co-
application of paxilline and GxTx produced a dramatic decrease in firing frequency, an increase in spike width, and a reduction in the AHP. Further experiments revealed that acute inhibition of either BK or Kv2 channels led to recruitment of additional current through the other channel type, minimizing changes in firing rate. While inhibition of either Kv2 or BK channels alone had no effect on spontaneous firing frequency, higher-frequency firing evoked by current injection was differentially altered. Blocking BK channels resulted in reduction in maximal firing frequency and flattening of the F-I curve, while inhibiting Kv2 channels resulted in faster firing, steeper F-I curves, and increased susceptibility to depolarization block. This suggests that BK and Kv2 channels have a high degree of “redundancy” during spontaneous pacemaking but differentially regulate evoked firing.
Introduction

Activity in the nervous system is finely tuned by the diverse populations of ion channels expressed by different types of neurons (Llinás, 1988; Hille, 2001). In general, the relationship between the ion channels found in a cell and the cell’s electrical input-output properties is complex and impossible to predict a priori. In some circumstances, very different sets of ion channel populations can produce similar outputs; in others, small modulations in even one conductance can lead to dramatic changes in overall excitability (Goldman et al., 2001; Swensen and Bean, 2005; Amendola et al., 2012). Dopaminergic neurons of the substantia nigra pars compacta exhibit two main modes of activity: spontaneous pacemaking and evoked burst firing (Grace and Bunney, 1984a, b). We investigated how different potassium currents interact to support tonic activity and burst-like firing in acutely dissociated SNc dopaminergic somata.

Recent work has identified large-conductance calcium-activated potassium (BK) channels in SNc dopamine cells (Su et al., 2010; Ramírez-Latorre, 2012). However, the physiological role of BK current in these cells remains unknown. In contrast to many other types of pacemaking neurons, the subthreshold depolarization of SNc dopamine cells is significantly supported by calcium current (Durante et al., 2004; Puopolo et al., 2007; Chan et al., 2007; Putzier et al., 2009; Guzman et al., 2009; Drion et al., 2011). This unusually large calcium influx has been proposed to contribute to these cells’ vulnerability to oxidative stress and to their degeneration in disease states such as Parkinson’s. It also positions BK current as a potentially key player in AP repolarization.

Like most central neurons, SNc dopamine cells also express Kv2 “delayed-rectifier” channels (Dufour et al., 2014; Khaliq and Bean, submitted). Kv2 channels have slow activation, deactivation, and inactivation kinetics (Misonou et al., 2005; Johnston et al., 2010). Nevertheless, they have been shown to contribute to the repolarization following action potentials in several neuronal types, with inhibition of Kv2 being associated with spike broadening and increased inter-spike voltages.
(Malin and Nerbonne, 2002; Tong et al., 2013; Liu and Bean, 2014). Until recently, investigation of Kv2 function was hampered by the lack of good pharmacological tools. However, Guangxitoxin-1E (GxTx), a peptide isolated from the venom of the Chinese tarantula *Plesiophriactus guangxiensis*, has been identified as a potent and relatively selective inhibitor of the channels (Herrington et al., 2006; Herrington, 2007). We made use of this new tool to additionally probe the contribution of Kv2 channels to the potassium current regulating SNc excitability.
**Methods and Materials**

**Preparation of acutely dissociated dopamine cells**

Dissociated dopamine neurons were prepared from 13- to 19-day-old male and female mice. After isoflurane anesthesia, mice were decapitated, and the brain was quickly removed into ice-cold solution containing (in mM) 110 NaCl, 2.5 KCl, 10 HEPES, 25 glucose, 75 sucrose, 7.5 MgCl2, pH adjusted to 7.4 with NaOH and bubbled with 95/5% O2/CO2. In the same solution, 200 µm coronal slices were cut using a vibratome (DSK model DTK-1000; Dosaka). The substantia nigra was dissected out of each slice and rinsed in dissociation solution containing (in mM) 82 Na2SO4, 30 K2SO4, 5 MgCl2, 10 glucose, 10 HEPES, pH adjusted to 7.4 with NaOH.

Following dissection, the pieces of the SNc were incubated for 7-8 minutes at 34°C in 3 mg/mL protease XXIII (Sigma Life Science) dissolved in dissociation solution. After enzymatic treatment, the tissue pieces were rinsed in ice cold dissociation solution containing 1 mg/mL trypsin inhibitor and 1 mg/mL BSA, where they were stored until immediately prior to recording. Cells were used within 8 hours of dissection. Just before recording, chunks of tissue were gently trititated in dissociation solution using a fire-polished Pasteur pipette to free individual cells. The resulting suspension was dispersed into the recording chamber. The cells were allowed to settle for 10-15 minutes, after which time the recording chamber was flooded with Tyrode’s solution.

Some recordings were made from mice expressing eGFP under control of the tyrosine hydroxylase promoter (Sawamoto et al., 2001), kindly provided by Dr. Kazuto Kobayashi, (Fukushima Medical University). This allowed unambiguous identification of dopaminergic neurons. Some recordings were also made from wild-type littermates or from wild-type Swiss-Webster mice, in which case dopaminergic neurons could be identified by their large size. Neurons from wild-type mice invariably had large I\textsubscript{A} currents and typically fired spontaneous actions potentials at low frequencies, consistent with their dopaminergic phenotype. Cells with
spontaneous firing rates higher than 10 Hz were excluded from the analysis. All cells used for current clamp experiments were spontaneously active.

**Electrophysiological recordings**

Experimental solutions were designed to approximate physiological conditions. Standard external solution was Tyrode’s solution containing (in mM) 151 NaCl, 10 HEPES, 13 glucose, 1.5 CaCl2, 1 MgCl2, 3.5 KCl, pH adjusted to 7.4 with NaOH. Internal solution contained (in mM) 122 K-methanesulfonate, 9 NaCl, 9 HEPES, 0.18 EGTA, 0.036 CaCl2, 0.27 MgCl2, 4 MgATP, 14 creatine phosphate (Tris salt), 0.3 GTP (Tris salt), pH 7.4.

Recording pipettes were pulled from borosilicate glass (VWR International) using a Sutter Instruments P-97 horizontal puller, and wrapped with Parafilm to reduce pipette capacitance. When filled with internal solution, typical pipette resistances ranged from 1-3 MΩ. Data have been corrected to reflect a -8 mV liquid junction potential between external and pipette solutions, measured using a flowing 3 M KCl reference electrode as described by Neher (1992).

After a GΩ seal and whole-cell configuration were established, cells were lifted off the bottom of the recording chamber and placed in front of a set gravity flow perfusion pipes in order to facilitate rapid solution exchange, which could be accomplished in less than 1 s. Solutions flowing through the pipes were heated using a Warner Instruments TC-344B temperature controller, measured to be 37°C at the location of the cell. All solutions were heated identically throughout the course of the experiment.

Whole-cell recordings were performed using an Axon Instruments 700B amplifier (Molecular Devices), a Digidata 1322A A/D converter (Axon Instruments), and pCLAMP9.2 software (Molecular Devices). For voltage clamp experiments, series resistance was compensated 70-90% using the amplifier circuitry. Signals were filtered at 10 kHz and sampled at 10 or 20 μs.
Data analysis was done in Igor Pro 6.12A (WaveMetrics, Lake Oswego, OR) using DataAccess (Bruxton Software) to read pCLAMP files into Igor Pro. Current records were corrected for linear capacitative and leak current using 5- or 10- mV hyperpolarizing steps to define linear capacitance and leak currents and then subtracting appropriately scaled currents. In some cases, correction for capacity transients was imperfect as a result of amplifier saturation for large voltage steps; in these cases, 100-200 μs of the current record is blanked in the displayed records. In some cases, current was digitally filtered with a low-pass filter corresponding to a 4-pole Bessel filter with a corner frequency of 2 kHz. Data are presented as mean ± standard error of the mean; p-values were calculated using a paired or unpaired Student’s t-test.
Results

BK current is activated during action potentials in SNc dopamine cells

Single channel currents through large conductance calcium-activated potassium (BK) channels have been described in SNc dopamine cells (Su et al., 2010; Ramírez-Latorre, 2012), but the properties of the macroscopic current in these cells is unknown. We explored whether BK channels are activated during action potentials using paxilline, a selective BK channel blocker. We first tested for BK activation during voltage steps. In voltage clamp experiments using quasi-physiological solutions, depolarizing steps evoked a large outward current. A sizeable fraction of this current was blocked by 300 nM paxilline, showing that BK channels contribute substantially to the overall potassium current (Figure 2.1A). In collected results, blocking BK channels reduced the outward current at the end of a 20 ms step from -88 mV to -8 mV by 28 ± 3%. Using paxilline to isolate the current through BK channels, we found the voltage dependence of BK activation to be well-fit by a Boltzmann function with midpoint -14.3 ± 1.2 mV and slope factor 9.2 ± 0.5 mV (Figure 2.1B-D; n=12).

We next used the action potential (AP) clamp technique to test whether BK channels contribute to action potential repolarization in dopamine neurons. We recorded pacemaking activity from a cell firing spontaneously at 37°C. We then used this waveform as a voltage command in voltage clamp mode, recording the currents that flowed in response to the waveform. By subtracting current flowing in control and in 300 nM paxilline, we isolated the current flowing through BK channels during the action potential (Figure 2.1E, F). We found that paxilline-sensitive current contributes a substantial fraction of the outward current during the action potential repolarization. On average, blocking BK channels decreased the peak outward current evoked by
**Figure 2.1 Macroscopic current through BK channels.**

(A) Outward currents evoked by depolarization from -68 to -8 mV before (black) and after (red) application of 300 nM paxilline.

(B) Paxilline-sensitive BK current evoked by depolarizing voltage steps.

(C) Conductance-voltage relationship for outward current in control (black) and after (red) application of 300 nM paxilline.

(D) G-V relation for paxilline-sensitive current.

(E) Outward current evoked by action potential waveform. Top, command waveform of an AP previously recorded from a cell firing spontaneous action potentials. Bottom, currents evoked by the AP waveform in control (black) and in paxilline (red). Voltage-gated sodium channels were blocked with 1 µM TTX.

(F) Paxilline-sensitive current, determined by subtracting traces from E. Dotted vertical lines and arrows in E and F correspond to the peak voltage during the AP waveform.
Figure 2.1 (Continued)
AP waveforms by 53±3% (n=23; p=3x10⁻⁶). The paxilline-sensitive component of outward current flowed relatively early during spikes: in some cells, activation was already underway at the peak of the action potential (e.g. Figure 2.1F).

**Block of BK current has little effect on pacemaking**

We next investigated how blocking BK channels affected spontaneous firing in SNc dopamine cells. Despite the prominent contribution of BK channels to action potential repolarization, 300 nM paxilline had surprisingly small effects on spontaneous firing (Figure 2.2A, B). Overall, blocking BK channels had no discernible effect on firing rate (6.0 ± 0.6 Hz in control; 6.1 ± 0.5 Hz in paxilline; n=14; p = 0.83). However, there were small but clear changes in action potential shape. As expected from a contribution of BK channels to spike repolarization, action potentials became slightly broader. AP width measured at half-maximal amplitude increased from 1.5 ± 0.2 ms in control to 1.9 ± 0.1 ms in paxilline (n=14; p = 0.0005). In most cells, the trough of the afterhyperpolarization (AHP) became more hyperpolarized, changing from -72.9 ± 0.6 mV in control to -75.4 ± 0.4 in paxilline (n=14; p=0.001). In several cells, the peak of the action potential became more positive in paxilline than in control, consistent with BK current already being partially activated at the peak of the AP.

**Kv2 channels are also activated during action potentials**

The enhancement of the AHP by paxilline was unexpected, as BK channels activated during the spike might be expected to contribute to the AHP. One possible explanation is that the broader APs after BK block recruit additional activation of other potassium channels whose kinetic properties provide more effective hyperpolarization. We next explored what other potassium channels contribute to action potential repolarization. Kv2 channels are widely expressed in the somatodendritic regions of most central neurons (Trimmer, 1991). They activate and deactivate
Figure 2.2. Effect of paxilline on spontaneous firing.

(A) Spontaneous pacemaking at 37°C in control (left, black) and after application of 300 nM paxilline (right, blue).

(B) Signal averaged, peak-aligned waveforms over several pacemaking cycles in control (black) and 300 nM paxilline (blue). Waveforms on the right are shown on an expanded timescale.

(C-E) Collected results of paxilline's effects on firing rate (C), width at half-maximal amplitude (D), and most negative voltage reached after the action potential (E) from 14 neurons.

Data from individual cells are in light blue; dark blue points are mean values ± S.E.M. Asterisks indicate $p \leq 0.001$. 


slowly, and make variable contributions to spike repolarization (Misonou et al., 2005; Johnston et al., 2010). We tested the contribution of Kv2 channels to overall potassium current using 100 nM GxTx. At this concentration, GxTx inhibits Kv2 channels nearly completely without much effect on Kv4 channels, which are also present in dopamine cells (Liu and Bean, 2014; Liss et al., 2001).

In voltage-clamp experiments, a fairly large fraction of the depolarization-evoked outward current in dissociated SNc cells was sensitive to GxTx. When GxTx was cumulatively applied after paxilline, it inhibited a majority of the remaining current (Figure 2.3A). GxTx applied alone decreased the total outward current evoked by a step depolarization to -8 mV by 36 ± 3% relative to control (n=19). During a repolarizing step to -68 mV, GxTx-sensitive tail currents were evident, deactivating with average time constant 8.1 ± 0.7 ms (n=20). This is consistent with the expected slow deactivation kinetics of Kv2 channels (McCrossan et al., 2003; Misonou et al., 2005; Liu and Bean, 2014). We used GxTx-subtraction to isolate the current through Kv2 channels at different voltages (Figure 2.3B-D); an example GV curve is shown in Figure 2.3D. Activation of the GxTx-sensitive conductance could be well-described by a Boltzmann curve with midpoint -8.6 ± 1.2 mV and slope factor 10.4 ± 0.3 mV (n=11).

We also tested whether Kv2 channels were activated during action potentials, using GxTx to isolate the Kv2 channel current evoked by waveforms of spontaneous action potentials (Figure 2.3E). Overall, inhibiting Kv2 channels reduced the peak outward current evoked by AP waveforms by 46 ± 5% (n=19; p=3x10^-6). Figure 2.3E (middle) illustrates the paxilline- and GxTx-sensitive components of currents overlaid on the same time axis. The paxilline-sensitive component was consistently activated much earlier during the AP waveform, beginning before the peak of the action potential. In contrast, the GxTx-sensitive current began to activate later during the repolarizing phase of the action potential and continued into the AHP, consistent with the slower
Figure 2.3. Kv2 channel activation during step depolarizations and action potentials.

(A) Outward current evoked by voltage steps from -68 mV to +22 mV in control (black), after application of 300 nM paxilline (blue), and with additional application of 100 nM GxTx-1E (red).

(B) GxTx-sensitive Kv2 current during multiple voltage steps.

(C) Conductance-voltage relations of outward current in paxilline (blue) and after additional application of GxTx (red).

(D) G-V relationship for Kv2 current.

(E) Left: Currents evoked by the waveform of a spontaneous AP in control (black), in paxilline (blue), and in both paxilline and GxTx (red). Inward sodium current has been truncated. Middle: Paxilline- (blue) and GxTx- (green) sensitive components of current evoked by the AP waveform. Right: Conductances sensitive to paxilline (blue) and GxTx (green) during the AP waveform.
Figure 2.3 (Continued)
activation and deactivation kinetics of Kv2 channels. In collected results, the current through BK channels reached its peak $0.65 \pm 0.04$ ms after the peak of the action potential, while the current through Kv2 channels reached its peak $0.96 \pm 0.04$ ms after the peak ($n=26; p=8\times10^{-6}$). The BK channel current decayed to 25% of its maximal value on average $1.64 \pm 0.04$ after the peak of the AP, while the Kv2 channel current decayed to 25% of maximum $2.92 \pm 0.61$ ms after AP peak ($n=26$, $p=0.04$).

Because BK channels were activated relatively earlier during the AP waveform, more of the current through these channels was flowing when the cell was more depolarized, and the potassium driving force was therefore larger. To more directly compare the contributions from BK and Kv2 channels, we calculated the conductances sensitive to paxilline and GxTx (Figure 2.3E, right). As was reflected in the timing of the currents, the conductance supplied by Kv2 channels reached its peak later during the AP and persisted longer into the AHP.

**Effect on pacemaking of Kv2 channel inhibition**

Next, we investigated how inhibiting Kv2 channels affects spontaneous firing (Figure 2.4). Like paxilline, GxTx had no clear effect on firing rate ($5.7 \pm 0.5$ Hz in control; $5.4 \pm 0.3$ Hz in GxTx; $n=18$, $p=0.38$) but produced changes in spike shape. Comparing signal-averaged waveforms recorded in control and in GxTx revealed an increase in AP width, from $1.6 \pm 0.1$ ms in control to $1.9 \pm 0.2$ ms in GxTx ($n=18$, $p=0.03$). This is consistent with Kv2 channels contributing to the repolarization following action potentials. The AHP became less hyperpolarized in GxTx, decreasing from $-71.7 \pm 1.0$ mV in control to $-66.7 \pm 0.9$ mV in GxTx ($n=18$, $p=7\times10^{-8}$), consistent with Kv2 channels contributing to the AHP. This might be expected given their slow deactivation kinetics, and it also supports the idea that the increased AHP observed in paxilline resulted from additional recruitment of Kv2 channels.
Figure 2.4. Effect of Kv2 channel inhibition on spontaneous firing.

(A) Spontaneous pacemaking in control (left, black) and after application of 100 nM GxTx-1E (right, green)

(B) Signal-averaged, peak-aligned waveforms over several pacemaking cycles from control (black) and GxTx (green). Waveforms on the right are shown on an expanded timescale.

(C- E) Summary of results of GxTx’s effect on firing parameters for 18 neurons. Light green points are individual cells; dark green points are mean values ± S.E.M. Asterisks indicate p ≤ 0.03.
Complementary recruitment of BK and Kv2 channels

Paxilline and GxTx each inhibited a large fraction of the AP-evoked outward current, so it seemed surprising that neither of them had much effect on spontaneous firing rate. We hypothesized that when either BK or Kv2 channels were inhibited, additional current was being recruited through the other channel type. We tested this using the AP clamp technique. First, we used waveforms of spontaneous activity recorded in control and after application of 300 nM paxilline, and we compared the amount of GxTx-sensitive Kv2 current flowing during each waveform (Figure 2.5). Consistent with our hypothesis, more Kv2 current flowed during the waveform recorded in paxilline (Figure 2.5B) than during the control waveform (Figure 2.5A). To quantify this, we integrated the current flowing during each AP waveform and compared total GxTx-sensitive charge. Overall, 0.8 ± 0.1 pC of GxTx-sensitive charge flowed during the control waveform, while 1.3 ± 0.2 pC flowed during the waveform recorded in paxilline (n=18; p=9x10^-6).

In complementary experiments, we used the waveforms of spontaneous pacemaking recorded in control and after application of 100 nM GxTx as voltage commands, and we calculated the paxilline-sensitive charge flowing during each of these waveforms (Figure 2.6). As before, more paxilline-sensitive charge was recruited by the waveform recorded in GxTx (1.8 ± 0.4 pC) than by the control waveform (0.9 ± 0.1 pC; n=19; p=0.01). We used two sets of command waveforms to compare paxilline-sensitive current: one from a cell whose AP shape changed very little in GxTx, and one from a cell with a more dramatic increase in AP width. With both pairs of waveforms, the BK current was larger in response to the waveform in GxTx, but the increase was larger in response to the pair of command waveforms with a larger increase in AP width. This suggests that BK and Kv2 channels fulfill broadly similar roles during spontaneous firing of dopamine cells, and that each channel type can at least partially compensate for the acute inhibition of the other.
Figure 2.5. Additional recruitment of Kv2 channels in paxilline.

(A) Top: waveforms used as voltage command, recorded in control (black) or in paxilline (red). Bottom: Kv2 current evoked by the waveforms recorded in control (black) and paxilline (red). Kv2 current was defined by GxTx subtraction.

(B) GxTx-sensitive charge flowing during the different waveforms from 18 neurons. Dark green points are population means ± S.E.M.
Figure 2.6. Additional recruitment of BK channels in GxTx.

(A) Top: AP waveforms recorded in control (black) or 100 nM GxTx (red). Bottom: BK current evoked by the control waveform (black) or the waveform recorded in GxTx (red). BK current was defined by paxilline subtraction.

(B) Summary of paxilline-sensitive charge flowing during control and GxTx waveforms in 19 neurons. Light blue points are individual cells; dark blue points are population means ± S.E.M.
Simultaneous inhibition of both BK and Kv2 channels

It appeared that when either BK or Kv2 channels were inhibited, recruitment of the other channel type was sufficient to maintain pacemaking activity at almost the same frequency. SNc dopamine cells express multiple other types of potassium channels, including SK, Kv3, Kv4, and K\textsubscript{ATP} channels (Wolfart et al., 2001; Liss et al., 2001; Rhodes et al., 2004; Schiemann et al., 2012). We wondered whether other potassium channels were capable of compensating for the simultaneous inhibition of both BK and Kv2 channels. In the presence of both paxilline and GxTx, spontaneous pacemaking typically continued but with dramatically different characteristics (Figure 2.7). Firing rate slowed from 6.1 ± 0.4 Hz in control to 3.4 ± 0.3 Hz in both toxins (n=25; p=7x10\textsuperscript{-6}). AP width increased from 1.6 ± 0.1 ms to 7.5 ± 1.2 ms (p=4x10\textsuperscript{-5}); and the AHP became shallower, from -71.9 ± 0.7 mV to -66.3 ± 0.8 mV (p=4x10\textsuperscript{-8}). In some cells, pacemaking ceased shortly after application of both toxins, apparently from entering depolarization block. When this happened, spiking typically resumed once the toxins were washed off.

Effects on evoked firing

While SNc dopamine cells fire spontaneous action potentials in the absence of stimulation, they also can be driven to fire bursts of action potentials by synaptic input or somatic current injection (Blythe et al., 2009). To explore how BK and Kv2 channels contribute to evoked firing, we compared responses of dopaminergic cells to 200 ms current injections in control, in GxTx, and in paxilline, all at physiological temperature. Small current injections evoked faster firing for the duration of the current; cells fired faster in response to larger current injections until they entered apparent depolarization block. When Kv2 channels were inhibited, the frequency vs. current (F-I) relation of cells was typically steeper than it was in control, as in the example cell shown in Figure 2.8A. However, cells in GxTx entered depolarization block and stopped firing in response to smaller
Figure 2.7. Simultaneous inhibition of BK and Kv2 dramatically alters pacemaking.

(A) Spontaneous firing in control (left) and after application of 300 nM paxilline and 100 nM GxTx (right).

(B) Signal averaged, peak-aligned waveforms of spontaneous activity in control (black), paxilline (blue), and paxilline + GxTx (red). Traces to the right are shown on an expanded time scale.

(C- E) Summary of the effects of paxilline plus GxTx on firing parameters for 25 neurons. Data from individual cells are shown in pink; population means ± S.E.M. are in red. Asterisks indicate $p \leq 5 \times 10^{-5}$
Figure 2.8. Inhibition of Kv2 of BK channels has contrasting effects on evoked firing.

(A) Burst-like activity in response to 140 pA current injections in control (top) and after application of 100 nM GxTx-1E (middle). Bottom: F-I relations in control (black) and 100 nM GxTx-1E (green).

(B) Responses to 140 pA current injection in control (top) and 300 nM paxilline (middle). Bottom: F-I in control (black) and in 300 nM paxilline (blue).
Figure 2.8 (Continued)
current steps. Conversely, cells tended to have shallower F-I relations after BK channels were blocked, and they also tended to reach a saturating firing frequency (e.g. Figure 2.8B). In paxilline, cells were frequently able to maintain firing in response to larger current injections than in control, but the maximum firing frequency achieved was lower.

In control, cells generally had F-I relations that were approximately linear, while in either toxin, the relations tended to saturate. To quantitatively compare frequency responses, we calculated the slope of the F-I based on the basal firing rate (with no current injection) and the maximal firing rate. Using this metric, the slope increased from $0.44 \pm 0.05 \text{ Hz/pA}$ in control to $0.77 \pm 1.5 \text{ Hz/pA}$ in GxTx ($n=11$; $p=0.04$). Conversely, the slope became shallower in paxilline ($0.43 \pm 0.04 \text{ Hz/pA}$ in control, $0.21 \pm 0.01 \text{ Hz/pA}$ in paxilline; $n=11$; $p=1x10^{-4}$). This suggests that inhibiting potassium channels can either increase or decrease excitability, depending on the kinetics of the channel.


Discussion

Our results show that currents through both BK and Kv2 channels are important for repolarization of action potentials in SNc dopamine cells. When either type of channel was inhibited, the resultant broadening of action potentials recruited additional current through the other channel type, and pacemaking was maintained at virtually the identical rate. When both channel types were inhibited simultaneously, pacemaking was altered more dramatically. Additionally, evoked responses to current injections appeared to rely on current through both channel types.

Importance of channel kinetics

In SNc dopaminergic somata, the voltage dependence and size of the currents through BK and Kv2 channels were similar. This helps explain the symmetry we observed in how inhibiting either channel type affected spontaneous firing. When either BK or Kv2 channels were inhibited, action potentials became slightly broader, as would be expected from inhibition of a potassium current involved in AP repolarization. Because neither Kv2 nor BK in these cells inactivated appreciably over the time course of action potentials, AP broadening resulted in additional current recruitment through the other type of channel. We can understand the different effects on the shapes of action potentials in light of the different channel kinetics. When blocking BK current, we decreased the contribution of quickly-deactivating K-current and increased the relative contribution of slowly-deactivating current. During the downstroke of the action potential, BK channels deactivated quickly. When relatively more of the repolarization was caused by current through Kv2 channels, the repolarizing potassium conductance would deactivate more slowly, leading to a more hyperpolarized AHP. Conversely, when Kv2 channels were inhibited and additional BK current was recruited to repolarize the cell, the faster deactivation of BK channels resulted in a less hyperpolarized AHP.
One potential consequence of the different channel kinetics might have been changes in spontaneous firing rate. We might have expected that blocking BK channels and increasing the relative contribution of Kv2 channels to AP repolarization would result in more potassium current flowing between spikes (again because of the relatively slow kinetics of Kv2 deactivation), and that this would lead to a decrease in pacemaker frequency. Conversely, we might have expected that inhibiting Kv2 channels would decrease the potassium current flowing between spikes, causing an increase in firing rate. In fact, however, spontaneous firing continued at the same rate when either BK or Kv2 channels was inhibited. While both BK and Kv2 channels appear to contribute to AP repolarization, neither has a significant effect on the interspike interval during pacemaking. This suggests that other ionic currents govern the slow depolarization leading up to spontaneous APs.

In contrast to the similar effects on spontaneous firing, inhibiting Kv2 and BK channels produced different and opposite effects on evoked responses, with inhibition of Kv2 producing steeper responses to excitatory current injections and inhibition of BK conversely producing shallower ones. This may also be a result of the different activation and deactivation properties of BK and Kv2 channels. Even during normal pacemaking, BK channels were activated much earlier during spikes than Kv2 channels. Additional recruitment of Kv2 channels in paxilline provided sufficient potassium current for spike repolarization, but it also caused a relative increase in potassium conductance later in the spike. During higher-frequency firing, this might put a lower bound on the inter-spike interval, thereby limiting overall firing rate. Conversely, inhibition of Kv2 channels would reduce the potassium conductance following each action potential, allowing more rapid subsequent depolarization and faster firing. While cells in GxTx fired at higher frequencies, they also had an increased tendency to stop firing altogether. Inhibition of Kv2 channels decreased the depth of the AHP, and this might have reduced sodium channel recovery from inactivation. Similar effects have been observed in other cell types, including cortical pyramidal cells, CA1 pyramidal cells, and superior cervical ganglion neurons (Guan et al., 2013; Liu and Bean, 2014).
Roles of BK channels in other cell types

BK channels are expressed in many types of neurons, but the roles they play in spike repolarization varies across cell types. In cerebellar Purkinje cells, substantial BK current has been observed during the falling phase of action potentials (Swensen and Bean, 2003). Acute inhibition of BK channels with iberiotoxin is associated with a variety of changes in Purkinje cell spikes, including increases in both spontaneous firing rate and burst firing (Womack et al., 2009). In contrast, in some neurons of the vestibular nucleus, BK channels also contribute to spike repolarization, but blocking them tends to decrease maximal firing rates (Gittis et al., 2010). In CA1 pyramidal cells, blocking BK channels broadens somatic action potentials but does not affect EPSPs (Hu et al., 2001). And at the rat mossy fiber bouton, although step depolarizations evoke BK current, purely voltage-dependent potassium channels make the dominant contribution to the post-spike repolarization (Alle et al., 2011).

Adrenal chromaffin cells share many characteristics with SNc dopaminergic neurons. Like dopamine cells, chromaffin cells spontaneously fire broad action potentials and secrete catecholamines. Additionally, in both cell types, pacemaking appears to be substantially supported by calcium influx through Cav1.3 channels (Nedergaard et al., 1993; Mercuri et al., 1994; Vandael et al., 2010). In chromaffin cells, blocking BK current affects firing pattern and spike shape, causing a decrease in the AHP (Solaro et al., 1995; Marcantoni et al., 2010). In dopamine neurons, while paxilline also caused AP broadening, it instead induced an increase in the depth of the AHP, probably because of additional recruitment of Kv2 channels, which deactivate more slowly than BK channels. This difference between the effects of blocking BK channels in chromaffin and in dopamine cells may be due to differences in the other potassium channels present, or to different kinetics of BK channels in the different cell types.
**Electrical roles of Kv2 channels**

Kv2 channels are widely expressed in the nervous system and are major contributors to the neuronal delayed rectifier current (Hwang et al., 1993; Scannevin et al., 1996; Murakoshi and Trimmer, 1999; Misonou et al., 2005; Johnston et al., 2010). In some cell types, studies have suggested that because of their slow kinetics, Kv2 channels are activated primarily during periods of sustained firing (Du et al., 2000; Misonou et al., 2005; Johnston et al., 2008, 2010). In others, Kv2 channels have been shown to contribute to spike repolarization even after individual action potentials (Malin and Nerbonne, 2002; Tong et al., 2013; Liu and Bean, 2014). Our results indicate that during spontaneous pacemaking in dopamine neurons, Kv2 channels make a large contribution to the outward current. Inhibiting these channels had a less striking effect on pacemaking not because the channels were not activated during individual action potentials, but because of the large reserve of other potassium current (namely BK) that could compensate for the inhibition. The wide action potentials characteristic of dopamine neurons may be particularly conducive to activation of Kv2 channels, and contributions from these channels during individual action potentials could be a general feature of broad-spooling neurons.

Proposed roles for Kv2 channels during sustained firing include providing hyperpolarizing drive to allow sodium channels to recover from inactivation (Johnston et al., 2008; Guan et al., 2013; Liu and Bean, 2014), and conversely limiting high-frequency firing (Du et al., 2000; Khaliq and Bean, submitted). Our results on evoked firing support both of these. The failure of repetitive spiking in response to current injections that we observed in GxTx is consistent with the Kv2-mediated AHP being important for sodium channel recovery. Meanwhile, when BK channels were blocked and the balance of potassium current was shifted to include a larger contribution through Kv2 channels, maximal evoked firing rates decreased, suggesting a dampening effect of the slowly-deactivating Kv2 current. This is also consistent with the recent observation that glutamatergic
enhancement of Kv2 current results in a decrease in evoked firing frequency (Khaliq and Bean, submitted).

Dynamic regulation of BK and Kv2 channels

Both BK and Kv2 channels are modulated by multiple intracellular signaling molecules. A variety of kinases and phosphatases have been shown to shift the voltage dependence of BK channel activation, including CAMKII, PKA, PKC, protein phosphatase 1, phosphatase 2A, and calcineurin (Liu et al., 2006; Tian et al., 2001; Reinhart and Levitan, 1995; Widmer et al., 2003; Loane et al., 2006; van Welie and du Lac, 2011). Because BK channel gating is also calcium-dependent, coupling to calcium sources and modulation of the calcium channels themselves may provide additional parameters for modifying properties of the BK current (Vandael et al., 2013). Kv2 channels are also subject to extensive regulation. Activity-dependent modulation of phosphorylation and SUMOylation can induce shifts in the voltage dependence of the channels (Murakoshi et al., 1997; Mohapatra and Trimmer, 2006; Park et al., 2006; Misonou et al., 2006; Cerda and Trimmer, 2011; Plant et al., 2011). In our results, we observed that acute inhibition of either BK or Kv2 channels had no effect on spontaneous firing rate, while having a larger effect on evoked responses. As BK and Kv2 channels may be targets of independent or overlapping second messenger pathways, which in turn may be activated by different or overlapping transmitters or modulators, this suggests a mechanism by which the responses of dopamine cells to synaptic input could be selectively fine-tuned with only minor effects on pacemaking.
Chapter 3
Sodium currents in substantia nigra dopamine neurons during pacemaking and burst firing

Abstract

Voltage-gated sodium current is a critical component of action potential generation, and accurate measurements of the underlying channels are important for ongoing efforts towards understanding and modeling SNc activity. Previous studies of SNc sodium current have been done using nucleated patches and ionic conditions that might alter channel gating, and at sub-physiological temperatures. We recorded voltage-gated sodium currents at 37°C using neurons acutely dissociated from mouse SNc, with solutions designed to resemble physiological conditions. We measured the voltage dependence of sodium channel activation and inactivation, as well as the rates of entry into and recovery from fast inactivated states at a number of voltages. Autonomous pacemaking is preserved in isolated cell bodies, and we had previously recorded spiking in a variety of conditions. Using these waveforms as voltage commands, we were able to measure the sodium current evoked by the voltage trajectories of pacemaking and evoked firing. Finally, we constructed a Markov model of sodium channels that predicted waveform-evoked currents similar to those we had observed experimentally.
Introduction

Electrical excitability in the nervous system is produced by the finely-coordinated activity of ion channels. In the mammalian nervous system, fast voltage-gated sodium channels, delayed rectifier potassium channels, and a variety of additional voltage- and ligand-gated ion channels interact to generate a wide diversity of spike shapes and firing patterns (Llinás, 1988; Hille, 2001). Dopamine neurons of the substantia nigra pars compacta (SNc) are spontaneously active, firing pacemaker-like action potentials even in the absence of synaptic input. In response to excitatory (or disinhibitory) synaptic events, current injections, and biologically salient stimuli, these cells also fire “bursts” of higher-frequency action potentials, which are associated with increased levels of dopamine release (Floresco et al., 2003). Dysregulation of dopaminergic signaling is associated with numerous psychiatric and neurological disorders, including schizophrenia (and some extrapyramidal effects of neuroleptic drugs), ADHD, and Parkinson’s disease (Grace, 1991; Oades, 1987; Dalley and Roiser, 2012; Koob and Le Moal, 1997; Schober, 2004). Consequently, substantial effort has gone into understanding the mechanisms contributing to spontaneous and evoked firing in these neurons.

In SNc DA neurons, both voltage-gated sodium current and L-type calcium current play important roles generating the depolarization underlying pacemaking (Durante et al., 2004; Chan et al., 2007; Puopolo et al., 2007; Guzman et al., 2009; Putzier et al., 2009). Recent computational studies have highlighted the relevance of voltage-gated sodium channels to depolarization block, in which accumulating sodium channel inactivation causes eventual spike failure and cessation of firing (Tucker et al., 2012; Qian et al., 2014). Because of the size and speed of voltage-gated sodium current, experimental characterizations of the channels are technically challenging. As a result, most modeling studies have been based on experimental data collected at either room temperature or slightly warmer from nucleated patches (Seutin and Engel, 2010; Ding et al., 2011). We sought to
investigate sodium channels and their contributions to spontaneous action potentials under conditions as similar as possible to physiological, using acutely dissociated dopaminergic neurons. Isolated cell bodies continue to fire in a pacemaker-like manner, suggesting that the essential components for electrical pacemaking are present within the soma. Furthermore, dissociated cells are electrotonically compact, which allows for excellent voltage control, even at physiological temperature.
Methods and Materials

Preparation of dissociated cells

Dopamine neurons were prepared from P14-P18 mice of both sexes. Some experiments used animals in which eGFP was expressed under control of the tyrosine hydroxylase promoter (Sawamoto et al., 2001), kindly provided by Dr. Kazuto Kobayashi, (Fukushima Medical University). Other experiments used wild-type littermates of the transgenic mice, or wild-type Swiss-Webster mice. Animals were anesthetized with isoflurane and decapitated, and the brain was quickly removed into ice-cold solution containing (in mM) 110 NaCl, 2.5 KCl, 10 HEPES, 25 glucose, 75 sucrose, 7.5 MgCl2, pH adjusted to 7.4 with NaOH, and oxygenated with 95/5% O2/CO2. In the same solution, 200 µm coronal slices were cut using a vibratome (DSK model DTK-1000, Dosaka), and the SNC was dissected out and rinsed in ice-cold dissociation solution containing (in mM) 82 Na2SO4, 30 K2SO4, 5 MgCl2, 10 glucose, 10 HEPES, pH adjusted to 7.4 with NaOH. Pieces of tissue containing the SNC were then incubated for 7-8 minutes at 34°C in dissociation solution with 3 mg/mL protease XXIII (Sigma Aldrich). The tissue was then placed in ice-cold dissociation solution containing 1 mg/mL trypsin inhibitor and 1 mg/mL BSA, where it was stored until immediately before recording. Cells were used within 10 hours of dissection. Just before recording, pieces of tissue were gently triturated with a fire-polished Pasteur pipette to free individual cells; the resulting suspension was then dispersed onto the bottom of the recording chamber, and the cells were allowed to settle for 15-20 minutes. The chamber was then flooded with standard Tyrode’s solution containing (in mM) 151 NaCl, 10 HEPES, 13 glucose, 1.5 CaCl2, 1 MgCl2, 3.5 KCl, pH adjusted to 7.4 with NaOH. Dopamine neurons could be identified unambiguously by eGFP expression or by their large size.
Electrophysiological recordings

Recording solutions were designed to be as physiological as possible. External solution was Tyrode’s as above; internal solution contained (in mM) 122 K-methanesulfonate, 9 NaCl, 9 HEPES, 0.18 EGTA, 0.036 CaCl₂, 0.27 MgCl₂, 4 MgATP, 14 creatine phosphate (Tris salt), 0.3 GTP (Tris salt), pH 7.4. Data [will be] corrected for an -8 mV junction potential between internal and external solutions, as measured according to the method in Neher, 1992. Pipettes for recording were pulled using a Sutter Instruments P-97 horizontal puller from borosilicate glass (VWR International), and wrapped in Parafilm (American National Can) to reduce pipette capacitance. After a GΩ seal and whole-cell mode were established, cells were lifted off the bottom of the recording chamber and placed in front of a set of gravity flow perfusion pipes, heated to 37°C (except in the experiments shown in Figure 1) with a feedback-controlled warmer (TC-344B; Warner Instruments). This perfusion system allowed rapid solution exchange in <1 s.

Whole cell voltage and current clamp recordings were made using an Axon Instruments 700B amplifier (Molecular Devices), a Digidata 1322A ADC (Axon Instruments), and pClamp 9.2 software (Molecular Devices). Signals were filtered at 10 kHz and sampled at 10 or 20 µs. Data were analyzed in Igor Pro 6.12A (WaveMetrics, Lake Oswego, OR), using DataAccess (Bruxton Software) to read in pClamp files. Data were corrected for linear capacitative and leak currents offline, using 5 mV hyperpolarizations to define linear capacitance and leak currents and subtracting appropriately scaled currents. In some cases, capacitative current correction was imperfect due to amplifier saturation for large voltage steps. In these cases, 100-200 µs of the current record are blanked in the figures. Data are presented as mean ± S.E.M.; p-values were calculated using a Student's t-test.

SNc dopamine cells express potassium channels that activate quickly over similar voltages as sodium channels do. To minimize potential contributions from these channels, we initially planned to do some experiments with Cs-based internal solution containing (in mM) 126 CsCl, 9
HEPES, 0.09 EGTA, 0.18 CaCl$_2$, 1.8 MgCl$_2$, 4 MgATP, 14 creatine phosphate (Tris salt), 0.3 GTP (Tris salt), pH 7.4, and external solution with TEA replacing potassium. However, in preliminary experiments at room temperature, we observed an apparent shift in the voltage dependence of sodium channel activation and inactivation (Figure 3.1). Activation and inactivation were fit with Boltzmann equations of the form $1/(1+\exp((V - V_{\text{half}})/k))$. For activation, the average midpoint using K-Mes internal solution was -27.1 ± 1.3 mV; using CsCl internal, it was -21.2 ± 2.2 mV (Figure 3.1A; 19 cells with K-Mes, 14 with CsCl; p=0.018). The average midpoint of inactivation using K-Mes internal was -70.6 ± 1.1 mV; using CsCl internal, it was -59.2 ± 1.0 mV (Figure 3.1B; 18 cells with K-Mes, 15 with CsCl; p=2.5x10$^{-8}$). Consequently, we used potassium-based solutions for all subsequent experiments. For some experiments on the development and recovery of inactivation, we added 100 nM GxTx-1E, 300 nM paxilline, and 500 nM SNX-482 to reduce contributions from Kv2, BK, and Kv4 channels, respectively. In control experiments, this combination of inhibitors did not affect the sodium channel voltage dependence or kinetics of sodium channels. Synthetic GxTx-1E and SNX-482 were purchased from the Peptide Institute (Osaka, Japan); paxilline was purchased from Tocris Bioscience (Bristol, UK).

**Modeling**

We constructed a model of voltage-gated sodium current based on those of Kuo and Bean (1994) and Carter et al. (2012). Parameters were adjusted using a simulated annealing algorithm minimizing the difference between the model’s predictions and experimental data of the voltage dependence of activation and inactivation, traces of TTX-sensitive current recorded at -28 mV and +2 mV, the times of the peak current at different voltages, the rate of the development of inactivation measured at -53 mV, and the rate of recovery from inactivation at -88 mV. For the times to peak current and the fits to the raw traces, we included a temporal offset to account for the
lag between the voltage command and the voltage “seen” by the ion channels in this type of experimental setup (e.g. Carter and Bean, 2011).
Figure 3.1. Cesium-based internal solution appears to shift the voltage dependence of sodium channel activation and inactivation.

(A) Voltage dependence of relative activation using K-Mes-based internal solution and 3.5 K Tyrode’s external (black) or CsCl-based internal and Tyrode’s solution with TEA replacing K (red) solutions. Each point corresponds to the average relative activation for 19 (K-Mes) or 14 (CsCl) cells.

(B) Voltage dependence of inactivation measured with the same solutions. Each point corresponds to average relative availability for 18 (K-Mes) or 14 (CsCl) cells. Recordings were made at room temperature.
Results

Voltage dependence of activation and inactivation

We began by exploring the voltage dependence of sodium channel activation in SNc dopamine neurons. We recorded currents in quasi-physiological solutions at 37°C and isolated the contribution from voltage-gated sodium (Nav) channels by subtracting records before and after application of 1 µM TTX, a selective blocker of Nav channels (Figure 3.2A). Sodium channels activated extremely rapidly and inactivated nearly completely after 1-5 ms. Activation was apparent at voltages above ~-40 mV and reached a maximum at ~-20 mV (Figure 3.2B). Based on ohmic open channel behavior and an estimated sodium reversal potential of +75 mV, we constructed conductance-voltage relations for Nav channels. In general, activation could be well-fit with a Boltzmann curve of the form 1/(1+exp((V_{half}-x)/k)), where V_{half} is the half-activation voltage and k is the slope factor. In collected results, the midpoint of activation was -29 ± 1 mV and the slope factor was 4.1 ± 0.4 mV (n = 10). An example G-V curve is shown in Figure 3.2C.

One of the most striking features of Nav channels is their rapid inactivation, which takes place over a similar voltage range as activation. Sodium channel inactivation generally follows an exponential time course. We fit the decaying phase of the TTX-sensitive current with exponential functions (Figure 3.2D). Nav channel inactivation is typically faster at more depolarized potentials (Hodgkin and Huxley, 1952d). This is consistent with what we observed when we plotted the time constants of current inactivation versus test voltage (Figure 3.2E).

We also investigated the voltage dependence of inactivation, using conditioning steps to various voltages followed by a depolarizing test pulse to -18 mV (Figure 3.3A). We calculated the fraction of channels available at each voltage by normalizing the peak inward current during the test pulse to the peak current during the test pulse after the most negative voltage tested. Availability was well-fit by a Boltzmann curve. In collected results, inactivation had midpoint -56 ±
Figure 3.2 Voltage dependence of sodium channel activation.

(A) Depolarization-evoked currents in control conditions (black) and TTX (red), recorded at 37°C.

(B) Peak TTX-sensitive current as a function of test voltage.

(C) Peak conductance calculated from data in (B) based on a sodium reversal potential of +75 mV.

(D) TTX-sensitive currents defined by subtracting currents recorded in TTX from those recorded in control. The inactivating phase of each current was fit with an exponential function (blue) with time constants as shown.

(E) Plot of time constants of transient current inactivation versus test pulse voltage.
Figure 3.3. Voltage dependence of sodium channel inactivation.

(A) Example traces of voltage-activated currents in control (black) and TTX (red).

(B) Availability of TTX-sensitive current at -18 mV as a function of the conditioning voltage. Dashed line corresponds to the average voltage during pacemaking (~-63 mV, corresponding to ~75% availability). Data were fit with a Boltzmann curve.
1 mV and slope factor 5.5 ± 0.3 mV (n = 10); an example availability-voltage curve is shown in Figure 3.3B.

**Kinetics of inactivation onset and recovery**

We investigated the development of inactivation using depolarized conditioning pulses of varying durations to induce inactivation, followed by a test pulse to -18 mV to assay availability (Figure 3.4A, top). The fraction of available channels was calculated by normalizing the current during each test pulse to the current evoked by a step to -18 mV with no preceding conditioning pulse. In general, the onset of inactivation could be well-fit with a single exponential function (e.g. Figure 3.4A, bottom). We also measured rates of recovery from inactivation, using a standard double-pulse protocol (Figure 3.4B, top). Inactivation was induced by a brief step to -18 mV; a step to the tested voltage for varying durations allowed channels to recover. Then, a second step to -18 mV was used to determine the fraction of channels that had recovered. Recovery from inactivation was sometimes well-fit by a single exponential (e.g. Figure 3.4B, bottom), but was often better fit as the sum of two exponential components. Figure 3.5 shows a summary of the time constants of the development of and recovery from inactivation as functions of voltage. Data for the development of inactivation were fit as sums of two exponential functions, and the value of the smaller time constant is plotted in the figure. Figure 3.5 also includes the time constants of inactivation of the transient current from the cell shown in Figure 3.2.

**Sodium channel activation during action potentials**

To determine the precise timing of sodium currents during action potentials, we used the action potential (AP) clamp technique. In current clamp mode, we recorded action potentials from a neuron firing spontaneously at 37°C; we later used this as a command waveform in voltage clamp mode (Figure 3.6A). We recorded the currents flowing in response to the AP waveform, and used TTX subtraction to isolate the contribution from voltage-gated sodium channels (Figure 3.6B).
Figure 3.4. Kinetics of the development of inactivation and recovery.

(A) Top: schematic of voltage protocol used to measure kinetics of the onset of inactivation. Middle: current traces of inactivating sodium current followed by delayed rectifier potassium currents. Trace in red corresponds to the protocol shown in the top panel with $V_{test} = -48$ mV. Bottom: Relative availability as a function of duration of the conditioning pulse. Points are fit with an exponential function.

(B) Top: schematic of voltage protocol to measure recovery from inactivation. Middle: current traces for $V_{test} = -78$ mV. Trace in red corresponds to the protocol illustrated above. Bottom: Relative availability as a function of recovery duration. Fit in blue is to a single exponential.
Figure 3.5. Time constants of development and recovery from inactivation.

Time constants for exponential recovery from inactivation are plotted in blue open circles. Development of inactivation was generally well-described as the sum of two exponential functions; the time constant of the smaller one is plotted in black circles. Circular points are the mean of 3-19 cells; error bars are S.E.M. Time constants of inactivation for the transient current from Fig. 2 are plotted as triangles.
Figure 3.6. Sodium current activation during waveforms of spontaneous action potentials.

(A) Action potential waveform recorded from a cell firing spontaneously at 37°C.

(B) Currents flowing in response to the AP waveform recorded in the same solutions (black) and with the addition of 1 μM TTX (blue). Voltage-gated sodium current defined by TTX-subtraction is shown in red. Dashed line corresponds to the peak of the action potential.
Sodium current was primarily activated during the rising phase of the action potential, but some continued to flow during the repolarization as well.

Typical values for the average membrane potential during spontaneous firing were ~-63 mV, which is in the voltage range where sodium channel inactivation is quite steep (Figure 3.3B). This suggests that during pacemaking, a large fraction of the sodium channels may be inactivated. To test this experimentally, we compared the spike-evoked sodium current to the maximal sodium current in the cell. We measured the TTX-sensitive current evoked during a step from -93 mV, where our availability curve predicts that inactivation is mostly removed, to +2 mV, close to the voltage that elicited the peak sodium current during AP waveforms (+5 ± 1 mV, n=60). We interpreted this as the maximal sodium current for the cell (Figure 3.7A). We also measured the TTX-sensitive current evoked by waveforms of spontaneous action potentials (Figure 3.7B) and of higher-frequency firing evoked by current injections (Figure 3.7C). Different AP waveforms have slightly different voltage trajectories. The peaks of action potentials are typically above 0 mV, which is in the voltage regime where stronger depolarization results in smaller current because of the decrease in driving force (Figure 3.2B). In order to evaluate the effects of the different waveforms on channel activation, we calculated the TTX-sensitive conductance by dividing the current by the difference between the command voltage and the sodium reversal potential (Figure 3.7D). The peak conductance during the step was 63 ± 7 nS for 11 cells. The fraction of maximal current and the fraction of maximal conductance were extremely similar for the different waveforms, typically within 1% for collected results.

Overall, we found the sodium conductance during the waveform of spontaneous action potentials to be 29 ± 4 nS, or 46 ± 6% of that during the step (n=11), suggesting that a sizeable fraction of sodium channels is indeed inactivated during spontaneous firing. Over the course of the burst-like waveform, we observed a decrease in spike-evoked conductance: typically, the
Figure 3.7. Sodium channel activation during spontaneous and evoked firing.

(A) Maximal sodium current was evoked by a step from -93 mV to +2 mV.

(B) Currents were recorded in response to the waveform of a spontaneous action potential, and TTX was used to isolate the component from voltage-gated sodium current.

(C) The contribution of voltage-gated sodium current during waveforms of evoked action potentials was measured the same way as in (B).

(D) TTX-sensitive conductance during the step (left), the spontaneous AP waveform (middle), and the waveform of evoked APs (right).
conductance during the first spike of the burst waveform was larger than during the waveform of the spontaneous AP (58 ± 6% of maximum, n=11; p=9.7x10^{-6} vs spontaneous). It then decreased over the course of the burst so that by the third spike, it reached a value similar to that during the spontaneous AP waveform (44 ± 6% of maximum; n=11). We quantified the decrease by measuring the difference in the conductance between the 1st and 3rd spikes, and dividing that value by the peak conductance (i.e. that during the step). For a burst recorded in control solutions, this fractional difference was 15 ± 2%.

In the previous chapter, we explored the contributions of large-conductance calcium-activation potassium (BK) channels and delayed rectifier Kv2 channels to spontaneous and evoked firing in SNc dopamine cells. When we applied paxilline, a selective BK channel blocker, or GxTx-1E, a peptide inhibitor of Kv2 channels, to spontaneously firing neurons, we observed small increases in AP width but no change in firing rate. Blocking BK channels resulted in increased current through Kv2 channels and vice versa; the two types of channels appeared to serve extremely similar roles during pacemaking with substantial functional redundancy. During evoked firing, distinct roles became apparent. Kv2 channels activate and deactivate substantially more slowly than BK channels do. When BK channels were blocked pharmacologically and the relative contribution from Kv2 channels to the overall potassium current increased, cells became more limited in their ability to fire at high frequencies. At the same time, they appeared to become more resistant to depolarization block and were able to continue firing in response to larger current injections. Conversely, when Kv2 channels were inhibited and the contribution from BK channels increased, we observed the opposite effect. Cells fired at higher frequencies, but were more susceptible to apparent depolarization block. This effect was likely the result of interactions between the potassium and sodium channels present in the cells. We investigated this more directly by recording and comparing the TTX-sensitive currents evoked by the different waveforms of spontaneous and evoked firing before and after potassium channel inhibition.
Neither of the K-channel blockers had had much effect on spontaneous firing rate or on upstroke velocity, so we expected the waveforms of spontaneous firing in control or in either toxin to activate similar sodium conductances. Indeed, there was very little difference in the sodium current or in the calculated conductance during waveforms of spontaneous firing. The change in conductance during pacemaking before and after BK channels were blocked was $3 \pm 1\%$ of the maximum ($n=11$); for inhibition of Kv2 channels, the difference was $0 \pm 1\%$ ($n=11$). In contrast, there were more pronounced differences during the waveforms of evoked firing (Figure 3.8). As voltage commands, we used the waveforms of burst-like events evoked by depolarizing current injections in control and after inhibition of either BK or Kv2 channels. During the burst recorded in control, the peak sodium conductance during each spike had undergone a modest reduction. This effect was greatly reduced after BK channels were blocked: using the waveform of evoked spikes recorded in paxilline, the fractional difference in conductance from the first to the third spike was $3 \pm 1\%$ ($n=11$; $p=2 \times 10^{-5}$ relative to the control burst) (Figure 3.8A, right). In contrast, the difference became larger after Kv2 channels were inhibited. In response to the waveform of evoked activity before application of GxTx, the conductance decreased by $19 \pm 2\%$ of maximum from the first to the third spike. After inhibition of Kv2 channels, this increased to $25 \pm 3\%$ ($n=11$; $p=0.001$) (Figure 3.8B). To summarize, waveforms of burst-like firing recorded under control conditions activated sodium conductances and currents that decreased moderately over the course of the burst. This reduction was reduced in response to waveforms recorded after BK channels were blocked, and it increased in response to waveforms recorded after Kv2 channels had been inhibited (Figure 3.8C).

**Modeling**

We constructed a Markov model of sodium channel gating based on those of Kuo and Bean (1994) and Carter et al. (2012) to describe properties of the sodium current at physiological temperature (Figure 3.9A). Rate constants were adjusted so that the model’s predictions for the
Figure 3.8. Decreasing sodium current during evoked action potentials.

(A) Top, waveforms of spikes evoked by a 140 pA current injection in control (left) and after blockade of BK channels with 300 nM paxilline (right). Bottom, currents elicited by the corresponding waveforms recorded in control (black) and in 1 μM TTX (blue). TTX-sensitive currents are shown in red.

(B) Top, waveforms of evoked spikes recorded in control (left) or after inhibition of Kv2 channels with GxTx-1E (right). Bottom, currents evoked by these waveforms in control (black) or TTX (blue). TTX-sensitive current is shown in red.

(C) Difference in the fraction of maximal sodium current (left) and conductance (right) from the first to the third spike of the waveforms of evoked APs recorded in different conditions.
Figure 3.9. Markov model of voltage-gated sodium current.

(A) Schematic of the model. Voltage-dependent transitions between successive closed states (top row) correspond to movement of the voltage sensors. The final opening step is voltage-independent. Transitions to the lower row correspond to inactivation.

(B) Family of currents predicted by the model in response to voltage steps from a holding potential of -98 mV to voltages from -48 mV to +2 mV in 10 mV increments.

(C) Voltage dependence of activation (filled circles) and inactivation (open circles) predicted by the model.

(D) Simulated currents in response to the waveform of a spontaneous action potential (left) and a burst of evoked action potentials (right).
voltage dependence of activation and inactivation and the kinetics of the predicted current were similar to what we observed experimentally (Figure 3.9B). The model predicts midpoints of activation and inactivation of -27.2 and -55.8 mV respectively, which correspond to typical experimental values (Figure 3.9C). We also calculated the sodium current that the model predicted during an action potential (Figure 3.9D). We tested the model using waveforms of evoked activity as in Figure 3.8, and calculated the current generated by the model in response to these waveforms. Like the experimental data, the model predicted a decrease in sodium current over the course of the burst waveform recorded in control. This reduction was almost completely eliminated in response to the waveform recorded in paxilline (Figure 3.10A), and it was slightly larger during the waveform recorded in GxTx (Figure 3.10B).
Figure 3.10. Model predictions for sodium current during waveforms of burst-like firing.

(A) Simulated sodium currents in response to the waveform of evoked firing in control (left), and after inhibition of BK channels with paxilline (right).

(B) Simulated currents in response to waveforms of evoked firing in a different cell in control (left) and after application of the Kv2 channel inhibitor GxTx (right).

(C) Summary of the predicted decrease in current over the course of each burst waveform.
Discussion

Comparisons with similar cell types

Two reports have recently been published on properties of the voltage-gated sodium current in SNc neurons with dramatically different results. Seutin and Engel (2010) recorded sodium currents in nucleated patches from rat SNc at room temperature. Using cesium-containing internal solution and extracellular cadmium, they observed a half-activation voltage of -9.6 mV and a half-inactivation voltage of -48.9 mV. These values have been used in a number of recent modeling studies (Tucker et al., 2012; Qian et al., 2014), but are quite different from sodium channel parameters reported in other cell types. For example, sodium channels in CA1 pyramidal neurons have a half-activation midpoint of -29.8 mV (Royeck et al., 2008). In another study using nucleated patches from rats, Ding et al. (2011) found midpoints of activation and inactivation of -22.0 mV and -56.6 mV, respectively (after +4.7 mV junction potential correction), which seem more typical. This variation could result from a number of potential sources, including recording temperature and experimental solutions. Our goal in this study was to make measurements under the most physiological conditions possible. Using whole-cell recordings with physiological ionic conditions at 37°C, we observed midpoints of activation and inactivation of -29 mV and -56 mV, respectively, which are even more negative than those reported by Ding and colleagues, and are similar to values reported for sodium channels in other cell types.

Several types of monoaminergic pacemaking neurons, including SNc dopamine neurons, have very broad action potentials. The parallels in pacemaking pattern suggest that there may be similarities in the underlying mechanisms, potentially including their sodium channels. In serotonergic neurons of the raphé nuclei, sodium channels were found to activate with a half activation potential of -34.2 mV for a third-power Boltzmann, (corresponding to -25.6 for a single Boltzmann fit) with slope factor 6.4 mV, and a half-inactivation potential of -48.6 mV with slope
factor -5.2 mV (Milescu et al., 2010). These values were obtained from recordings at room
temperature from cells in slices from juvenile rats, but are nonetheless similar to the values found
in dopaminergic neurons both in these experiments and in those of Ding and colleagues (2011).
Histaminergic neurons in the tuberomammillary nucleus of the hypothalamus are also spontaneous
pacemakers with broad action potentials. Sodium channels in these cells have activation and
inactivation midpoints of -27 mV and -58 mV, also generally similar (Taddese and Bean, 2002).

Tonic inactivation of sodium channels

The sodium conductance activated by steps from hyperpolarized potentials was
substantially larger than that activated by waveforms of action potentials. This suggests that during
physiological firing, a significant fraction of sodium channels are either inactivated or remain closed
during individual action potentials. In cerebellar nuclear neurons, which like SNc neurons are
autonomous pacemakers, Raman et al. (2000) estimated sodium channel availability to be less than
25% immediately preceding spontaneous action potentials and showed that DC hyperpolarization
could recruit additional channels. SNc neurons receive numerous inhibitory inputs, so transient
synaptic hyperpolarizations might similarly relieve some tonic inactivation of sodium channels.
While it appears that the fraction of inactivated channels is substantial even immediately before
spikes, there appear to be available channels at all times during the pacemaking cycle: in several
cells, we observed some sodium influx even during the downstroke of the action potential, when
availability tends to be low (Carter and Bean, 2011), indicative of non-inactivated channels (e.g.
Figure 3.6).

Both activation and inactivation of sodium channels increase with membrane
depolarization. The rate of depolarization, therefore, can modulate the effective spike threshold
(Raman et al, 2000; Platkiewicz and Brette, 2011). Threshold variations have been documented in
several neuronal types, including those in cortex, the deep cerebellar nuclei, and the medial
superior olive (Azouz and Gray, 2000; Raman et al., 2000; Svirskis et al., 2004). We observed that the waveform of the first current-evoked spike activated a larger sodium conductance than the waveform of a spontaneous action potential. One possible explanation is that the depolarization in response to the current injection was faster than the spontaneous depolarization, leaving fewer sodium channels inactivated at the time of the spike. Sodium channels are not the only ion channels that inactivate with depolarization: dopamine neurons also express Kv4.3 channels, which mediate fast-activating and fast-inactivating potassium current (Liss et al., 2001). In dopaminergic VTA neurons, I_A is preferentially recruited by rapid depolarizations, and this negative feedback stabilizes pacemaking at low frequencies (Khaliq and Bean, 2008). Faster depolarizations, which might result from excitatory synaptic inputs, could cause decreased inactivation (and therefore increased availability) of both sodium and Kv4.3 channels, which would exert generally opposite effects. Independent modulation of these two channel types could shift the balance of which exerts the stronger effect.

Typical interspike voltages for pacemaking SNc neurons are ~-63 mV, where our availability curve predicted ~25% of channels to be inactivated. The spike-evoked current was only about 45% of the step-evoked current, suggesting that more inactivation is accumulating than the availability curve alone predicts. Multiple factors may contribute to this discrepancy. The membrane potential is slowly increasing between spikes, so immediately before each spike, it is depolarized relative to the mean interspike voltage. Additionally, the measured availability curve describes the extent of inactivation following 200 ms pulses at the tested voltage. This duration was selected to be substantially longer than the time constants of recovery from fast inactivated states, but was not meant to capture potential contributions from slow inactivation. Slow inactivation is well-documented in sodium channels (e.g. Do and Bean, 2003; Jo and Bean, 2011), so it is likely that it contributes to the apparent decreased activation.
Sodium channel modulation

The voltages traversed between spikes are in the range where sodium channel inactivation is steeply voltage-dependent. Several drugs and endogenous signaling molecules are capable of shifting the voltage dependence of inactivation, which in turn could affect the availability of sodium channels during spontaneous and evoked firing. Sodium channel alpha subunits can be phosphorylated by both PKA and PKC, and can conversely be dephosphorylated by calcineurin and by phosphatases 1 and 2A (reviewed in Cantrell and Catterall, 2001). Phosphorylation by PKA appears to increase channel entry into slow inactivated states, decreasing overall availability without affecting the voltage dependence of activation. Activation of PKC, meanwhile, decreases peak current while simultaneously slowing fast inactivation. Dopamine neurons express D2/D3 dopamine autoreceptors. Tonic somatodendritic release from within the SNc may therefore influence basal sodium channel phosphorylation states. Dopamine neurons also express GPCRs activated by GABA (Koyrakh et al., 2005), glutamate (Testa et al., 1994), and acetylcholine (Nastuk and Graybiel, 1991), whose activation may also interact with biochemical pathways upstream of sodium channel phosphorylation. Therefore, modulating sodium channels may be one way in which dopaminergic signaling is regulated by other neuromodulators.
Chapter 4
Inhibition of A-type potassium current by the peptide toxin SNX-482


Acknowledgements

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Abstract

SNX-482, a peptide toxin isolated from tarantula venom, has become widely used as an inhibitor of Cav2.3 voltage-gated calcium channels. Unexpectedly, we found that SNX-482 dramatically reduced the A-type potassium current (I_A) in acutely dissociated dopamine neurons from mouse substantia nigra pars compacta (SNc). The inhibition persisted when calcium was replaced by cobalt, showing that it was not secondary to a reduction of calcium influx. Currents from cloned Kv4.3 channels expressed in HEK-293 cells were inhibited by SNX-482 with an IC50 of less than 3 nM, revealing substantially greater potency than for SNX-482 inhibition of Cav2.3 channels (IC50 20-60 nM). At sub-saturating concentrations, SNX-482 produced a depolarizing shift in the voltage-dependence of activation of Kv4.3 channels and slowed activation kinetics. Similar effects were seen on gating of cloned Kv4.2 channels, but the inhibition was less
pronounced and required higher toxin concentrations. These results reveal SNX-482 as the most potent inhibitor of Kv4.3 channels yet identified. Because of the effects on both Kv4.3 and Kv4.2 channels, caution is necessary when interpreting the effects of SNX-482 on cells and circuits where these channels are present.
Introduction

Mammalian neurons express many different types of voltage-dependent sodium, calcium, and potassium channels. The diversity of ion channel properties and expression patterns allows for complex and finely-tuned regulation of activity in the nervous system (Llinás, 1988; Hille, 2001). Specific inhibitors of ion channels are critical tools for understanding the functions of different channel types. Among voltage-dependent calcium channels, a variety of small molecule inhibitors and peptide toxins have allowed discrimination of different components of overall calcium current and functional roles of particular calcium channel types (Lacinová et al., 2000; Doering and Zamponi, 2003; McDonough, 2007), and some calcium channel blockers are of clinical interest (Lewis et al., 2012; Nimmrich and Gross, 2012; Vink and Alewood, 2012). SNX-482 is a peptide toxin originally isolated from venom of the tarantula Hysterocrates gigas and found to inhibit Cav2.3 channels with an IC$_{50}$ of about 30 nM, with at least 10-fold selectivity relative to effects on other calcium channel types (Newcomb et al., 1998). Subsequently, SNX-482 has been widely used to identify currents from Cav2.3 channels and to investigate their functions in cells and circuits (Pringos et al., 2011).

In initial studies on selectivity of the toxin, SNX-482 was found to have no effect on a variety of potassium currents, including native potassium currents in retinal ganglion neurons, IMR-32 neuroblastoma cells, and cloned Kv1.1 and Kv1.4 channels (Newcomb et al., 1998). However, in exploring possible contributions of calcium entry through Cav2.3 channels to activation of potassium currents in midbrain dopamine neurons, we found that SNX-482 completely eliminated a large component of transient potassium current corresponding to $I_A$. Further experiments showed that this effect of SNX-482 was not calcium-dependent and was consistent with direct inhibition of Kv4.3 channels. Cloned Kv4.3 channels were inhibited with an IC$_{50}$ of less than 3 nM. Kv4.2 channels were also affected, but to a lesser degree and at higher toxin concentrations.
Materials and Methods

Acute dissociation of substantia nigra dopaminergic neurons

Dissociated dopamine neurons were prepared from 14- to 19-day-old male and female mice. Mice were anesthetized with isoflurane and decapitated; the brain was quickly removed into ice-cold solution containing (in mM) 110 NaCl, 2.5 KCl, 10 HEPES, 25 glucose, 75 sucrose, 7.5 MgCl2, pH adjusted to 7.4 with NaOH and bubbled with 95/5% O2/CO2. In the same solution, 200 µm coronal slices were cut using a vibratome (DSK model DTK-1000; Dosaka). The substantia nigra pars compacta (SNc) was subsequently dissected out of each slice and rinsed in dissociation solution containing (in mM) 82 Na2SO4, 30 K2SO4, 5 MgCl2, 10 glucose, 10 HEPES, pH adjusted to 7.4 with NaOH.

Following dissection, the pieces of the SNc were incubated for 8 minutes at 34°C in 3 mg/mL protease XXIII (Sigma Life Science) dissolved in dissociation solution. After enzyme treatment, the tissue pieces were rinsed in ice cold dissociation solution containing 1 mg/mL trypsin inhibitor and 1 mg/mL BSA, where they were stored until immediately prior to recording. Cells were used within 8 hours of dissection. Just before recording, chunks of tissue were gently triturated in dissociation solution using a fire-polished Pasteur pipette to free individual cells. The resulting suspension was dispersed into the recording chamber. The cells were allowed to settle for 10-15 minutes, after which time the recording chamber was flooded with Tyrode's solution. Most recordings were made from neurons isolated from mice expressing eGFP under control of the tyrosine hydroxylase promoter (Sawamoto et al., 2001), kindly provided by Dr. Kazuto Kobayashi, (Fukushima Medical University). This allowed unambiguous identification of dopaminergic neurons. Some recordings were also made from neurons from wild-type Swiss-Webster mice, in which case dopaminergic neurons could be identified by their large size and prominent I\textsubscript{A} currents.
I_A in neurons from wild-type mice were inhibited by SNX-482 identically to those of I_A in
dopaminergic neurons from the TH-eGFP mouse line.

**Heterologous expression of Kv4.3, Kv4.2, and Cav2.3**

HEK-293 cells stably expressing Kv4.3 (Eghbali et al., 2002) were kindly provided by Dr.
Ligia Toro (David Geffen School of Medicine at UCLA). Cells were maintained in DMEM with L-
glutamine (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100
units/mL penicillin/streptomycin, and 400 µg/ml G418. Cells were kept at 37°C in a humidified 5%
CO2 incubator and passaged every 4-7 days. Prior to electrophysiological experiments, cells were
treated with Trypsin-EDTA, plated onto uncoated glass coverslips, and allowed to settle for 4-30
hours. Immediately before recording, coverslips were transferred to the recording chamber
containing Tyrode’s solution.

Experiments on cloned Kv4.2 and Cav2.3 channels were done using transient transfection of
HEK-293 cells. HEK-293 cells were maintained in DMEM with L-glutamine supplemented with 10%
HI-FBS and 100 units/mL penicillin/streptomycin. Cells were transiently transfected 1-2 days
before recordings using the PolyJet™ DNA Transfection Reagent (SignaGen) according to
manufacturer’s instructions. Kv4.2 channels were transiently expressed in HEK-293 cells using
plasmids generously provided by Dr. Jeanne Nerbonne (Washington University School of Medicine),
and Cav2.3 channels were transiently expressed in HEK-293 cells using plasmids for α1E, α2δ, and
β1b generously provided by Dr. Gerald Zamponi (University of Calgary). In both cases, eGFP was
included as a transfection marker.

**Electrophysiological recording**

Most experiments used solutions designed to approximate physiological conditions.
Standard external solution was Tyrode’s solution containing (in mM) 151 NaCl, 10 HEPES, 13
glucose, 1.5 CaCl$_2$, 1 MgCl$_2$, 3.5 KCl, pH adjusted to 7.4 with NaOH. For experiments on potassium currents in the absence of calcium entry, we used an external solution in which calcium was replaced by 1.5 mM cobalt and in some experiments (as noted in figure legends) 1 µM TTX was added to block sodium currents. Experiments on potassium currents in both native neurons and HEK-293 cells used an internal solution containing (in mM) 122 K-methanesulfonate, 9 NaCl, 9 HEPES, 0.18 EGTA, 0.036 CaCl$_2$, 0.27 MgCl$_2$, 4 MgATP, 14 creatine phosphate (Tris salt), 0.3 GTP (Tris salt), pH 7.4. In experiments on cloned Cav2.3 channels, recordings were done in an external solution containing (in mM) 155 TEA Cl, 5 BaCl$_2$, 10 HEPES, 13 glucose, pH adjusted to 7.4 with TEA-OH and an internal solution containing (in mM) 126 CsCl, 1.8 MgCl$_2$, 0.09 EGTA, 0.018 CaCl$_2$, 9 HEPES, pH 7.4. Most experiments using dopaminergic neurons were done at 34°C, with a few done at 37°C (including those in Figure 4.5A) and a few at room temperature. Results were the same at different temperatures except that block of I$_A$ was faster at 34°C or 37°C (complete within ~ 5 sec) than room temperature (~ 15 sec). Experiments testing the effect of SNX-482 on total non-inactivating potassium current (Figure 4.5A) were done using cobalt substituted for calcium (to eliminate possible effects from blocking calcium current) and with 1 µM TTX to block sodium currents; these experiments were done at both room temperature and at 37°C and results were combined in reported statistics since there was no obvious difference. Recordings from cloned channels in HEK-293 cells were done at room temperature.

Recording pipettes were pulled from borosilicate glass (VWR International) using a Sutter Instruments P-97 horizontal puller and wrapped with Parafilm to reduce pipette capacitance. Pipette resistances ranged from 1-3 MΩ. Data recorded with the potassium methanesulfonate-based internal solution have been corrected to reflect a -8 mV liquid junction potential between this solution and the Tyrode’s solution in which the current was set to zero at the beginning of the experiment, measured using a flowing 3 M KCl reference electrode as described by Neher (1992).
Whole-cell voltage clamp recordings were performed using an Axon Instruments 700B amplifier (Molecular Devices), a Digidata 1322A A/D converter (Axon Instruments), and pCLAMP9.2 software (Molecular Devices). Pipette capacitance and series resistance (70-90%) was compensated using the amplifier circuitry. Signals were filtered at 10 kHz and sampled at 10 or 20 μs.

In most experiments, after a GΩ seal and whole-cell configuration were established, the cell was lifted off the bottom of the recording chamber and placed in front of a set of gravity flow perfusion pipes in order to facilitate rapid solution exchange. In experiments using toxin concentrations less than 100 nM, 1 mg/mL cytochrome C or bovine serum albumin was added to all experimental solutions to reduce toxin loss from binding to the tubing. In some experiments on HEK-293 cells, recordings were made with cells attached to the coverslip and toxin was applied directly in the bath.

Synthetic SNX-482 was purchased from the Peptide Institute (Osaka, Japan), and purified recombinant SNX-482 was purchased from Alomone Labs (Jerusalem, Israel). Peptide from the two sources gave identical results and inhibited Cav2.3 channels with similar potency as originally reported (Newcomb et al., 1998).

Analysis

Data analysis was done in Igor Pro 6.12A (WaveMetrics, Lake Oswego, OR) using DataAccess (Bruxton Software) to read pCLAMP files into Igor Pro. Current records were corrected for linear capacitative and leak current using 5 or 10 mV hyperpolarizing steps to define linear capacitance and leak currents and then subtracting appropriately scaled currents. In some cases, correction for capacity transients was imperfect as a result of amplifier saturation for large voltage steps and 100-200 μs of the current record is blanked in the displayed records. In some figures, current records were digitally filtered with a low-pass filter corresponding to a 4-pole Bessel filter.
with a corner frequency of 2 kHz. Data are presented as mean ± standard error of the mean; p-values were calculated using paired or unpaired Student’s t-test or the Mann-Whitney-Wilcoxon test.
Results

SNX-482 inhibits $I_A$ in acutely dissociated SNc dopaminergic neurons

We began by examining total potassium current in acutely dissociated SNc dopaminergic neurons using whole-cell voltage clamp recordings. Prominent A-type current is a hallmark of SNc dopaminergic neurons (Liss et al., 1999; Liss et al., 2001; Hahn et al., 2003; Durante et al., 2004). With physiological ionic conditions, a voltage step from -88 mV to -38 mV elicited a transient inward sodium current followed by a large outward current that inactivated over tens of milliseconds, typical of $I_A$ in SNc neurons (Figure 4.1). In testing for a possible contribution to overall potassium current from calcium-activated potassium channels, we examined the effects of a series of calcium channel blockers. Surprisingly, 500 nM SNX-482, a commonly-used inhibitor of Cav2.3 calcium channels, inhibited the transient outward current almost completely (Figure 4.1A). In collected results, 500 nM SNX-482 reduced the peak of the total outward potassium current evoked by a step from -88 mV to -23 mV to 36 ± 4% its control value (n=8, p=9.1x10^{-7}). The inactivating component of outward current (defined as the peak current minus the current remaining at the end of a 1-sec test pulse) was nearly abolished by 500 nM SNX-482 (reduced to 7 ± 3% of the control value (n=8, p=1.5x10^{-8})). The dramatic effect of SNX-482 on potassium current was unexpected because R-type calcium channels account for at most ~15% of overall calcium current in these cells (Cardozo and Bean, 1995; Durante et al., 2004). Also, it would be very surprising if so much of the overall potassium current were calcium-activated, and the inactivation kinetics of the current inhibited by SNX-482 seem to identify it as $I_A$.

A defining characteristic of $I_A$ is its sensitivity to inactivation by moderately-depolarized holding or conditioning voltages (Jerng et al., 2004). Consistent with this property, the transient component of outward current evoked by a step to -23 mV was completely eliminated by a 1-second conditioning step to -58 mV (Figure 4.1B). Application of 500 nM SNX-482 almost
Figure 4.1. SNX-482 inhibits $I_A$ in dissociated dopaminergic neurons.

(A) Currents evoked by depolarization from -88 mV to -38 mV in a dopaminergic neuron at 34°C before (black) and after (red) application of 500 nM SNX-482.

(B) Currents evoked by a step to -23 mV from -88 mV (black) or -58 mV (green) or from -88 mV after application of 500 nM SNX-482 (red), showing inhibition of holding potential-sensitive $I_A$ by SNX-482.

(C) Currents evoked in an extracellular solution with calcium replaced with cobalt, before (black) and after (red) application of 500 nM SNX-482. Recordings at 34°C.
Figure 4.1 (Continued)
completely inhibited the transient outward current that was sensitive to the depolarized conditioning voltage (Figure 4.1B). This suggests that $I_A$ is selectively reduced by SNX-482.

$I_A$ in SNc dopamine neurons is mediated by Kv4.3 channels (Liss et al., 2001; Hahn et al., 2003). Kv4 channels associate with multiple auxiliary subunits, including Kv4 channel interacting proteins (KChIPs), which regulate the functional properties and magnitude of macroscopic Kv4 current (An et al., 2000; Holmqvist et al., 2002; Shibata et al., 2003). SNc dopaminergic neurons contain mRNA for both Kv4.3 and KChIP3.1T, but for no other Kv4 alpha subunits or for KChIPs 1, 2, or 4 (Liss et al., 2001). KChIPs are calcium binding proteins, and their modulation of currents through Kv4 channels includes both calcium-dependent and calcium-independent actions (Patel et al., 2002). In cerebellar stellate cells, calcium entry through T-type (Cav3) calcium channels appears to be selectively coupled to KChIP regulation of Kv4 channels (Anderson et al., 2010), and in CA1 pyramidal neurons, blocking calcium entry through SNX-482-sensitive R-type calcium channels has been reported to enhance EPSPs by reducing availability of Kv4.2 channels (Wang et al, 2014). Accordingly, the reduction of $I_A$ by SNX-482 could in principle be explained if calcium entry through R-type calcium channels regulated Kv4 function via KChIP3, although as noted R-type calcium channels account for only a small fraction of total calcium current in these cells.

To test whether the effects of SNX-482 on $I_A$ reflect inhibition of calcium entry, we performed experiments using external solutions in which calcium was replaced with equimolar cobalt. In the absence of calcium influx, $I_A$ was still present, and it was still completely blocked by the application of 500 nM SNX-482 (Figure 4.1C). In collected results using external solutions with cobalt completely replacing calcium, the inactivating component of outward current evoked by a step from -88 mV to -18 mV was reduced by 500 nM SNX-482 to 10 ± 3% of its control value (n=11; $p=P_3\times 10^{-11}$). Thus, the reduction of $I_A$ by SNX-482 is not secondary to inhibition of Cav2.3 channels.
SNX-482 inhibits current through cloned Kv4.3 channels

To test the effects of SNX-482 on Kv4.3 channels in isolation, we examined currents in HEK-293 cells stably expressing Kv4.3 channels (Eghbali et al., 2002). The transient outward current in these cells was dramatically reduced by low concentrations of SNX-482 (Figure 4.2A). In collected results, 3 nM SNX-482 reduced the peak current evoked by a step from -88 mV to -18 mV to 37 ± 8% of its control value (n=6; p=0.0004). Application of 60 nM SNX-482 reduced peak current to 7 ± 2% of its control value (n=5; p=4x10^{-7}).

To verify that the samples of toxin we used had the expected effect on Cav2.3 channels, we examined its effects on currents in HEK-293 cells expressing Cav2.3 α-subunits and the auxiliary calcium channel subunits α2δ and β1b. Consistent with previous reports (Newcomb et al., 1998; Bourinet et al., 2001), SNX-482 inhibited the current through Cav2.3 channels (Figure 4.2B) with moderate potency. Under our conditions, 60 nM SNX-482 reduced the current to 52 ± 14% of its control value (n=3). Thus, SNX-482 blocks Kv4.3 channels ~20 times more potently than Cav2.3 channels.

SNX-482 slows activation and shifts the voltage dependence of Kv4.3 channels

To explore the mechanism by which SNX-482 inhibits Kv4.3, we examined the effect of the toxin on the kinetics and voltage dependence of channel gating. Figure 4.3A shows currents evoked before and after application of 3 nM SNX-482. In this cell, current evoked by a depolarization to -23 mV was inhibited nearly completely by 3 nM SNX-482, but current evoked by a large depolarization to +32 mV was inhibited only partially (to about 22% of control). As suggested by this comparison, SNX-482 shifted the voltage-dependence of activation of Kv4.3 channels to more depolarized voltages (Figure 4.3B). In collected results, 3 nM SNX-482 shifted the half-activation potential from +1±1 mV in control to +31±3 mV in toxin (n=5; p=0.001), with little change in slope factor (15.7 ± 0.6 mV in control, 16.7 ± 0.6 mV in SNX-482; p=0.18). However, the reduction of current is not only
Figure 4.2. SNX-482 inhibition of cloned Kv4.3 and Cav2.3 channels.

(A) Effect of 3 nM (top) and 60 nM (bottom) SNX-482 on current carried by cloned Kv4.3 channels expressed in HEK-293 cells.

(B) Effect of 3 nM (top) and 60 nM (bottom) SNX-482 on current carried by cloned Cav2.3 channels expressed in HEK-293 cells. Recordings at 23 °C.
Figure 4.3. Altered gating of Kv4.3 channels induced by SNX-482.

(A) Effect of 3 nM SNX-482 on current carried by Kv4.3 channels for test pulses to -23 mV (left) or to +32 mV (middle). Currents at +32 mV are superimposed with single exponential functions fit to the inactivating phase (blue traces). Right, currents at +32 mV shown on expanded time scale to illustrate slowing of activation by SNX-482. Dashed lines with arrows illustrate time of peak current (5.5 msec in control and 25.7 msec with SNX-482).

(B) Conductance-voltage relation of cloned Kv4.3 channels in control (black) and in 3 nM SNX-482 (red). Conductance was measured from peak current evoked from a holding potential of -88 mV using a reversal potential of -90 mV and normalized to the maximum value in control. Solid black line is fit of control data to Boltzmann function, \( G_{\text{max}}/(1+\exp(-(V-V_h)/k)) \), where \( G_{\text{max}} = 26.3 \text{ nS} \), \( V_h = -9.8 \text{ mV} \) is the midpoint and \( k = 16.2 \text{ mV} \) is the slope factor. Solid red line is fit of the data in 3 nM SNX-428, with \( G_{\text{max}} = 10.2 \text{ nS} \), \( V_h = +25.4 \text{ mV} \), and \( k = 16.1 \text{ mV} \).

(C) Voltage-dependence of inactivation of Kv4.3 channels determined using 1-sec prepulses and a test pulse to +12 mV. Peak test pulse current is plotted versus prepulse voltage for recordings in control (black) and after adding 250 nM SNX-482 (red). Solid black line is fit of control data to Boltzmann function, \( I_{\text{max}}/(1+\exp(-(V-V_h)/k)) \), where \( I_{\text{max}} = 6.2 \text{ nA} \), \( V_h = -56.8 \text{ mV} \) is the midpoint and \( k = 6.7 \text{ mV} \) is the slope factor. Solid red line is fit of the data in 250 nM SNX-428, with \( I_{\text{max}} = 1.3 \text{ nS} \), \( V_h = -30.0 \text{ mV} \), and \( k = 8.3 \text{ mV} \). Recordings at 23 °C.
Figure 4.3 (Continued)
due to the shift in the voltage-dependence of activation, because there was also a substantial 
reduction in the peak conductance evoked at strongly depolarized voltages (Figure 4.3B). On 
average, the peak conductance evoked by a step to +82 mV was reduced to 56 ± 7% of its control 
value by 3 nM SNX-482 (n=5; p=0.004).

Activation of Kv4.3 current was slowed by SNX-482 (Figure 4.3A). The current evoked by a 
step from -88 mV to +32 mV reached a peak at 8 ± 2 ms in control but at 27 ± 2 ms in 3 nM SNX-482 
(n=12, p=2.1x10^-6). Inactivation was also slower in the presence of SNX-482. In control, decay of 
the current at +32 mV could be fairly well-fit by a single exponential, with average time constant 46 
± 4 ms and in 3 nM SNX-482 the time constant was increased to 154 ± 23 ms (n=12, p=0.0007). 
SNX-482 also shifted inactivation to more depolarized voltages. In control, the midpoint of 
inactivation assayed by a test step to +12 mV following 1-sec prepulses was -61 ± 1 mV, while in 
250 nM SNX-482, it was shifted to -32 ± 4 mV (n=6, p=0.0017).

**SNX-482 inhibits Kv4.2 channels**

Kv4.2 channels are expressed in many brain regions and make the major contribution to 
 somatodendritic I_A in hippocampal and neocortical pyramidal cells, some cerebellar granule cells, 
and other cell types (Serôdio and Rudy, 1998; Song et al., 1998; Rhodes et al., 2004; Chen et al., 
2006; Kim et al., 2007). Because of its enrichment in dendrites, Kv4.2 is thought to participate 
especially in integration of synaptic inputs and regulation of action potential backpropagation 
(Sheng et al., 1992; Hoffman et al., 1997). We tested the effects of SNX-482 on cloned Kv4.2 
channels expressed in HEK-293 cells. Kv4.2 current was inhibited by SNX-482 in a manner 
qualitatively similar to Kv4.3 (Figure 4.4). However, higher toxin concentrations were required, and 
the resulting inhibition was less pronounced. Peak current evoked by a step to -18 mV was reduced 
to 84 ± 4% of its control value by 60 nM SNX-482 (n=9; p=0.002). This value, however, 
underestimates the effect of SNX-482 early in the depolarization, because SNX-482 markedly
Figure 4.4. Effect of SNX-482 on cloned Kv4.2 channels.

(A) Effect of 60 nM SNX-482 on Kv4.2-mediated current evoked by a depolarization from -98 to -18 mV.

(B) Effect of 500 nM SNX-482 on Kv4.2 current evoked by steps to -28 mV (left) or +32 mV (right).

(C) Conductance-voltage relation of cloned Kv4.2 channels in control (black) and in 500 nM SNX-482 (red). Conductance was measured from peak current evoked from a holding potential of -98 mV using a reversal potential of -90 mV and normalized to the maximum value in control. Recordings at 34 °C.
slowed the kinetics of activation of Kv4.2 currents (Figure 4.4A, B), as seen for Kv4.3 channels. In control conditions, current evoked by a step to -18 mV reached its peak at 21 ± 1 ms while in 60 nM SNX-482, peak current was at 34 ± 1 ms (n=9; p=4x10^-5).

As for the effects of SNX-482 on Kv4.3, inhibition of Kv4.2 channels by SNX-482 was also voltage dependent (Figure 4.4B), resulting in a shift in the voltage dependence of activation (Figure 4.4C). However, the change in the midpoint of activation was smaller for Kv4.2 channels than for Kv4.3 channels, even with 500 nM SNX-482. On average, 500 nM SNX-482 shifted the midpoint of Kv4.2 activation by +13.7 ± 1.9 mV (n=6, p=0.0009).

For Kv4.2 currents evoked by large depolarizations, SNX-482 had relatively little effect on peak current but slowed activation (Figure 4.4B, right). Inactivation was also slowed, and the result was a “crossover” of the current, so that late in the depolarizing step, the current in SNX-482 was actually larger than it had been in control (Figure 4.4B, right). Decay of the current with inactivation could be described with a single exponential function. For a step to +32 mV, the time constant of decay increased from 52 ± 4 ms in control to 101 ± 13 ms in 500 nM SNX-482 (n=6, p=0.0059).

Selectivity for I_A

To explore the selectivity of SNX-482 for inhibiting I_A versus other native potassium currents, we examined the effects of SNX-482 on other voltage-activated currents present in SNc dopaminergic neurons, using cobalt-containing external solution to avoid contributions from calcium or calcium-activated currents. SNX-482 was highly selective for inhibiting the low-threshold, rapidly-inactivating component of current attributable to I_A (Figure 4.5A). After application of 500 nM SNX-482, the sustained current evoked by a step from -88 mV to +12 mV was 95 ± 4% its control magnitude, similar to the small changes that took place independent of toxin application (98 ± 1%; n=5; p=0.15). Similarly, 250 or 500 nM nM SNX-482 had no effect on the
Figure 4.5. Selectivity of SNX-482 for $I_A$ in SNc dopamine neurons.

(A) Currents evoked by depolarizations from -88 mV to -48 mV (left) and +12 mV (right) before (black) and after (red) application of 500 nM SNX-482, showing selective inhibition of $I_A$. External solution contained cobalt (replacing calcium) to avoid possible effects from inhibition of calcium channels and 1 μM TTX to block sodium channels. Recording at 37 °C.

(B) Currents evoked by a depolarization from -88 mV to -18 mV before (black) and after (red) application of 250 nM SNX-482 showing lack of effect on sodium current. External solution contained cobalt (replacing calcium). Recording at 34 °C.
transient sodium current (Figure 4.5B). In collected results, peak sodium current in 250 or 500 nM SNX-482 was 94 ± 3% its control value (n=7), little different from toxin-independent variation in currents recorded in control only (97 ± 1%, n=3; p=0.52). Thus, at least among the native sodium and potassium currents present in SNc neurons, the effect of SNX-482 appeared to be highly selective for I_A.
**Discussion**

We found both native A-type current in midbrain dopamine neurons and cloned Kv4 channels to be potently inhibited by the tarantula toxin SNX-482, which is currently widely used as a selective inhibitor of Cav2.3 channels. Native I_A in SNc dopamine neurons, thought to be mediated by Kv4.3 channels (Liss et al., 2001), was inhibited completely by 250-500 nM SNX-482. SNX-482 inhibited cloned Kv4.3 channels with an IC50 of <3 nM, both by shifting the voltage dependence of channel opening to more depolarized voltages and by reducing current elicited by maximal depolarizations. The toxin also inhibited cloned Kv4.2 channels by shifting the voltage-dependence of gating to depolarized voltages, although the effect was less potent and less dramatic than on Kv4.3 channels. In addition to the shift in voltage dependence, the activation kinetics of both Kv4.2 and Kv4.3 were slowed by the toxin. The shift of voltage-dependence and slowing of activation are similar to the effects of SNX-482 on gating of Cav2.3 channels (Bourinet et al., 2001). For both Kv4.3 and Cav2.3, inhibition additionally involves substantial reduction of currents evoked by the strongest depolarizations. This effect was both more potent and more complete for SNX-482 action on Kv4.3 currents.

In contrast to the striking reduction of I_A in dopamine neurons, the sodium current and the other potassium currents in these cells did not appear to be affected by the toxin. This is consistent with previous data showing a lack of effect on a wide variety of native potassium currents in other cell types (Newcomb et al., 1998). Thus, the effect of SNX-482 on Kv4 family channels appears to be highly selective among potassium channels.

Structurally, SNX-482 belongs to the family of “inhibitory cystine knot” (ICK) peptide toxins that includes hanatoxin and grammotoxin. These peptides contain six cysteine residues that form three disulfide bonds at the core of the molecule and have substantial amino acid similarity in the rest of the molecule (Swartz, 2007). Most ICK toxins that inhibit voltage-dependent ion channels
are gating-state modifiers that partition into the cell membrane and stabilize closed states of the channels through interactions with the voltage-sensing domains and the lipid environment (Escoubas and Rash, 2004; Lee and MacKinnon, 2004; Jung et al., 2005; Swartz, 2007). Many peptides in this family inhibit activation of more than one type of channel, consistent with general conservation of the voltage-sensing regions of voltage-dependent ion channels. Several, including hanatoxin and grammotoxin, are known to inhibit gating of individual types of potassium channels and calcium channels (Li-Smerin and Swartz, 1998). Interestingly, while SNX-482 interacts fairly potently with both Kv4.3 and Cav2.3 channels, it has substantial selectivity against other subtypes of both potassium and calcium channels. Among calcium channels, SNX-482 has about 10-fold selectivity for Cav2.3 over other types of calcium channels (Newcomb et al., 1998; Bourinet et al., 2001); among potassium channels, SNX-482 fails to have significant activity against the mixture of non-I\textsubscript{A} potassium currents in native midbrain dopamine neurons or any of the other native potassium currents tested in other cell types (Newcomb et al., 1998). The precise structural elements of channels and toxin proteins that determine sensitivity and selectivity are still unclear. However, recent work has identified the “face” of the toxin peptide of one member of the ICK family that appears to be most critical for channel interaction (Wang et al., 2010; Jung et al., 2010).

A number of peptide inhibitors of Kv4 channels have previously been identified. Remarkably, SNX-482 has higher potency against cloned Kv4.3 than any of the other inhibitors yet described, including phrixotoxins (Diochot et al., 1999), AmmTX3 (Vacher et al., 2002), and heteropodatoxin (Sanguinetti et al., 1997). Like SNX-482, heteropodatoxin is an ICK toxin that acts by shifting activation to more depolarized potentials and by slowing inactivation (Zarayskiy et al., 2005; DeSimone et al., 2009). SNX-482 seems like an excellent candidate to explore the possibility of separating actions on potassium and calcium channels by systematically altering the peptide sequence in a manner similar to the alanine-scanning approach used to define residues important for binding of the structurally-related tarantula toxin SGTx to Kv2 channels (Wang et al., 2004). If it
proves possible to selectively eliminate actions on Cav2.3 by altering specific residues of SNX-482, the resulting peptide could be very useful as a Kv4.3 inhibitor. Alternatively, substitutions to eliminate Kv4 actions while retaining Cav2.3 inhibition would be equally useful.

The finding that SNX-482 inhibits Kv4 channels makes some experiments using the toxin as an R-type calcium channel blocker difficult to interpret. The effects on Kv4 channels are not problematic in cases where the toxin is used to identify components of calcium current in voltage-clamp experiments in which potassium currents are blocked. Experiments using SNX-482 to study the role of R-type calcium channels in cellular or network activity under physiological conditions are more problematic. Concerns about possible direct effects on Kv4 channels can be addressed to some extent by showing that the effect of SNX-482 requires signaling by intracellular calcium (Wang et al., 2014). Even stronger controls can be done by showing a lack of effect of SNX-482 in Cav2.3 -/- mice, which offers a strong argument that the relevant effect is indeed mediated by Cav2.3 channels (Bloodgood et al. 2007; Giessel et al. 2011). However, development of a modified blocker engineered to remove Kv4 inhibiting activity would be far more convenient for most purposes.
Chapter 5
Conclusions

This thesis focused on ion channels that help sculpt excitability in dopaminergic neurons of the SNC. In earlier studies, substantial effort went into identifying the channels that contribute to the spontaneous depolarization of dopamine neurons, but little was known about the potassium channels active during action potential repolarization. Chapter 2 describes an investigation of two of these channels: large-conductance calcium-activated potassium (BK) channels, and high-threshold delayed rectifier Kv2 channels. During spontaneous firing, they fulfil strikingly similar roles. Based on the components of the waveform-evoked outward current sensitive to paxilline or to GxTx-1E, selective inhibitors of BK and Kv2 channels respectively, each of these channels makes a substantial contribution to AP repolarization during spontaneous pacemaking. In current clamp, however, inhibition of either type of channel had surprisingly little effect on firing behavior. Action potentials became slightly wider when measured at half their maximal amplitude, as would be expected following inhibition of a potassium conductance, but there was no clear effect on pacemaking rate. The afterhyperpolarization was slightly smaller after Kv2 channels were inhibited and slightly larger when BK channels were blocked, but otherwise blocking either channel alone had little effect.

We hypothesized that the small changes in AP shape accompanying inhibition of either channel type led to increased recruitment of current through other, non-inhibited potassium channels. We tested this using APs recorded before and after inhibitor application. As predicted, waveforms recorded after Kv2 channels had been inhibited activated more current through BK channels, and waveforms recorded when BK channels had been blocked conversely activated more
current through Kv2 channels. The ability of each of the two channel types to be activated during action potentials allowed them to effectively compensate for each other during spontaneous firing.

During evoked firing, distinct roles for each of these channels became more apparent. Following inhibition of Kv2 channels, depolarizing current injections elicited higher-frequency firing than in control, but cells entered depolarization block more readily. In contrast, when BK channels were blocked, spiking persisted in response to stronger current injections, but firing rates saturated at lower frequencies. This differential regulation of evoked firing appeared to result from kinetic differences between the two currents, especially the fact that BK channels deactivate much faster than Kv2 channels do. One potential explanation for the increased susceptibility to depolarization block after Kv2 inhibition is that those channels are particularly important for generating the AHP following both spontaneous and evoked activity, and that the AHP is critical for allowing voltage-gated sodium channels to recover from inactivation, allowing generation of subsequent spikes.

Chapter 3 described a series of experiments on voltage-gated sodium channels, using physiological ionic conditions and temperatures to investigate channel voltage dependence and kinetics. In previous studies on sodium channels in SNc dopaminergic neurons, recordings were made from nucleated patches using non-physiological ionic conditions at room temperature or 30°C. The voltage dependence of ion channels is likely shifted during recordings from nucleated patches (Zayd Khaliq and Bruce Bean, unpublished observations). We were able to take advantage of the fact that isolated dopaminergic somata both continue pacemaking and offer excellent voltage control to investigate properties of sodium channels and spiking behavior in the same preparation.

We measured the voltage dependence of sodium channel activation and inactivation, and the rates of the onset and recovery from fast inactivated states. We also measured sodium currents evoked during the waveforms of spontaneous and evoked firing in control or after inhibition of BK
or Kv2 channels. Voltage waveforms of spontaneous firing recorded in control or in either K-channel inhibitor activated similar amounts of sodium current, which is consistent with the minor effects that the blockers had on spontaneous firing. In response to the waveform of evoked firing recorded in control, we observed a progressive reduction in the sodium current. This reduction was almost completely absent in response to the waveform recorded after BK channel blockade, while it was increased in response to a waveform recorded after inhibition of Kv2 channels. Blocking BK channels with paxilline resulted in increased current through Kv2 channels, an increase in the depth of the AHP, a reduction in maximal evoked firing rate, and a decreased susceptibility to depolarization block. The maintained levels of sodium current observed during the corresponding waveform is consistent with Kv2 channels playing an important role in generating the AHP, and with the increased AHP observed in paxilline (i.e. with a larger contribution from Kv2) allowing sodium channels to recover from inactivation more effectively. Conversely, sodium currents decreased more during the waveform of evoked firing recorded after Kv2 channels had been inhibited by GxTx. This again is consistent with Kv2 channels enabling sodium channels to recover from inactivation.

Chapter 4 described a series of experiments on the effects of SNX-482, a peptide toxin isolated from tarantula venom that has been widely used as a selective inhibitor of Cav2.3 calcium channels. During experiments exploring possible contributions of different voltage-gated calcium channels to activation of BK current in SNc dopamine neurons, we were surprised to observe that SNX-482 potently inhibited the A-type potassium current, mediated by Kv4.3 channels. In further experiments using cloned channels heterologously expressed in HEK-293 cells, we found that both Kv4.3 and Kv4.2 channels were inhibited by SNX-482: the voltage dependence of activation was shifted to more depolarized potentials and the kinetics of activation were slowed. The inhibition of Kv4 channels presents potential problems for studies using SNX-482 as a selective agent in systems where R-type calcium and A-type potassium currents interact. An intriguing possibility is that a
more selective inhibitor of either Cav2.3 or Kv4 channels could be generated by amino acid substitutions in SNX-482.

**Advantages and caveats of AP clamp and dissociated cells**

The present experiments on potassium channels highlight one of the pitfalls of examining effects of channel blockers exclusively in current clamp mode: small changes in AP shape can lead to compensation from other channels, resulting in underestimation of the contribution provided by the inhibited current. This problem is ameliorated by the combined use of both current and voltage clamp experiments. By using waveforms of action potentials as voltage commands, the currents evoked during firing can be recorded and pharmacologically isolated without any associated change in voltage trajectory. In contrast, some types of currents are extremely prominent during voltage protocols consisting of long-lasting steps, but their activation kinetics or voltage dependences might cause them not to be activated significantly during action potentials. Again, the use of voltage stimuli that resemble AP waveforms are useful for determining which currents normally contribute to shaping membrane potentials, and which are only activated during more extreme circumstances.

Voltage clamp experiments, especially those investigating currents that are large and fast, are much more feasible in dissociated neurons. The process of dissociation removes most of the cellular processes, leaving an approximately single-compartment cell. This makes it possible to have excellent control over the transmembrane voltage, even when recording large voltage-dependent currents. This is particularly an issue with at warmer temperatures, where channel kinetics are faster and where the currents themselves are larger than at room temperature. At the same time, this technique avoids the shifts in voltage dependence observed in nucleated patches.

Acutely dissociated somata are also ideal for allowing quick and complete exchange of extracellular solutions. In slices, solution exchange can take several minutes. Drug concentrations at
the recording site must be inferred based on timing and the expected rates of diffusion into the surrounding membranes and tissue. In contrast, we were able to exchange solutions bathing dissociated cells in less than ~1 second, and drug concentrations surrounding the cell were well-defined.

Finally, dopamine cells are spontaneous pacemakers in vivo, and they maintain their pacemaking properties in both slice and dissociated preparations. This suggests that the essential pacemaking machinery is still present in the isolated cell bodies, and it allowed us to do current and voltage clamp experiments under the same experimental conditions.

At the same time, the dissociated cell preparation is not without its limitations. The dendrites and axon are not passive compartments, and may contain different complements of ion channels from the cell bodies. These lie outside the scope of experiments on dissociated cells. Second, part of the dissociation procedure involves enzymatic digestion, but it is unclear whether or how this affects the ion channels in cell membranes. It is also likely that there is some organization of neurons within the SNc, and that some properties of the neurons vary with location within the nucleus. The information regarding cells’ locations is necessarily lost during dissociation. Next, while we attempted to use solutions that closely resembled physiological conditions, a perfect replica of the CSF bathing cells in vivo is of course lacking. We attempted to minimize disruptions of the intracellular environment by including in our internal solution ATP and GTP, but dialysis of the cytosol may disrupt intracellular signaling cascades, potentially altering properties of the ion channels in the cell. Finally, while pacemaking continues in the isolated cell body, it is unclear how similar the pacemaking arising from the isolated soma is to that produced by the intact neuron.

Identification of dopamine neurons

Traditionally, the presence of the hyperpolarization-activated current $I_h$ was often used to electrophysiologically identify dopamine neurons recorded in brain slice. In more recent studies,
however, subpopulations of dopamine cells lacking $I_h$ have been identified (Neuhoff et al., 2002). In many of these experiments, I used neurons from a mouse line in which eGFP was expressed under control of the tyrosine hydroxylase promoter, allowing unambiguous identification of dopaminergic neurons independent of the presence of $I_h$ (Sawamoto et al., 2001). Following dissection of the region containing the SNc and trituration of the tissue, I found that dopaminergic neurons could reliably be identified based on their large size alone. Even in experiments using neurons from wild-type mice, therefore, it is likely that the vast majority of recorded neurons were dopaminergic.

But how similar are dopamine neurons to each other? SNc neurons have classically been described as a homogeneous population. More recently, sub-populations have been identified based on their responsiveness to different sensory stimuli, electrophysiological characteristics, projection patterns, and molecular identities. For example, some SNc cells express calbindin and have $I_h$, while others do not (Neuhoff et al., 2002). And while most SNc neurons do not respond to aversive stimuli, a subset is inhibited (Brown et al., 2009). Among the dissociated cell bodies, we observed substantial variation in the sizes of currents and the firing rates. While some of this may have originated in the cell preparation procedure, it seems likely that at least some of it represents heterogeneity within the population. Among serotonergic neurons, genetic fate mapping tools have allowed identification of distinct neuronal subtypes with unique properties (Dymecki et al., 2010; Bang et al., 2012). In the future, it would be interesting to apply similar techniques to identify well-defined subpopulations of dopamine neurons.

**Mechanisms of pacemaking and burst firing**

What does this mean for our understanding of pacemaking mechanisms in dopamine cells, and in general? In part because of their relevance to Parkinson’s disease and the potential connection between calcium influx and neurodegeneration, SNc dopamine cells and their pacemaking mechanisms are well-studied, and many observations have already been made
concerning the currents that shape firing patterns and waveforms in these neurons. In spite of this, a unified narrative of SNc pacemaking has yet to emerge (Drion et al., 2011). Previous studies have identified multiple currents contributing to the spontaneous depolarization, which together allow maintenance of robust pacemaking during pharmacological challenges (Guzman et al., 2009). In these experiments, we observed that BK and Kv2 channels serve largely redundant roles during tonic firing. These are only two of several types of potassium channels present in SNc neurons, many of whose roles are still unexplored. For example, SNc dopamine neurons express Kv3 channels, which in some cell types make dominant contributions to AP repolarization (Martina et al., 2003; Alle et al., 2011). We might therefore expect these channels to also be active during the broad spikes of dopamine neurons. Kv1 channels are also present in SNc cells, and blocking these channels with dendrotoxin was able to restore firing after it had been stopped by dihydropyridine application (Guzman et al., 2009). This suggests that the kinetics and voltage dependence of these channels in dopamine neurons may also be well-suited to activation during spontaneous firing. While a role for SK channels during pacemaking has already been established, the precise timing of their contribution is still unknown. SK channels are activated by calcium, and in SNc neurons have been reported to be coupled selectively to influx from T-type calcium channels (Wolfart and Roeper, 2002). Changes in waveform trajectory or calcium influx could alter the contributions of SK during the pacemaking duty cycle. These potassium channels likely have substantial functional overlap with BK and Kv2 channels and might add yet more layers of robustness to AP repolarization.

As with spontaneous pacemaking, multiple channels appear to make important contributions to burst firing. Previous studies have implicated ionotropic glutamate receptor, SK, $K_{ATP}$, and other channels in bursting, suggesting that there are multiple combinations of currents that can cooperate to produce fast firing as well. While BK and Kv2 channels appeared to make largely overlapping contributions to spontaneous firing, their roles during higher-frequency firing
were distinct. Inhibiting Kv2 channels allowed for faster firing, but simultaneously appeared to increase depolarization block; inhibiting BK channels limited firing frequency but increased the ability of cells to continue firing in response to stronger current injections. Manipulating the balance of current through BK and Kv2 channels could increase or decrease the slope of the frequency-current relationship without affecting pacemaker rate. One potential consequence of having multiple types of potassium channels with similar but non-identical properties appears to be the independent modulation of evoked and spontaneous firing. Modulating potassium channels by separate or overlapping biochemical cascades could allow fine-tuning of the input-output properties of these neurons without necessarily altering spontaneous firing rate.

The contributions of different conductances to overall firing pattern depend critically on their context (Goldman et al., 2001). During spontaneous firing, the contributions of BK and Kv2 channels were similar enough that they could compensate for each other. In response to moderate current injections, BK channels enhanced firing frequency. And during strong current injections, Kv2 channels allowed firing to continue by promoting sodium channel recovery from inactivated states. It seems likely that there is not one unique way that firing emerges even within the population of SNc dopamine cells, but rather multiple context-dependent solutions to the problem of action potential generation.
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