# THE SUBTYPE SPECIFIC ROLES OF SK2 AND SK3 CHANNELS IN DOPAMINE NEURONS OF THE SUBSTANTIA NIGRA

by

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CERTIFICATE OF APPROVAL

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# Abbreviations

| | |
|---|---|
| AHP | afterhyperpolarization |
| AP | action potential |
| BK | $K_{Ca}1$, large-conductance calcium-activated potassium channel |
| CaM | calmodulin |
| $Ca_V$ | voltage-dependent calcium channel |
| CICR | $Ca^{2+}$ induced $Ca^{2+}$ release |
| CNS | central nervous system |
| CV | coefficient of variation |
| DA | dopamine |
| EBIO | 1- ethyl-2-benzimidazolinone |
| ER | endoplasmic reticulum |
| iEM | immuno-electron microscopy |
| IK | $K_{Ca}3$, intermediate-conductance calcium-activated potassium channel |
| ISI | inter-spike interval |
| ISI-CV | coefficient of variation of the inter-spike interval |

| | |
|---|---|
| $I_{SK}$ | SK mediated current |
| $K_{Ca}$ | $Ca^{2+}$-activated $K^+$ channel |
| $K_V$ | voltage-dependent $K^+$ channel |
| LTD | long term depression |
| LTP | long term potentiation |
| $Na_V$ | voltage-dependent $Na^+$ channel |
| NS309 | 6,7-dichloro-1H-indole-2,3-dione- 3-oxime |
| PKA | protein kinase A |
| PP2a | protein phosphatase 2A |
| PSD | postsynaptic density |
| RCK | regulator of $K^+$ conductance domain |
| SK | $K_{Ca}$ 2, small-conductance calcium-activated potassium channel |
| $SK2_L$ | long isoform of SK2 |
| $SK2_S$ | short isoform of SK2 |
| SN | substantia nigra |

TH tyrosine hydroxylase

TTX tetrodotoxin (voltage-dependent sodium channel antagonist)

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Mom & Dad. What don't I owe to you? Well some stuff sure, but most of the good stuff I owe to you. You're still my role models.

# *Abstract*

The activity of dopamine neurons in the substantia nigra (SN) is potently influenced by the activity of small-conductance calcium-activated potassium channels (SK channels). This is thought to be due solely to the expression of a single SK family member, SK3. We show that SK2 is also expressed in dopamine neurons of the substantia nigra, and unlike SK3 channels that are homogenously distributed, SK2 is selectively targeted to the dendrites, with the highest expression in the distal dendrites. Blockade of SK2 channels selectively decreased the regularity of pacemaker activity without altering the frequency. Unlike some other cell types in which SK2 is trafficked to the dendrites, SK2 channels in DA neurons did not regulate fast, glutamatergic synaptic transmission.

# *Chapter I*

## Introduction

### *$Ca^{2+}$ as a second messenger*

Cytosolic $Ca^{2+}$ concentrations are tightly controlled by a combination of soluble and fixed buffer proteins, ion pumps, transporters, and sequestration into the endoplasmic reticulum (ER). $Ca^{2+}$ signaling due to transient increases in intracellular $Ca^{2+}$ is regulated by the rapid activation of voltage- and $Ca^{2+}$-dependent ion channels. The dynamic regulation of basal and transient $Ca^{2+}$ levels limits the temporal and spatial range of $Ca^{2+}$ signals thus establishing distinct $Ca^{2+}$ signaling domains. $Ca^{2+}$ transients drive diverse neuronal processes such as vesicular neurotransmitter release, axonal outgrowth, and the induction of synaptic plasticity (Ghosh and Greenberg, 1995). These processes are often the result of signaling cascades initiated by $Ca^{2+}$-dependent effectors. $K_{Ca}$ channels are among the earliest responding effectors of increased $Ca^{2+}$ levels, and the feedback they exert inhibits $Ca^{2+}$ influx, regulating the time course of $Ca^{2+}$-dependent signaling.

### *An overview of $Ca^{2+}$-activated $K^+$ channels*

Gardos (1958) demonstrated $Ca^{2+}$-dependent increases in membrane $K^+$ permeability in red blood cells (RBCs) providing the first evidence of $Ca^{2+}$-activated $K^+$-channels ($K_{Ca}$ channels, $K_{Ca}$ ). It is now clear that the underlying channel observed in RBCs is the intermediate-conductance calcium-activated $K^+$ channel (IK channel, $K_{Ca}$ 3). Subsequently, electrophysiological experiments in

cultured muscle cells identified two other $K_{Ca}$ channel subtypes: the large-conductance $Ca^{2+}$-activated $K^+$-channels (BK channels, $K_{Ca}$ 1) and the small-conductance $Ca^{2+}$-activated $K^+$-channels (SK channels, $K_{Ca}$ 2). $K_{Ca}$ channels have now been observed in many tissues, but $K_{Ca}$1 and $K_{Ca}$2 are expressed at particularly high levels in the CNS, each type distinguished by distinct biophysical properties and pharmacological sensitivities. SK and IK channels exhibit structural and functional similarities that place them into the same gene family (Ishii et al., 1997; Joiner et al., 1997), whereas BK channels are only distantly related. Despite their functional differences, the unifying aspect of these channels is that they act physiologically as negative feedback regulators of $Ca^{2+}$ signaling and membrane depolarization.

## $K_{Ca}$1, BK channels

*Molecular biology*

BK (Big K) channels have large unitary conductances (150-225 pS), are gated by the combined influence of voltage and $Ca^{2+}$, and are present in a wide variety of cell types (Blatz and Magleby, 1984; 1987). Their large conductance and the relative ease of incorporating these channels into planar lipid bilayers facilitated their biophysical characterization (Moczydlowski and Latorre, 1983; Vergara et al., 1984; Kapicka et al., 1994). Clones encoding functional BK channels were first reported in 1992 (Adelman et al., 1992). The BK channel has a 7 transmembrane domain structure with an extracellular N-terminus and a cytoplasmic C-terminal tail (Atkinson et al., 1991; Adelman et al., 1992). Like

voltage-gated $K^+$ channels ($K_V$ channels), voltage-dependence is conferred by positively charged residues on the S4 transmembrane domain, and a P loop connecting S5 and S6 contains the ion selectivity filter as well as the channel pore (Meera et al., 1997). The first transmembrane domain, S0 that is absent in $K_V$ and SK channels, mediates interactions with auxiliary beta subunits (Wallner et al., 1996). BK channels can be divided into three functional domains. The voltage-sensing domain (VSD) includes S1-S4, whereas the pore and gate domain (PGD) comprised of S5 and S6 forms the gate, permeation path, and ion selectivity filter (Yellen, 2002). Finally, the large cytosolic C-terminal domain (CTD) contains two *regulator of $K^+$ conductance domains* (RCK1 and RCK2) domains (Yuan et al., 2010) and a '*$Ca^{2+}$ bowl*' (Schreiber and Salkoff, 1997) all of which bind $Ca^{2+}$ to modulate BK channel gating.

### *BK channel gating*

BK channels are voltage-dependent and $Ca^{2+}$ modulates that voltage-dependence (Moczydlowski and Latorre, 1983). Under physiological conditions BK gating requires elevated $Ca^{2+}$ levels as well as depolarization (Blatz and Magleby, 1984). S4, in the VSD, moves in response to depolarization whereas $Ca^{2+}$ binding to the two RCK domains modulates BK channel gating (Yuan et al., 2010). The $Ca^{2+}$ affinity of RCK1 increases at depolarized potentials, such that at resting membrane potential, $Ca^{2+}$ binding to the $Ca^{2+}$ bowl contributes more to BK channel activation than binding to the RCK1 domain. However, this relationship is reversed at the AP peak where the $Ca^{2+}$ affinity of the RCK1 domain exceeds

that of the $Ca^{2+}$ bowl (Sweet and Cox, 2008). Structure-function experiments show that removal of the region of the CTD containing the RCK2 domain reduces $Ca^{2+}$ sensitivity and increases the open probability of BK channels (Moss and Magleby, 2001). This suggests that $Ca^{2+}$ binding mediated by these domains relieves constitutive inhibition of gating (Salkoff et al., 2006).

*Molecular diversity and modulation*

Alternative mRNA splicing of the single BK channel gene gives rise to an enormous number (potentially >1000) of structurally and functionally distinct isoforms (Adelman et al., 1992; Tseng-Crank et al., 1994). Phosphorylation, and association with auxiliary beta subunits further modulate the biophysical properties of BK channels (Dworetzky et al., 1996; Meera et al., 2000) The open probability of BK channels is directly regulated by the opposing actions of protein kinase A (PKA) and protein phosphatase 2A (PP2A) (Reinhart et al., 1991; Dworetzky et al., 1996; Sansom et al., 1997). Similar to SK channels (see below) BK channel activity is dynamically tuned by reciprocal kinase/phosphatase activity.

Four beta subunits of BK channels have been identified (Knaus:1994ve; Tanaka et al., 1997). Co-assembly with beta subunits occurs through interactions with the S0 domain (Wallner et al., 1996; 1999). The association of up to four beta subunits with the tetrameric pore-forming complex alters functional properties such as surface expression, gating kinetics, voltage-dependence, calcium sensitivity, and the pharmacological profile of the channel (McManus et al., 1995;

Brenner et al., 2000; Meera et al., 2000). Co-expression of pore-forming alpha subunits with beta 1 or beta 4 subunits produce non-inactivating currents, whereas beta 4 alone is reported to slow the activation time (Brenner et al., 2000). Beta 2 or Beta 3 subunits convey rapid inactivation and enhanced sensitivity to $Ca^{2+}$ (Wallner et al., 1999). The tissue specific expression of beta subunits is a potent means of tuning BK channel properties to meet specific functional demands.

*Pharmacology*

BK channels are blocked by low mM concentrations of tetraethyl ammonium (Meech and Standen, 1975; Vergara et al., 1984), as well as two peptide antagonists have been isolated from scorpion toxins, iberiotoxin and charybdotoxin (Miller et al., 1985; Galvez et al., 1990). The results of experiments using antagonists must be interpreted carefully because of altered sensitivities conveyed by beta subunit association. For example, the beta 4 subunit is prominently expressed in the brain and co-expression with alpha subunits results in iberiotoxin and charybdotoxin insensitive channels (Behrens et al., 2000; Meera et al., 2000). Similarly, coexpression with beta 2 or beta 3 subunits confers sensitivity to the BK channel agonist dihydrosoyasaponin-1 and increases charybdotoxin sensitivity (McManus et al., 1995).

*BK channel function*

BK channels have many roles, all of which rely on the unique ability to respond to the coincident increase in membrane potential and intracellular $Ca^{2+}$. In some

neurons such as CA1 pyramidal neurons, BK channels regulate spike frequency adaptation and intrinsic excitability by repolarizing the membrane during action potentials (APs) and may contribute to the fast component of the after-hyperpolarization (fAHP) (Lancaster and Nicoll, 1987; Storm, 1987). BK channels are also negative feedback regulators of dendritic excitability in CA1 pyramidal neurons (Golding et al., 1999). Presynaptic BK channels on hippocampal CA3-CA3 synapses and nerve terminals of the frog neuromuscular junction are activated by AP-mediated depolarization and $Ca^{2}$ (Robitaille et al., 1993; Raffaelli et al., 2004). BK channel activation narrows the AP and decreases the probability of release by limiting presynaptic $Ca_V$ channel activation (Raffaelli et al., 2004).

## $K_{Ca}3$, IK channels

The single gene that encodes the IK channel is very closely related to SK channels, but distinguished by pharmacology and distinct expression profile (Ishii et al., 1997). IK channels share the 6 transmembrane structure of voltage-gated $K^+$ channels and SK channels and share the gating mechanism of SK channels (see below) with a comparable $Ca^{2+}$ sensitivity; the reported $EC_{50}$ ranging from ~ 95 nM (Joiner et al., 1997) to ~ 300 nM (Ishii et al., 1997). Expressed primarily in red blood cells, smooth muscle endothelium, and epithelial tissue, IK channel expression is largely absent from brain (Jensen et al., 1998). IK channel pharmacology shares traits of both SK and BK channels. IK channels are blocked by the BK antagonist charybdotoxin, but not iberiotoxin (Jensen et al., 1998). IK channels are insensitive to the SK channel antagonist apamin, but both

SK and IK currents are potentiated by 1-ethyl-2-benzimidazolinone (EBIO) and NS309 (Jensen et al., 1998; Strobaek et al., 2004).

## $K_{Ca}2$, SK channels

*Cloning and molecular biology*

The three SK channel genes were identified in 1996, KCNN1-3, corresponding to $K_{Ca}2.1$ (SK1), $K_{Ca}2.2$ (SK2) and $K_{Ca}2.3$ (SK3) (Köhler et al., 1996). SK subunits have similar structure to voltage-gated $K^+$ channels, but share only ~ 40% homology. Each subunit has a 6 transmembrane domain topology. Although the S4 TM domain contains two vestigial positive charges, SK channels lack voltage-dependence (Blatz and Magleby, 1986; Köhler et al., 1996; Hirschberg et al., 1998). TM domains are similar among the SK subunits, but the most conserved region is the proximal C-terminus, adjacent to the intracellular face of the membrane. The similar biophysical properties of SK channels suggests that distinct physiological roles arise in part from less well conserved domains in the distal N- and C-termini (Köhler et al., 1996; Xia et al., 1998; Strassmaier et al., 2005).

Several mechanisms enhance SK channel diversity. Most notably in mammalian SK1 genes, the mRNA undergoes extensive alternative splicing, generating as many as 32 different mRNAs (Shmukler et al., 2001). A single SK2 splice variant and two splice variants of SK3 have also been reported (Kolski-Andreaco et al., 2004; Wittekindt et al., 2004; Murthy et al., 2008). Although the extent to which these mRNAs are expressed and act to modify channel properties remains

largely unexplored, the function of a distinct SK2 isoform, generated from an alternative promoter, has begun to be defined (Strassmaier et al., 2005; Allen et al., 2011).

The SK2 gene contains two promoters. An upstream promoter produces a long SK2 ($SK2_L$) isoform that extends the N-terminus by 207 amino acids (Strassmaier et al., 2005) compared to SK2 short ($SK2_S$), generated from a distinct promoter and entirely contained within the $SK2_L$ isoform. $SK2_L$ subunits form functional homomeric channels and co-assembles in-vivo with $SK2_S$ and SK3 subunits (Strassmaier et al., 2005). The extended N-terminal domain of $SK2_L$ contains putative sites for MAP kinase phosphorylation, serine threonine kinase phosphorylation and protein-protein interactions. Additionally, multiple cystine residues are thought to form disulfide bonds and mediate formation of macromolecular complexes.

SK channels are formed by both homomeric and heteromeric subunit assembly. Functional heteromeric SK channels have been demonstrated using all three subunits in heterologous expression systems (Benton et al., 2003; Mongan et al., 2005; Strassmaier et al., 2005). Immuno-precipitation and pharmacological studies have concluded that in some cell types SK2 (Purkinje cells; JPA personal communication) and SK3 (DA neurons; see below) form homomeric channels (Pedarzani et al., 2000; Stocker and Pedarzani, 2000; Wolfart et al., 2001). However, it is also clear that native SK channels may be heteromeric as well (Stocker et al., 1999; Strassmaier et al., 2005).

*Pharmacology*

Apamin, an 18 amino acid peptide isolated from bee venom was the first described and remains the most characterized SK channel antagonist (Habermann and Reiz, 1965). When tested for blocking ability, apamin shows the highest efficacy on homomeric SK2 channels, with an $IC_{50}$ of 107 pM, 6.1 nM for SK3 channels, and ~10 nM for homomeric human SK1 channels (Lamy et al., 2010). Interestingly, both SK2 and SK3 have a much higher affinity for apamin binding ($K_D$ ~ 5 pM) than blocking sensitivity (Lamy et al., 2010). The difference between the binding sensitivity and blocking affinity indicates that following apamin binding, blockade requires further interactions with the channel. Structure-function studies show that high affinity binding is mediated by three amino acid residues in the extracellular loop between transmembrane domains S3 and S4 (Nolting et al., 2007), but actual block requires the subsequent interaction with residues in the outer pore of the adjacent subunit (Weatherall et al., 2011). This suggests that apamin acts as an allosteric inhibitor, rather than a pore blocker as originally thought (Lamy et al., 2010; Weatherall et al., 2011).

One of the major experimental drawbacks of apamin is that its high affinity renders block largely irreversible. In contrast, the less specific agents, d-tubocurarine and bicuculline salts reversibly block SK channels, although both compounds affect other targets (Köhler et al., 1996; Khawaled et al., 1999; Strøbaek et al., 2000). N-methyl-laudanosine, a more recently developed antagonist appears to be both selective for SK channels and reversible (Scuvée-Moreau et al., 2004). Lei-dab 7, a peptide derived from leiurotoxin, a component

of scorpion venom, is reportedly selective for SK2 channels (Shakkottai et al., 2001). The agonist-like compounds EBIO and 6,7 dichloro-1H-indol-2,3-dione 3-oxime (NS309) enhance activity by increasing the apparent $Ca^{2+}$-sensitivity of SK channels (Cao et al., 2001) but are not pure activators.

*SK channel gating*

SK channels are gated solely by increases in intracellular $Ca^{2+}$. The apparent $K_D$ is ~ 0.3 μM, considerably more $Ca^{2+}$ sensitive than BK channels (Blatz and Magleby, 1986; Xia et al., 1998). Additionally, SK channels do not desensitize; their probability of being in the open state depends only on the $Ca^{2+}$ concentration and is not affected by voltage or prolonged exposure to $Ca^{2+}$ (Hirschberg et al., 1998). On the macroscopic scale the strict $Ca^{2+}$-dependence of gating means that SK channels will remain active for as long as $Ca^{2+}$ levels in their environment remain sufficiently high.

Investigations into the $Ca^{2+}$ gating mechanism of SK channels revealed that the exquisite $Ca^{2+}$ sensitivity of is not an inherent property of the pore-forming subunits. SK channels exist as multi-protein complexes (Figure 1), where gating is endowed by the constitutive association with calmodulin (CaM) that serves as the $Ca^{2+}$ sensor (Xia et al., 1998), with $Ca^{2+}$ sensitivity tuned by the opposing actions of protein kinase CK2 and protein phosphatase PP2A (Bildl et al., 2004; Allen et al., 2007). Gating is accomplished when $Ca^{2+}$ binds CaM, which may trigger subunit dimer formation with the adjacent subunit (Schumacher et al., 2001). This molecular rearrangement transmits energy to the activation gate

located in deep in the vestibule of the channel (Bruening-Wright et al., 2007). $Ca^{2+}$ gating is modified by the phosphorylaton state of CaM. In the closed state, CK2 can phosphorylate SK-bound CaM to decrease the apparent $Ca^{2+}$ affinity of the channel. When open, PP2A can remove the phosphate, increasing the $Ca^{2+}$-sensitivity. This provides the only known example of strictly state-dependent modulation of ion channel gating and because the state of the channel is determined by the presence or absence of $Ca^{2+}$ bound to CaM, the $Ca^{2+}$ sensitivity of SK channels is itself $Ca^{2+}$ sensitive (Allen et al., 2007).

The association with CaM is also required for the expression of SK channels on the plasma membrane (Joiner et al., 2001). Mutation studies disrupting CaM binding show that in the absence of bound CaM, SK subunits are retained intracellularly (Joiner et al., 2001; Lee et al., 2003). CaM binding is also a critical component of the non-desensitizing nature of SK channels. CaM is constitutively associated with a highly conserved, CaM binding domain on each subunit (Xia et al., 1998). Typically, the affinity of CaM for a substrate varies such that CaM-substrate complex is stable in only the $Ca^{2+}$-bound state (Klee, 1984) The stability of the SK-CaM interaction in both the $Ca^{2+}$ bound and unbound states explains the lack of desensitization. The strict $Ca^{2+}$ dependence of gating and dynamic $Ca^{2+}$ sensitivity ideally suits SK channels to act as negative feedback regulators of both $Ca^{2+}$ mediated 2nd messenger cascades and electrical activity associated with $Ca^{2+}$ entry.

*The afterhyperpolarization (AHP)*

The AHP is a prominent hyperpolarization that follows APs (Abel et al., 2004). Evoked in part by $Ca^{2+}$ influx, the AHP can have several underlying components defined by the kinetics of onset and decay (Lorenzon and Foehring, 1992). In many neurons, BK and SK channels mediate the fast and medium components, respectively. The molecular identity of the channels underlying the slow phase of the AHP is, in most cases, still unknown, but it too appears to be due to a $Ca^{2+}$-dependent $K^+$ conductance. Together, the three components of the AHP provide a pronounced feedback period in which $Ca^{2+}$ influx associated with APs is transduced to a hyperpolarizing influence on the membrane potential, limiting further activity (Lorenzon and Foehring, 1992). The sensitivity to apamin shows that SK channels contribute to the AHP in at least 15 different cell types, including dopamine neurons of the substantia nigra, pyramidal neurons in the hippocampus, cortex and amygdala, cerebellar Purkinje neurons, serotonergic neurons of the dorsal raphe, vagal motoneurons, and neurons of the subthalamic nucleus (Nedergaard et al., 1993; Meis and Pape, 1997; Womack and Khodakhah, 2003; Bond et al., 2004; Adelman et al., 2012).

The techniques used to the AHP are important to consider when interpreting results. Whole-cell current clamp experiments dialyze soluble molecules, including $Ca^{2+}$ buffers, which may alter native $Ca^{2+}$ handling and thus the AHP, a caveat avoided by intracellular, sharp electrode recordings. To characterize the currents underlying the AHP, voltage-clamp experiments are used to depolarize the soma and elicit $Ca^{2+}$ influx. The resulting tail-current or I-AHP has been

ascribed to the channels contributing to the AHP. However, voltage clamp experiments combine the caveats of altered $Ca^{2+}$ handling with the activation of $Ca^{2+}$ sources in a very different way than would occur during an AP. Thus, the I-AHP is not necessarily a good surrogate for the AHP (Faber, 2009).

*Synaptic transmission & plasticity*

In many neurons, SK channels in dendritic spines shape EPSPs. Apamin increases the amplitude of EPSPs and spine $Ca^{2+}$ transients in hippocampal CA1 pyramidal neurons, pyramidal neurons of the basolateral amygdala, and layer 5 neurons in the medial prefrontal cortex (Faber et al., 2005; Ngo-Anh et al., 2005; Faber, 2010). Thus, synaptically-evoked $Ca^{2+}$ influx activates synaptic SK channels that repolarize the spine head to reduce the EPSP and associated spine $Ca^{2+}$ transient. Immuno-gold labeling of dendritic spines in CA1 pyramidal neurons has shown that SK channels and NMDARs co-localize at the post-synaptic density (PSD) (Lin et al., 2008). There is some controversy regarding the $Ca^{2+}$ source for SK channel activation in CA1 spines. Although NMDAR-mediated $Ca^{2+}$ influx is necessary for SK channel activation, single spine uncaging experiments suggest that $Ca^{2+}$ entry through R-type channels activates synaptic SK channels (Bloodgood and Sabatini, 2007). In the hippocampus, blocking NMDARs and possibly R-type channels occludes the effects of apamin, whereas in the prefrontal cortex, blocking $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) from intracellular stores, L-type $Ca^{2+}$ channels or R-type $Ca^{2+}$ channels occludes the effect of apamin (Ngo-Anh et al., 2005; Bloodgood and Sabatini, 2008; Faber, 2010). These results suggest that distinct $Ca^{2+}$ signaling domains are present

even within single dendritic spines.

Synaptic plasticity, the activity-dependent change of synaptic strength, is induced in many neurons by postsynaptic $Ca^{2+}$ influx, mainly through NMDAR (Malenka and Nicoll, 1993). In the amygdala and hippocampus, apamin decreases the threshold for the induction of long-term potentiation (LTP) (Faber et al., 2005; Lin et al., 2008). SK channels regulate LTP induction by facilitating $Mg^{2+}$ block of NMDARs achieving a concomitant reduction of EPSP amplitude and associated $Ca^{2+}$ influx (Ngo-Anh et al., 2005). Therefore in the presence of apamin the $Ca^{2+}$ level required for triggering plasticity is more easily reached. LTP expression is mediated by both the addition of AMPA receptors to the PSD (Isaac et al., 1995; Durand et al., 1996) and the removal of SK channels (Lin et al., 2008), two components of LTP expression that appear to be linked (Lin et al., 2008). This activity-dependent SK channel trafficking is requires the PKA-mediated phosphorylation of SK channels. It also appears to require the concomitant insertion of GluA1-containing AMPA receptors because blocking their insertion prevented SK channel removal and rescued the apamin enhancement of EPSPs (Lin et al., 2010). Apamin applied following LTP induction does not enhance EPSP amplitude suggesting that most, if not all, synaptic SK channels undergo endocytosis following LTP induction. This result is supported by immunogold electron-microscopy (iEM) of synapses treated with a chemical LTP protocol (Lin et al., 2008). In the hippocampus, SK channel regulation of synaptic transmission requires the expression $SK2_L$ (Allen et al., 2011). In $SK2_S$-only mice, lacking $SK2_L$, apamin has no effect on EPSP amplitude and iEM results show that SK

channels are excluded from the PSD, but are still present in dendritic spines.

LTP is proposed to be a cellular substrate for some forms of learning and memory (Pastalkova et al., 2006; Whitlock et al., 2006). Consistent with this idea, systemic modulation of SK channel activity alters learning tasks. Systemic injection of apamin accelerates learning in Morris water maze (Stackman et al., 2002), enhances working memory (Brennan et al., 2008), and accelerates contextual fear conditioning (Phillips and LeDoux, 1992). Conversely, enhancing SK channel activity by over expression of SK2 or infusion of EBIO, an SK channel activator, impairs task performance as well as the induction of synaptic plasticity (Hammond et al., 2006; Vick et al., 2010).

SK channels have been proposed as therapeutic targets for the treatment of ataxia, addiction, Parkinson's disease, and schizophrenia (Werkman et al., 2006; Wang et al., 2008; Alviña and Khodakhah, 2010; Hopf et al., 2010). For Parkinson's disease and schizophrenia, SK channels are targeted for their ability to influence DA signaling by modulating the activity of DA neurons. Importantly, SK channel activity affects dopamine firing in several ways, including increasing the firing rate, the transitions to burst firing and mGluR receptor signaling, all of which influence DA signaling.

## Dopamine neurons

The patterned activity of DA neurons is centrally important to DA signaling throughout the brain. In vivo, DA neurons typically exhibit both low frequency

regular and irregular APs that maintain a tonic level of DA in target areas. Occasional transitions to high frequency AP bursts result in a dramatic and transient increase in DA levels (Grace and Bunney, 1983; Gonon, 1988; Tepper et al., 1995). Normal variations in DA neuron activity patterns are associated with voluntary movement, reward based learning, and cognition (Clatworthy et al., 2009; Kravitz et al., 2010; Flagel et al., 2011), whereas addiction, Parkinsonism and schizophrenia are associated with hypo- or hyper-dopaminergic states in the striatum – the primary target of midbrain DA neurons (Bernheimer et al., 1973; Grace, 1991a; Graybiel, 1997; Maia and Frank, 2011). The prevalence and severity of these diseases underscores the need to define the determinants of DA neuron activity that may lead to interventional strategies for a wide range of disorders.

*Pacemaking in dopamine neurons*

An intrinsic pacemaking mechanism drives autonomous APs in nigral DA neurons. Pacemaker firing is characterized by regularly timed APs at frequencies ranging from 1 - 8 Hz (Grace and Bunney, 1983). In the presence of TTX, pacemaker-like activity is maintained in adult DA neurons by a slow oscillation of the membrane potential (SOP) and high threshold $Ca^{2+}$ spikes (Fujimura and Matsuda, 1989; Harris et al., 1989; Kang and Kitai, 1993). In freely firing DA neurons, low concentrations of $Ni^{2+}$ and the substitution of $Ba^{2+}$ or $Co^{2+}$ for $Ca^{2+}$, increase the firing rate and decrease AP width, whereas nifedipine, which abolishes the SOP, blockes firing entirely (Harris et al., 1989; Yung et al., 1991; Nedergaard et al., 1993). $Ca_V1.3$, L-type $Ca^{2+}$ channels are a necessary

component of the pacemaker as their blockade abolishes spontaneous APs and the SOP (Nedergaard et al., 1993; Mercuri et al., 1994; Durante et al., 2004). $Ca_V1.3$ channels are well suited to drive pacemaking activity because they are activated at subthreshold potentials and undergo slow inactivation (Scholze et al., 2001; Xu and Lipscombe, 2001; Helton et al., 2005). The reliance of DA neurons on $Ca_V1.3$ channels to drive autonomous APs differs from many other types of spontaneously active neurons, including juvenile DA neurons which rely on the cyclic interplay of $I_h$ and $Na_V$ channels (Nedergaard et al., 1993; Amini et al., 1999; Chan et al., 2007).

The SOP is generated by the sequential activation of depolarizing $Ca_V1.3$ L-type $Ca^{2+}$ channels and repolarizing SK channels (Figure 2) (Nedergaard et al., 1993). Blocking $Ca_V1.3$ channels abolishes the APs and the SOP, whereas blocking SK channels with apamin reduces the characteristic regularity of pacemaker activity and following TTX, converts the SOP into broad plateau potentials. The effect of apamin on the SOP indicates that $Ca_V1.3$ and SK channels are functionally coupled, but in the absence of TTX, during normal pacemaker activity, the effect of this coupling is unknown.

Underscoring the importance of the pacemaker mechanism are studies showing that continued activity is necessary for DA neuron survival. In two models of Parkinson's disease, the loss of spontaneous activity preceded and contributed to the death of nigral DA neurons (Liss et al., 2005). APs were inhibited by the activation of K-ATP channels that hyperpolarized the membrane. Manipulations

that inhibited K-ATP channel activation spared nigral DA neurons (Liss et al., 2005). In cell cultures, DA neuron death occurs spontaneously during the maturation of the culture (Michel et al., 1999). DA neurons in post-natal or embryonic cultures are rescued by several manipulations that enhance activity (Douhou et al., 2001; Salthun-Lassalle, 2004). AP-mediated $Ca^{2+}$ influx is important for DA neuron survival because blockade of $Ca_V$ channels occludes rescue (Douhou et al., 2001; Santi et al., 2002; Salthun-Lassalle, 2004). Intracellular $Ca^{2+}$ levels between 35-80% of control values were maximally protective, suggesting that $Ca^{2+}$ levels outside of a narrow range are unable to support DA neuron survival (Douhou et al., 2001). By supplying negative feedback onto both N- and T-type $Ca_V$ channels in DA neurons, SK channels may play an important role in regulating intracellular $Ca^{2+}$ levels (Nedergaard et al., 1993; Wolfart and Roeper, 2002).

*SK channel regulation of AP frequency*

Apamin has been employed to identify the roles SK channels play in DA neurons of the substantia nigra (Shepard and Bunney, 1988; 1991; Ping and Shepard, 1996). The results show that SK channel activity influences both the frequency and regularity of spontaneous APs, as well as firing mode, as apamin may induce AP bursts. These effects are mediated by SK channel contributions to the pacemaker in which SK channel activity is driven by $Ca^{2+}$ influx through $Ca_V1.3$ $Ca^{2+}$ channels, and by contributions to the AHP, driven by $Ca^{2+}$ influx through $Ca_V3$ T-type $Ca^{2}$ channels and CICR (Grace, 1991b; Shepard and Bunney, 1991). The AHP is a potent regulator of intrinsic excitability and limits the

maximum frequency at which the cells can fire APs (Shepard and Bunney, 1991; Grace, 1991b). Voltage clamp studies of the I-AHP show that current amplitudes recorded from nigral DA neurons correlate with the intensity of SK3 immuno-labeling in those cells and single-cell rtPCR results detected SK3 in all nigral DA neurons tested, but SK1 or SK2 in only a small fraction (Wolfart et al., 2001). Further, apamin and D-tubocurarine dose-response curves were consistent with the blockade of homomeric SK3 channels, leading to the conclusion that nigral DA neurons only express SK3 channels (Wolfart et al., 2001). Two $Ca^{2+}$ sources contribute to the I-AHP in nigral DA neurons. APs elicit $Ca^{2+}$ influx through $Ca_V3$, T-Type $Ca^{2+}$ channels that directly activates SK3 channels. $Ca_V3$ $Ca^{2+}$ influx also induces CICR that further contributes to the SK3 activation (Wolfart and Roeper, 2002; Cui et al., 2007). Their contribution to the I-AHP suggests that SK3 channels also contribute to the AHP. Because apamin has a greater effect on cells that fire faster in control, SK3 is said to act as a low pass filter (Wolfart et al., 2001).

*Synaptic transmission*

DA neurons express AMPARs, NMDARs and nAChRs, as well as mGluRs mAChRs that mediate responses from glutamergic and cholinergic afferents (Grace and Bunney, 1984; Clarke et al., 1987; Johnson et al., 1992; Blaha and Winn, 1993). DA neurons also receive GABAergic projections from several brain areas and responses to agonist application show that DA neurons express $GABA_A$ and $GABA_B$ receptors (Smith and Bolam, 1989; Bolam and Smith, 1990).

Irregular and burst firing modes are induced by synaptic inputs that perturb the intrinsic pacemaker of nigral DA neurons (Grace and Bunney, 1984; Shepard and German, 1988). Glutamatergic inputs from the cortex, and subthalamic nucleus, project to nigral DA neurons (Kita and Kitai, 1987; Naito and Kita, 1994). A third input from the pedunculopontine nucleus releases both glutamate and ACh (Clarke et al., 1987; Futami et al., 1995). Activation of these excitatory pathways increases firing rates (Grenhoff et al., 1986; Johnson et al., 1992; Mameli-Engvall et al., 2006), or induces NMDAR- dependent transitions to burst firing (Lacey et al., 1988; Johnson et al., 1992; Deister et al., 2009). Notably, synaptic stimulation of mGluRs or muscarinic AChRs, induces an IPSP in DA neurons (Fiorillo and Williams, 1998; 2000). Both mGluR and mAChR stimulation induces $Ca^{2+}$ release from the ER that activates SK channels to generate the IPSP (Morikawa et al., 2000). It is thought that these IPSPs serve as a feedback mechanism to terminate bursts using the excitatory signals that generate them (Morikawa and Paladini, 2011).

It is estimated that 70% of synapses impinging on DA neurons release GABA (Smith and Bolam, 1989; Bolam and Smith, 1990; Tepper et al., 1995). GABAergic synapses are concentrated at the soma and proximal dendrites. In vivo, the local application of the $GABA_A$ antagonist gabazine induces transitions to burst firing, indicating that tonic GABA tone influences DA neuron activity (Paladini and Tepper, 1999). Sudden decreases in GABA signaling, result in disinhibition-induced AP bursts (Lobb et al., 2011). GABAergic projections onto DA neurons originate in the striatum, and the output nuclei of the basal ganglia –

the substantia nigra pars reticulata and the internal globus palidus (Chang et al., 1984; Smith and Bolam, 1989; Bolam and Smith, 1990). DA neurons themselves project to the striatum where DA levels influence activity in the straitonigral pathway as well as striatal projections to the external globus palidus. The inhibitory projections from the external globus palidus to the subthalamic nucleus modulate glutamate release back onto nigral DA neurons, thus forming a large, interconnected GABAergic feedback network (Bolam et al., 2000).

*Dopamine signaling in the striatum*

In addition to dopaminergic projections, the striatum receives inputs from sensory, association, and motor areas of the cortex (Figure 3). By integrating cortical and thalamic inputs, the striatum relays information into the basal ganglia circuit and is instrumental in linking reward, emotion and motivation in goal directed actions (Balleine et al. 2007, Cisek & Kalaska 2010, Nambu 2008). DA signaling in the striatum is mediated predominantly by D1 and D2 receptors expressed on GABAergic medium spiny neurons (MSNs) , the output neurons of the striatum (Gerfen et al., 1990). MSNs comprise ~ 90% of the striatal neuron population and are segregated into two output pathways. The direct pathway expresses excitatory, $G_s$-coupled D1 receptors, and the indirect pathway that expresses inhibitory $G_i/G_o$-coupled D2 receptors (Surmeier et al., 1996; Gong et al., 2003). The degree of excitatory D1 and inhibitory D2 receptor signaling balances the activation of the direct and indirect pathways and modulates the information processed by the striatum (Kreitzer and Malenka, 2008).

*Striatal synaptic plasticity mediated by dopamine signaling*

Consistent with the proposal that DA signaling acts to reinforce rewarding behaviors, DA modulates the induction of long-term changes in synaptic strength at excitatory corticostriatal synapses (Schultz et al., 1997; Schultz, 1998; Shen et al., 2008). In the direct pathway MSNs, spike-timing dependent LTP is induced by the coincident activation of D1 and NMDA receptors with dendritic depolarization while LTD is induced by the activation of mGluRs and dendritic depolarization that activates $Ca_V1.3$ channels (Shen et al., 2008). In the indirect pathway, LTD is induced when mGluRs, $Ca_V1.3$ channels and D2 receptors are simultaneously activated, and LTP is induced by coincident dendritic depolarization with the activation of adenosine A2a receptors and NMDA receptors (Shen et al., 2008). Conceptually, these findings suggest a model where a rewarding action induces DA neuron bursts. The resulting elevated striatal DA levels and simultaneous excitatory cortical input promote the induction of LTP in the direct pathway and LTD in the indirect pathway. Conversely, when an animal encounters aversive stimuli that inhibit DA neuron activity, this promotes indirect pathway LTP and LTD in the direct pathway. There is some precedent for this model. Intracranial self-stimulation experiments in which rats learned to press a lever that electrically stimulated their own substantia nigra showed potentiated corticostriatal synapses (Reynolds et al., 2001), and actions yielding unanticipated reward promote DA neuron bursts (Schultz et al., 1997). The difficulty is that in vivo, the reward signal necessarily occurs later in time than the actions that produce it and later still than the synaptic activation that produces the action. This is known as the distal

reward problem, and although the answer is unknown, it is clear that DA signaling influences moment-to-moment striatal function as well as long-term regulation of synaptic strength. Understanding the determinants of DA neuron activity and dynamics is necessary to understand DA signaling in healthy and pathological states.

*Somatodendritic dopamine release*

Midbrain DA neurons also release DA locally via somatodendritic release (Geffen et al., 1976; Cheramy et al., 1981). Bursts increase somatodendritic DA release that acts as a negative feedback regulator of DA neuron activity through several mechanisms. The most direct form of feedback is via D2 receptors expressed on the DA neurons. By coupling to GIRK channels, D2 activation hyperpolarizes DA neurons to decrease excitability and DA release (Lacey et al., 1988). Indirect feedback is provided by presynaptic D1 receptors expressed on GABAergic terminals (Cameron and Williams, 1993). DA levels sufficient to activate D1 receptors enhance GABA release to further inhibit DA neurons (Aiso et al., 1987; Floran et al., 1990; Cameron and Williams, 1993; Acosta-Garcia et al., 2009).

It is clear that SK channels in nigral DA neurons perform several physiological roles. SK3 channels are coupled to T-type $Ca^{2+}$ channels and CICR to contribute to the AHP and regulate AP frequency (Wolfart and Roeper, 2002). SK channels mediate IPSPs induced by activation of mGluRs and AChRs (Fiorillo and Williams, 1998; Paladini and Williams, 2004). In addition, SK channels interact with the pacemaking mechanism of DA neurons by providing feedback to CaV1.3

mediated $Ca^{2+}$ influx and influence the precision of AP firing (Nedergaard et al., 1993). All of these roles have been ascribed to SK3 channels, yet the coupling of SK channels to $Ca_V1.3$ channels, $Ca_V3$ channels, and $Ca^{2+}$ released from intracellular stores suggests several functionally distinct populations of SK channels in DA neurons that differentially contribute to their physiological roles. We show that nigral DA neurons express functional SK2 containing channels that are preferentially trafficked to the distal dendrites, and that SK2-containing and SK3-containing channels differentially modulate firing frequency and precision.

Chapter 3 characterizes the roles of SK channels in regulating the firing properties of DA neurons. By using wild type, $SK2^{-/-}$, and $SK3^{-/-}$ mice distinct functional roles for SK2 and SK3 channels were discriminated. The final experiment demonstrated that the preferential block of SK2 channels was able to selectively alter AP timing in wild type DA neurons. Chapter 4 presents the details of a series of control experiments that were not published with the results presented in chapter 3. These experiments, using $SK3^{-/-}$ mice, demonstrate that the block of homomeric SK2 channels with 300 pM apamin was sufficient to alter AP timing in DA neurons. Chapter 5 summarizes experiments investigating the possibility that SK channels influence AMPAR/NMDAR-mediated synaptic transmission onto DA neurons. Each of the chapters presenting data are concluded with a discussion of the results. The final chapter is a more expansive discussion of the possible significance of the results presented in this thesis.

**Figure 1. Structure and modulation of SK channels**

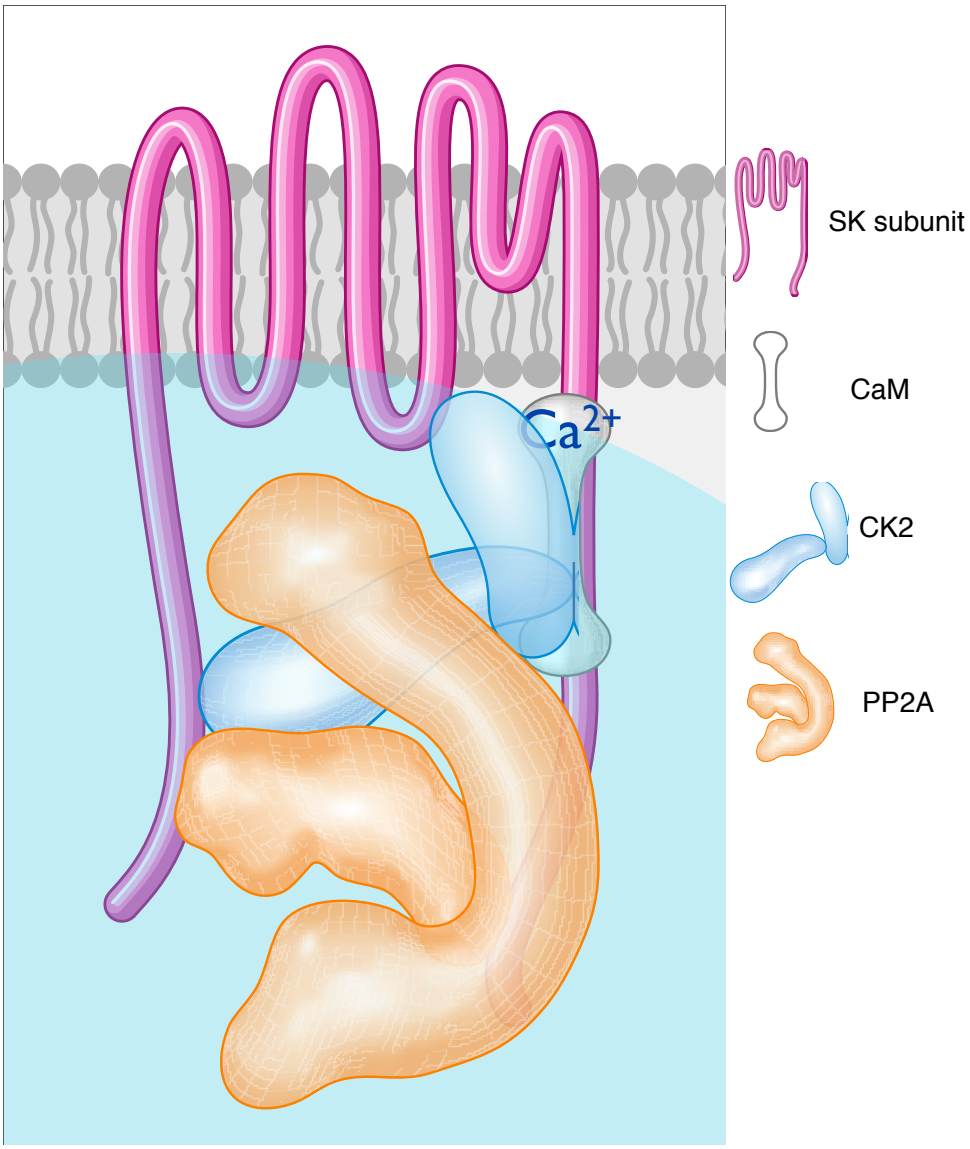


Figure1. Functional SK channels are multi-protein complexes, formed by tetrameric assemblies of subunits and modulatory proteins. $Ca^{2+}$ sensitivity is conferred by CaM, constitutively bound to the C-terminal domain of each subunit. CK2 and PP2A regulate the apparent $Ca^{2+}$ affinity by controlling the phosphorylation state of SK channels.

**Figure 2. TTX reveals the SOP of nigral dopamine neurons**

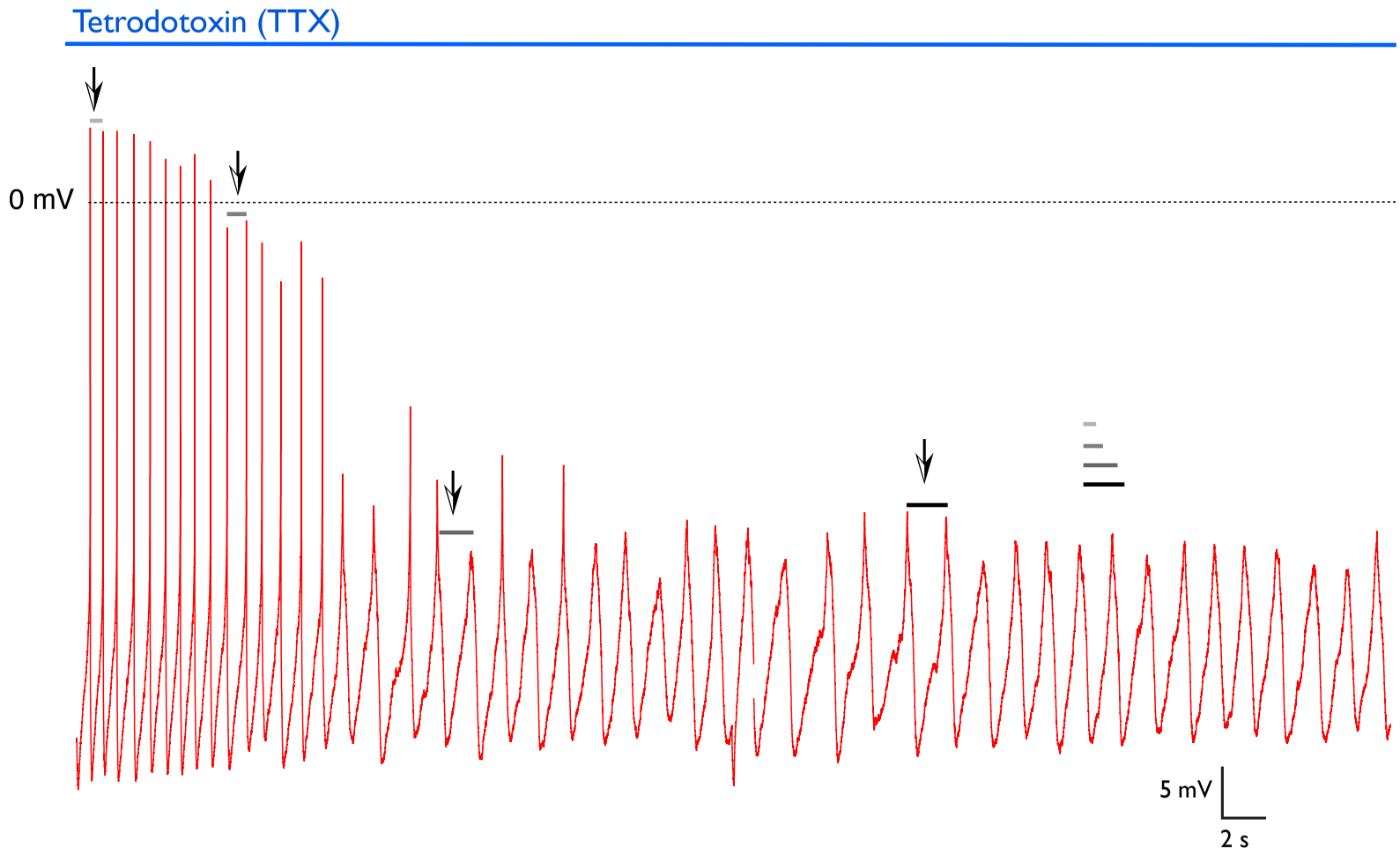

Figure 2. A whole-cell current clamp recording of spontaneous activity in a nigral DA neuron at the onset of the TTX application (blue line). Arrow heads mark bars indicating the duration between successive peaks. Bars are aligned on the (right) to illustrate the increase in interval duration as APs stop influencing the SOP. Dashed line indicates 0 mV.

**Figure 3. Dopamine signaling in the striatum**

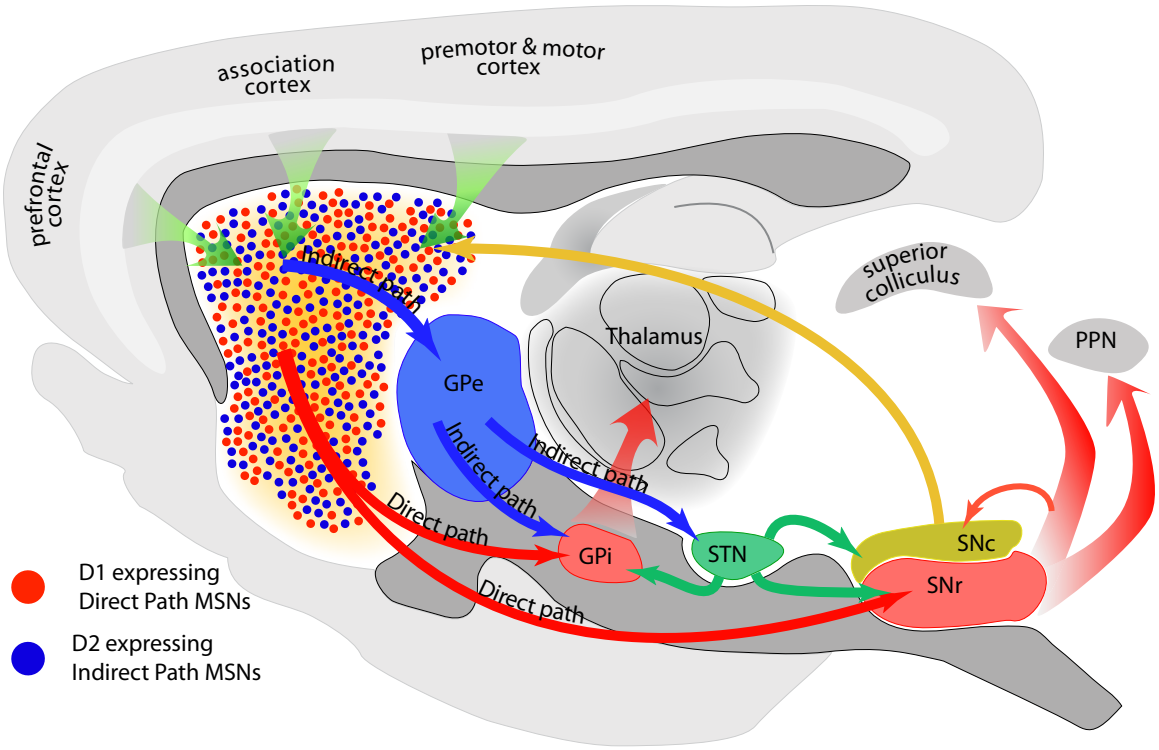


Figure 3. A sagital view of the striatum and basal ganglia. Striatal output neurons, MSNs (red and blue circles), receive excitatory inputs from the cortex (large green arrows). MSN activity is modulated by DA released from projections arising in the SNc (yellow arrow). DA increases excitability in MSNs expressing excitatory D1 receptors (red circles). The associated direct pathway activity removes cortical and thalamic inhibition (large red arrows) by GPi and SNr, the output nuclei of the basal ganglia. Whereas the direct pathway is stimulated, DA reduces indirect pathway output by inhibiting activity in the D2-expressing MSNs. Diminished DA release relieves D2-mediated inhibition of the indirect pathway. When disinhibited, the indirect pathway increases inhibitory drive to the thalamus and cortex by increasing activity in the GPi and SNr. GPe-globus palidus externus, GPi-globus palidus internus, STN-subthamic nucleus, SNc-substantia nigra.

# *Chapter II. Materials and Methods*

All experiments utilized C57/BL6 mice that were a minimum of 21 days old. The Institutional Animal Care and Use Committee approved all animal handling and protocols.

## Generation of SK null mice

### *SK2$^{-/-}$ mice*

The generation of SK2 null mice has been previously described (Bond et al., 2004).

### *SK3$^{-/-}$ mice*

A single lox P site was introduced into the first exon of the mouse SK3 gene, 200 bp 5' of the start of translation. The coding sequence for the neomycin resistance gene (neo), flanked by frt sites and followed by a single loxP site, was inserted into intron 1. Five chimeric mice derived from implantation of the same ES cell clone gave rise to germ line founders. Each of these lines have been crossed to an FLP-expressing mouse to remove the neo coding sequence, and subsequently backcrossed to C57Bl/6J for > 10 generations. Floxed mice were crossed to a global Cre expressing mouse (Schwenk et al., 1995) resulting in SK3$^{-/-}$ mice. The removed protein sequence constitutes the intracellular N-terminus and the first transmembrane domain. Probing Western blots prepared using protein extracts from wild type and SK3$^{-/-}$ mouse brains with anti-SK3 antibody (Alomone Labs) failed to detect a signal in the null mice (not shown).

## Real-time PCR

Total RNA from microdisections of 600 µm midbrain slices was isolated using Tri-reagent according to manufacturer's protocol. Total RNA was reverse-transcribed by MMLV reverse transcriptase (Invitrogen) in the presence of random hexamers but without dithiothriotol. Real-time PCRs were performed in triplicate for each SK transcript in each genotype, and expression levels were determined by comparison to 18S rRNA. The amplicon for 18S was 76 bp (primers: CCGCAGCTAGGAATAATGGA, CCCTCTTAATCATGGCCTCA); for SK1, 118 bp (primers: GCTCTTTTGCTCTGAAATGCC, CAGTCGTCGGCACCATTGTCC); for SK2, 151 bp (primers: GTCGCTGTATTCTTTAGCTCTG, ACGCTCATAAGTCATGGC); for SK3, 148 bp (primers: GCTCTGATTTTTGGGATGTTTG, CGATGATCAAACCAAGCAGGATGA). All SK amplicons span an intron. The efficiencies of the primer pairs were tested in a validation experiment using serial dilutions of a wild type cDNA (slope of ΔCt ($SK_{Ct}$-$18S_{Ct}$) <0.1; not shown). Ct, the threshold cycle, indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The reaction master mix, consisting of 10X buffer, Mg ($C_f$ = 4mM), dNTPs ($C_f$ = 200 mM), Platinum taq polymerase (Invitrogen, Carlsbad, CA) (0.6 units/ 20 µl reaction) and SYBR Green (Molecular Probes) (0.5X manufacturer's recommended concentration), was aliquoted, the cDNA substrates added, and then further aliquoted and primers added ($C_f$ = 200 nM). Reactions were then split into triplicates for amplification in an MJ Research Opticon DNA Engine with cycling parameters 95˚C, 2 minutes 1X; 95˚C, 30 seconds/ 64˚C, 45 seconds,

with fluorescence read at 78˚C for 40 cycles. A melting curve and gel electrophoresis analysis verified that a single product was amplified in all reactions. For each run, the relative mRNA level was determined by the expression $2^{-\Delta\Delta Ct}$ ($\Delta Ct$ ($SK_{Ct}$-$18S_{Ct}$) within each genotype, $\Delta\Delta Ct$ ($\Delta Ct_{SK\ transgene}$-$\Delta Ct_{wildtype}$) (ABO Prism 7700 Sequence Detection System, user bulletin 2). The mean and standard error of the value $2^{-\Delta\Delta Ct}$ for each SK mRNA in each genotype, across all runs, were plotted. Statistical significance was determined by 1-way ANOVA of $\Delta Ct$ values across all genotypes followed by Bonferroni t-test.

## Immuno-electron microscopy

Mice were transcardially perfused with 0.1 M phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde, 0.05% glutaraldehyde and 15% picric acid. Brains were removed from the skull and 60 µm thick sections were cut using a Vibratome. The sections were then processed for immunohistochemical detection of SK2 or SK3 and TH using double labelling pre-embedding techniques, as described previously (Koyrakh et al., 2005).

### *Antibodies*

The primary antibodies used were: rabbit anti-SK2 polyclonal antibody (custom), rabbit anti-SK3 polyclonal antibody (Alomone Labs), and mouse anti-TH monoclonal antibody (Calbiochem). The characteristics and specificity of the antibodies anti-SK2 subunit have been described elsewhere (Cueni et al., 2008; Lin et al., 2008).

## Electrophysiology

Electrophysiological recordings were made using 220 µm, horizontal, midbrain slices from wild type, $SK2^{-/-}$ (Bond et al., 2004) or $SK3^{-/-}$ mice. Mice anesthetized with isoflurane were decapitated and the brains rapidly removed. Acute horizontal sections were made using a Leica VT1000 vibratome (Leica Microsystems) in an ice slurry of cutting solution composed of (in mM): 119 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1 $NaH_2PO_4$, 1.3 $MgCl_2$, 2 $CaCl_2$, 25 dextrose, 3 ascorbate, and 1 pyruvate and equilibrated with 95%$O_2$/5%$CO_2$. The NMDAR antagonist, MK801(10 µM) was included in the cutting solution of experiments not measuring EPSPs. Slices recovered at 34ºC in cutting solution equilibrated with 95%$O_2$/5%$CO_2$ for at least 30 min until used for recording. Slices were then transferred to a heated recording chamber (33ºC) and perfused (3 ml/min) with ACSF composed of (in mM): 119 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1 $NaH_2PO_4$, 1.3 $MgCl_2$, 2 $CaCl_2$, 25 glucose, equilibrated with 95%$O_2$/5%$CO_2$. DA neurons were visualized with a CCD camera mounted on an Olympus BX-51 microscope equipped with a 60x, 0.9 N.A., water immersion objective and modified Dodt contrast enhancement optics. To minimize heterogeneity among DA neurons, cells from the medial SN were targeted for recording. DA neurons were identified by their location within the slice, spontaneous activity of 1 - 8 Hz, extracellular AP duration > 2 ms, and the presence of $I_h$ activated at a holding potential < -70 mV when in whole-cell mode (Grace and Bunney, 1983; Kita et al., 1986; Grace and Onn, 1989). Control ACSF was supplemented with the following antagonists to isolate intrinsic properties of DA neurons: SR 95531 (5 µM; $GABA_A$), CGP55845 (2 µM; $GABA_B$),

Sulpiride (100 nM; $D_2$ receptors), D-AP5 (50 µM; NMDA receptors), and CNQX (25 µM; AMPA receptors).

Patch pipettes were pulled from borosilicate glass capillary tubing (Sutter, Novato CA), and had tip resistances between 1.5-3.5 MΩ. Whole-cell voltage clamp recordings of tail-currents were conducted with an internal solution containing (in mM): 115 $KMeSO_4$, 20 NaCl, 1.5 $MgCl_2$, 10 HEPES, 0.1 EGTA, 2 Mg-ATP, 0.2 $Na^+$-GTP, and 10 Na-phosphocreatine and adjusted to pH 7.3 with KOH and 290 mOsm. Tail-current recordings were made using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA) and AxoGraph X software (AxoGraph, Sydney, Australia). Data were digitized at 10 KHz with a Digidata 1322A digitizer (Molecular Devices, Sunnyvale, CA) and filtered at 2 KHz. Uncompensated, series resistances ranged from 5 to 20 MΩ. Series resistance was compensated at 75-80% and cells in which the series resistance changed by more than 20% were discarded.

Recordings of evoked synaptic responses were made with a MultiClamp 700B amplifier run by Axograph X (AxoGraph, Sydney, Australia), data acquisition software. Data were acquired at 10 kHz and digitized with an ITC-18 (HEKA, Bellmore, NY). Synaptic stimulation was delivered with a Digitimer, constant current isolated stimulator (Welwyn, Garden City, England) connect to a fine (12.5 µM tip separation) bipolar stimulating electrode, (FHC, Bowdoinham, ME). EPSCs were recorded using an internal solution containing in mM: 130 $Cs^+$ methanesulfonate, 10 CsCl, 10 HEPES, 2 $Mg^{2+}$-ATP, 0.4 $Na^+$-GTP, 3.35 QX314,

0.02 D600, 5 BAPTA. Series resistance, but not whole cell capacitance, was compensated for. EPSPs were recorded with an internal solution containing (in mM) 133 $K^+$-gluconate, 10 $K^+$-HEPES, 10 NaCl, 1 MgCl, 0.05 EGTA, 4 $Mg^{2+}$-ATP, 0.04 $Na^+$-GTP.

*Cell attached recordings.* Cells exhibiting spontaneous activity were selected for recording. Recordings were made using either an Axopatch 200A or Multiclamp 700B amplifier (Axon Instruments). Records were digitized at 5 KHz and filtered at 2 KHz. *Analysis and Statistics.* Data were analyzed with Igor Pro (Wavemetrics, Lake Oswego, OR) and statistics were calculated using the statistical software environment R (R Foundation for Statistical Computing). Mean ISI measurements were not significantly different than normal (Shapiro Wilks test $p > 0.1$). CV measurements were log-transformed to enhance symmetry and approximately equalize within-group variance. The transformed CV measurements and ISI measurements were analyzed using ANOVA, and significant differences identified with Fisher's least significant difference tests. P values $< 0.05$ were considered significant. ANOVA analyses of log-CV data identified the same significant effects as non-parametric tests of CV measurements, but were preferred because ANOVA provided an estimate of the effect magnitude. Data were presented as mean ± S.E.M.

# *Chapter III*

# *SK channel regulation of DA neuron firing properties*

## Preface

The contents of chapter III have been published (Deignan et al., 2012). The introduction and methods sections have been moved to chapters I and II, respectively.

## Results

*SK2 and SK3 are expressed in DA neurons*

Whole cell voltage clamp recordings were performed on brain slices prepared from wild type, $SK2^{-/-}$ and $SK3^{-/-}$ mice. No differences in input resistance (MΩ) were detected between the genotypes (wild type 180 ± 17, n = 9; $SK2^{-/-}$ 242 ± 45, n = 5; $SK^{-/-}$ 164 ± 45; n = 5) Voltage steps from -70 mV to 0 mV, for 50 ms, followed by repolarization to -50 mV elicited outward tail currents that were predominantly blocked by apamin (Figure 4A-C). SK channel-mediated currents ($I_{SK}$) were obtained by subtracting the residual current recorded following apamin (200 nM) application from that recorded under control conditions. Apamin-Sensitive currents, $I_{SK}$, were observed in all three genotypes and the decay of the $I_{SK}$ currents were well described by single exponential functions. The peak of the $I_{SK}$ from wild type DA neurons (1175 ± 117 pA, n = 9) was significantly larger than either the $I_{SK}$ from $SK2^{-/-}$ DA neurons (490 ± 132 pA; n = 5; $P < 0.01$) or the $I_{SK}$ from $SK3^{-/-}$ DA neurons (122 ± 23 pA; n = 5; $P < 0.001$; Figure 4D). No significant differences were found between the decay kinetics of the three groups ($\tau_{decay}$

(ms): wild type, 199 ± 16, n = 8; $SK2^{-/-}$,188 ± 22, n = 5; $SK3^{-/-}$, 258 ± 71; n = 5).

Apamin-sensitive SK channel activity strongly influences the firing characteristics of DA neurons (Shepard and Bunney, 1988; 1991; Nedergaard et al., 1993; Ping and Shepard, 1996; Chan et al., 2007). Previous work showed that SK3 channel activity regulates AP frequency in DA neurons, but did not find evidence of functional SK2 channel expression (Wolfart et al., 2001). Therefore the finding of apamin-sensitive currents in $SK3^{-/-}$ mice might reflect compensatory expression of SK2. To determine whether SK2, as well as SK3 is expressed in wild type DA neurons iEM was performed. The specificity of the SK2 antibody has been previously demonstrated using sections from $SK2^{-/-}$ mice (Lin et al., 2008). Similarly, the SK3 antibody did not stain sections prepared from $SK3^{-/-}$ mice (not shown). DA neurons were identified by tyrosine-hydroxylase (TH) immunoreactivity (Figure 5 A,B,D,E). In TH-positive neurons, SK2 immunoparticles were sparsely distributed and almost exclusively found in the dendritic plasma membrane (Figure 5 A,B). For SK2 1.7% (2 immunoparticles) were in the soma, 26.3% (31 immunoparticles) were in thick dendrites (> 1μm2; presumably primary or secondary dendrites) and 72% (85 immunoparticles) were in thin dendrites (< 1μm; presumably distal dendrites). In contrast, SK3 immunoparticles labelled both the somatic plasma membrane and the plasma membrane of dendrites (Figure 5 D,E). For SK3, 29.2% (102 immunoparticles) were in the soma, 36.7% (128 immunoparticles) were in thick dendrites (> 1μm2), and 34.1% (119 immunoparticles were in thin dendrites (< 1μm2). These results demonstrate that SK2 is expressed in wild type DA neurons of the SN in a

pattern that partly overlaps the subcellular distribution of SK3.

To further characterise SK expression levels, qPCR was performed using RNA extracted from each genotype. Tissue samples containing the ventral tegmental area and SN were micro-dissected from 0.6 mm horizontal slices of mouse midbrain. The results showed that SK2 expression in $SK3^{-/-}$ mice was not different from wild type ($P > 0.1$). In contrast, SK3 expression was increased to 142% in $SK2^{-/-}$ mice compared to wild type ($P < 0.01$).

*SK channels influence both the rate and timing of action potentials*

To investigate the roles of SK2 and SK3 in regulating intrinsic firing properties, loose cell-attached recordings were made from spontaneously active wild type DA neurons. DA neurons were identified by the presence of pacemaker firing, broad extracellular APs (> 2 ms), and sensitivity to bath application of DA following experiments. All experiments were performed in the presence of synaptic blockers to isolate the intrinsic properties of recorded cells (see Methods). After obtaining at least 15 min of stable control recording, the SK channel blocker, apamin (200 nM), was bath applied. In wild type neurons, apamin application decreased the interspike interval (ISI) (Figure 6A). On average apamin decreased the ISI in 6 out of 7 of the recorded cells (control: 452 ± 38 ms; apamin: 325 ± 35 ms; $P < 0.01$, $n = 7$), corresponding to a 28 ± 13% decrease in the ISI. The leftward shift of the bottom portion of the cumulative probability histograms shows that apamin selectively increased the proportion of short intervals. (Figure 6 C).

The regularity of ISIs in wild type DA neurons was also affected by the blockade of SK channels as evidenced by the obvious change in regularity of AP firing in the representative traces and in the increased range of ISIs plotted versus time in Figure 6A. Histograms of the ISI (Figure 6B) reveal that apamin application both decreases the mean and increases the standard deviation of ISIs as shown by the increased width of the histogram. To quantify the precision of firing the coefficient of variation (CV) was determined, which increased in 7 of 7 cells (control: 0.05 ± 0.01; apamin: 0.30 ± 0.16, $P < 0.01$) demonstrating decreased regularity of firing after apamin application (Figure 6D).

To determine the relative contribution of SK2 and SK3 to these firing properties recordings were made from $SK^{-/-}$ mice. In slices prepared from $SK2^{-/-}$ mice the mean ISI was significantly longer than that of wild type DA neurons (wild type: 452 ± 38 ms; $SK2^{-/-}$: 644 ± 76 ms; $P < 0.05$). In $SK2^{-/-}$ mice, the effects of apamin can be attributed to the blockade of SK3-containing channels. As in wild type DA neurons, apamin application to $SK2^{-/-}$ DA neurons decreased the ISI (Figure 7A). On average apamin decreased the mean ISI by 22 ± 16% (control: 644 ± 76 ms; apamin: 492 ± 52 ms, $n = 9$; $P < 0.01$; Figure 7A). While apamin decreased the mean ISI in $SK2^{-/-}$ DA neurons, it remained significantly greater than that of wild type DA neurons (wild type, apamin: 325 ± 35 ms; $SK2^{-/-}$, apamin: 492 ± 52 ms; $P < 0.05$). In the absence of SK2 expression, blocking SK3-containing channel activity decreased firing precision as shown by the increase of the width of the ISI histogram and increase in CV (control: 0.08 ± 0.02; apamin: 0.34 ± 0.16, $P < 0.05$; Figure 7 B,D). Similar to wild type DA neurons, the cumulative probability

histogram of ISIs revealed a selective effect on the proportion of shorter intervals. Apamin application significantly decreased 72% of ISIs ($P < 0.05$; Figure 7C).

Experiments were performed on slices prepared from $SK3^{-/-}$ mice where the effects of apamin can be attributed to the blockade of SK2-containing channels. In $SK3^{-/-}$ DA neurons, apamin did not affect the mean ISI (control: 490 ± 72 ms; apamin: 466 ± 84 ms, n = 8; P = 0.38; Figure 8). The decreased regularity of the ISIs in apamin is evident in both the example traces and ISI diary plot (Figure 8A). SK2 channel blockade selectively broadened the distribution of ISIs as indicated by the increase of the ISI histogram width and increased CV (control: 0.08 ± 0.01; apamin: 0.17 ± 0.04; p = 0.01; Figure 8 B,D). The effects of apamin on mean ISI and CV are summarized the bar graph in Figure 9, which clearly shows that apamin decreases ISI only in WT and $SK2^{-/-}$ mice whereas it decreases precision in both $SK2^{-/-}$ and $SK3^{-/-}$ mice.

The results presented above using 200 nM apamin to block SK channels in $SK3^{-/-}$ DA neurons suggest that SK2 containing channels may selectively influence the regularity of action potentials while the results from $SK2^{-/-}$ DA neurons suggest SK3 channels may selectively influence firing frequency. Homomeric SK2 channels are ~50-fold more sensitive to block by apamin compared to homomeric SK3 channels ($EC_{50}$ ~100 pM vs ~5 nM; Lamy et al., 2010). Therefore, application of 300 pM apamin should block greater than 90% of SK2 channels while blocking less than 10% of SK3 channels. As shown in Figure 10, 300 pM apamin increased the CV (control: 0.04 ± 0.006; 300 pM apamin 0.07 ±

0.02; $p<0.05$; n=15), resulting in a 154% ± 19% increase in the CV, but did not alter isi (control: 411 ± 41 ms; 300 pM apamin 419 ± 41 ms; $p>0.05$; n=15). In control conditions, the ISI and CV values were not different from those found in the previous series of experiments using 200 nM apamin (expt 1- ISI: 452 ± 38 ms, CV: 0.05 ± 0.01; expt 2- ISI: 411 ± 41 ms, CV: 0.04 ± 0.006)

## Discussion

The results presented here show that both SK2 and SK3 are expressed in DA neurons of the SN. The iEM results demonstrate that SK2 and SK3 have partially overlapping subcellular distributions within DA neurons. Electrophysiological recordings show that SK2 channels selectively contribute to AP timing while SK3 channels impact both AP frequency and timing.

The iEM clearly establishes that SK2 is expressed exclusively in the dendrites of DA neurons. Since we cannot unambiguously identify each dendrite with the parent soma this raises the question of whether SK2 is expressed in every DA neuron. However, apamin (200 nM) blocked tail currents in all $SK3^{-/-}$ DA neurons tested, and applying a lower concentration of apamin (300 pM) that selectively blocks SK2 channels to wild type DA neurons decreased the precision of AP firing in 13 out of 15 DA neurons tested, suggesting that functional SK2-containing channels are expressed in most, if not all, DA neurons in the medial SN.

To gain insight into the distinct roles of SK2 and SK3, studies were performed on

the respective null mice. In principle this should allow a clear delineation of the roles for SK2 and SK3, however, the mice show indications of compensatory alterations. This was first apparent from the tail current measurements, as the sum of apamin-sensitive tail currents from $SK2^{-/-}$ and $SK3^{-/-}$ DA neurons is less than the apamin-sensitive tail current in wild type DA neurons, despite a likely upregulation of SK3 expression in $SK2^{-/-}$ mice as suggested by qPCR for SK3 mRNA. This might, in part, reflect a more dendritic expression profile for the remaining SK channels in each of the null mice, that would minimize their contribution to somatically recorded currents (i.e. lack of dendritic space clamp). It is also possible that the absence of SK2 or SK3 induces compensatory changes that reduce $Ca^{2+}$ influx during the voltage protocol, resulting in a smaller apamin-sensitive tail current.

The effects on firing frequency (mean ISI) in the null mice clearly show that SK3 is required for apamin to affect the mean ISI; apamin reduced the mean ISI in wild type but did not affect the mean ISI in $SK3^{-/-}$ DA neurons. This result corroborates previous conclusions that SK3 regulates AP frequency by contributing to the AHP (Wolfart et al., 2001). Moreover, cumulative probability distributions show that blocking SK3 (in wild type and $SK2^{-/-}$ DA neurons) preferentially increases the proportion of short intervals, supporting the conclusion that, by contributing to AHP, SK3 acts as a low pass filter (Wolfart et al., 2001). The notable difference with previous studies is the remaining apamin-sensitive component of the I-AHP in the absence of SK3. However, the dominant contribution of SK3 to the I-AHP and the lack of highly subtype selective

antagonists preclude discriminating an SK2 contribution to the I-AHP. In addition, it is possible that the single cell PCR approach used by Wolfart, et al. efficiently extracted the somatic mRNA population but might have underestimated the SK2 mRNA content if the SK2 mRNA is localized to the dendrites where it undergoes local translation.

The results also indicate that there are compensatory changes in the $SK3^{-/-}$ mice. For example, the mean ISIs for $SK3^{-/-}$ and wild type DA neurons are not different, even though loss of SK3 would be expected to decrease the mean ISI to that of wild type DA neurons in the presence of apamin. There are also indications from measurements of the mean ISI that there have been compensatory changes in the $SK2^{-/-}$ mice. The mean ISI of $SK2^{-/-}$ DA neurons is larger than that for wild type. While this might reflect the upregulation of SK3, it is unlikely, as apamin application should normalize the mean ISI to that of wild type in apamin, but it does not. Apamin reduces the mean ISI in $SK2^{-/-}$ DA neurons, but it remains larger than the mean ISI in wild type DA neurons in the presence of apamin.

The data suggest that both SK2 and SK3 channels affect the precision of AP firing (ISI-CV) as apamin reduced AP precision (increased the ISI-CV) in all genotypes, even though the ISI-CV values were not different in control conditions among the three genotypes. SK2 channels may selectively affect the precision of AP firing (ISI-CV) as apamin reduced AP precision (increased the ISI-CV) in wild type and $SK3^{-/-}$ DA neurons. This is supported by the finding that apamin, while increasing the ISI-CV, did not affect the mean ISI in $SK3^{-/-}$ DA neurons. The effect

of apamin, increasing the ISI-CV in $SK2^{-/-}$ DA neurons, suggests that SK3-containing channels also affect firing precision. However, this might reflect compensation in the $SK2^{-/-}$ mice as indicated by the upregulation of SK3 mRNA.

Previous single cell PCR studies detected SK1 mRNA expression in DA neurons (Wolfart et al., 2001). While evidence for native protein expression of rat or mouse SK1 is lacking, heterologous expression studies have shown that rat or mouse SK1 subunits do not form functional homomeric channels in the plasma membrane. However, expression of chimeric subunits containing the transmembrane core of rat SK1 did result in functional homomeric channels that were not blocked by apamin (D'hoedt et al., 2004). Moreover, co-expression of mouse or rat SK1 with SK2 or SK3 (Benton et al., 2003); unpublished) did yield functional heteromeric channels that were apamin-sensitive. Therefore, while we cannot rule out a contribution of SK1 subunits to the SK channels in $SK2^{-/-}$ and $SK3^{-/-}$ mice, the functional channels must contain the remaining apamin-sensitive subunits.

Several findings using $SK2^{-/-}$ and $SK3^{-/-}$ mice support the conclusions that SK2-containing channels are functionally expressed in DA neurons and that they selectively influence the precision, but not the frequency, of AP firing. First, apamin blocked the tail currents in $SK3^{-/-}$ DA neurons. Second, apamin decreased the precision of AP firing in $SK3^{-/-}$ mice. Third, the effect of apamin on the precision of firing in $SK3^{-/-}$ mice occurred despite the lack of apamin effect on AP frequency, also showing that SK2 does not compensate for the loss of SK3.

Fourth, the deletion of SK2 affected both the mean ISI and tail current amplitudes. Nevertheless, there are clearly complications arising from compensatory changes in each of the null mice.

To directly test for the role of SK2 channels in DA neurons, a concentration of apamin (300 pM) was applied to wild type DA neurons that will selectively block >90% of SK2, but <10% of SK3 channels. The results support the conclusion that SK2 channels primarily influence the regularity of action potential firing, as the ISI CV increased while the frequency of action potentials was unaltered. Though unlikely, we cannot, however, formally exclude the possibility that the low dose of apamin is mediating its effects on the regularity of firing by blocking a small fraction (<10%) of SK3 channels that selectively contribute to action potential precision. Also, SK2 and SK3 subunits are capable of forming heteromeric channels in brain (Strassmaier et al., 2005), and heteromeric SK2/SK3 channels will have intermediate apamin sensitivity depending upon the precise subunit stoichiometry (Weatherall et al., 2011). Both SK2 and SK3 are present in the dendrites of DA neurons, therefore, we cannot rule out a contribution of dendritic SK3 subunit containing channels to the regularity of firing. Regardless, the selective modulation of CV by low doses of apamin support the conclusion that SK2 containing channels regulated action potential precision. Taken together with the effects of higher concentrations of apamin that also decreased the ISI, increased action potential frequency, these results suggest that SK2 and SK3 channels may serve complementary roles in regulating the activity of DA neurons.

SK2 and SK3 channels are similar in their $Ca^{2+}$ gating properties (Köhler et al., 1996; Keen et al., 1999). SK2-containing channels in hippocampal CA1 neurons are subject to activity-dependent regulation and trafficking via protein kinase A phosphorylation of serine residues that are not conserved in the SK3 subunit sequence (Lin et al., 2008). This may endow SK2-containing channels in the dendrites of DA neurons with the ability to serve as selective targets for activity-dependactivity-dependent changes and/or signal transduction mechanisms that affect AP precision.

Finally, the blockade of SK2 channels influences the precision of the pacemaker mechanism. Yet, the continued activity of DA neurons in apamin indicates that SK channels are not a necessary component for actual pacemaking. In contrast, $Ca_V1.3$ (L-type) $Ca^{2+}$ channel current is necessary for continued pacing (Putzier et al., 2009) and generates $Ca^{2+}$ oscillations peaking in fine dendrites (Wilson and Callaway, 2000; Chan et al., 2007; Kuznetsova et al., 2010). In the presence of TTX, SK channels are activated by $Ca_V1.3$ mediated $Ca^{2+}$ influx (Nedergaard et al., 1993). This interaction may still occur during normal pacemaker activity, offering a possible mechanism by which apamin influences the ISI-CV. SK channels provide a strong hyperpolarizing influence following an action potential that de-inactivates voltage gated channels, standardizing the number of channels available for the subsequent pacemaker potential and AP. Dendritic SK2 channels are positioned to fulfill this role for the $Ca_v$ channels implicated as part of the pacemaker (Putzier et al., 2009; Kuznetsova et al., 2010).

**Figure 4. Apamin-sensitive currents recorded from wild type, SK2$^{-/-}$ and SK3$^{-/-}$ DA neurons.**

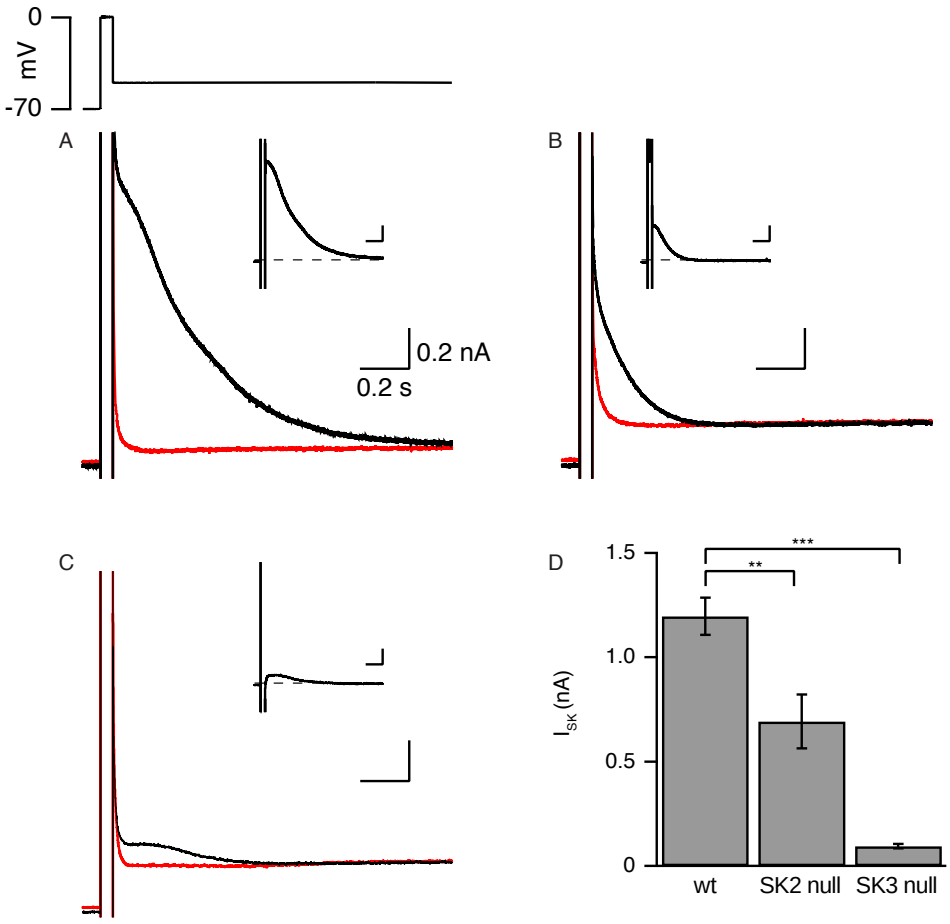


Figure 4. Apamin-sensitive currents recorded from wild type, SK2$^{-/-}$ and SK3$^{-/-}$ DA neurons. Pharmacological isolation of the apamin-sensitive SK current in wild type (A), SK2$^{-/-}$ (B) and SK3$^{-/-}$ (C) DA neurons. The currents were evoked by a 50 ms depolarizing pulse to 0 mV from -70 mV holding potential, followed by a return to -50 mV. The voltage protocol is schematically presented above the data trace in panel A. The black traces are currents recorded in control solution, while the red traces are currents recorded after apamin (200 μM) application. To obtain the subtracted, apamin-sensitive current (inset), each record was zeroed to the mean, steady state current following repolarization to -50 mV. Each trace is the average of four sequential trials. (D) Averages ± S.E.M. for the apamin-sensitive currents for each genotype.

**Figure 5. SK2 and SK3 are expressed in wild type DA neurons.**

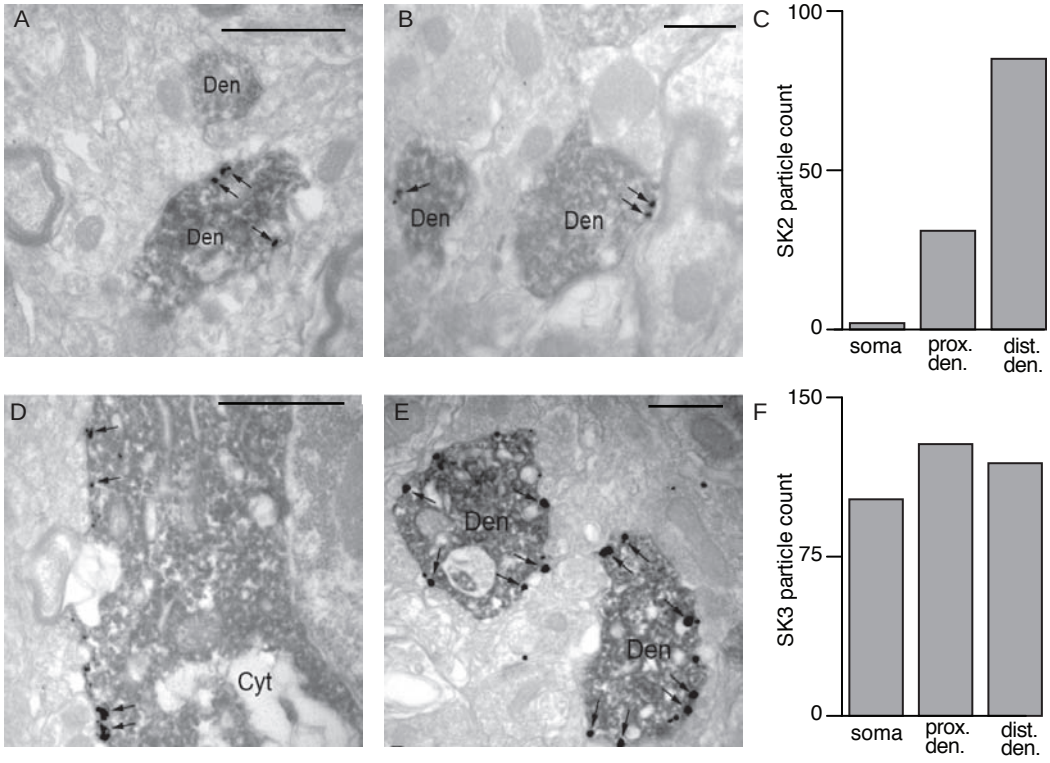


Figure 5. Electron micrographs from SN showing immunoparticles for SK channel subunits and TH immunoreactivity as detected using double labeling immunogold-HRP. (A, B) Immunoparticles for SK2 protein (arrows) were found along the plasma membrane in dendrites (Den) of TH-positive neurons. (D) Immunoparticles for SK3 (arrows) were detected along the plasma membrane of the soma (Cyt, cytoplasm; N, nucleus) and (D) the dendrites (Den) of TH-positive neurons labeled with the HRP reaction product. (C,F) Summary graphs showing the sub-cellular distribution of SK2 and SK3 immunoparticles, respectively. Scale bars: 500 nm.

**Figure 6. SK channel activity regulates the timing and frequency of APs in wild type DA neurons.**

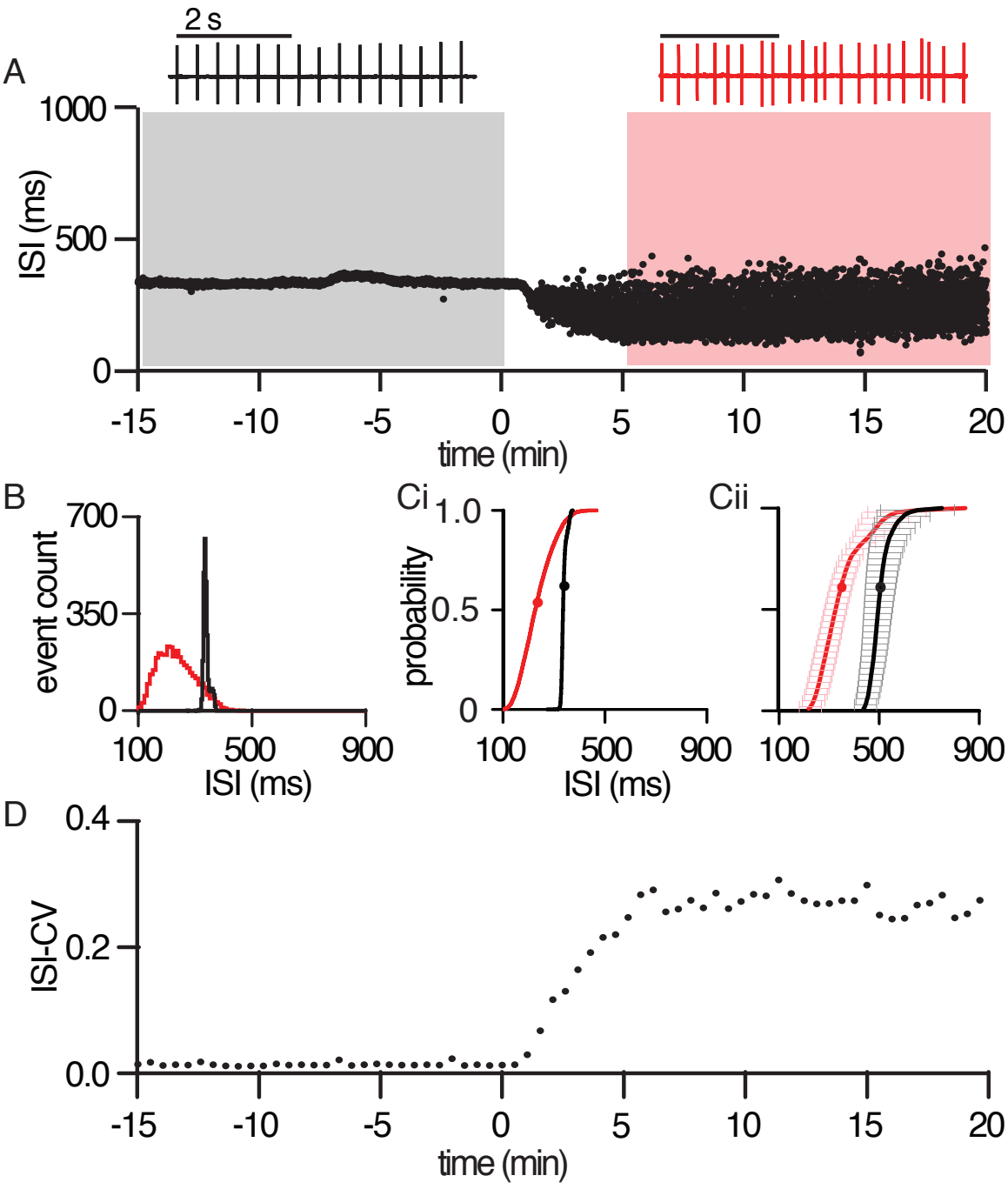

Figure 6. Panels A,B,Ci, and Di are derived from the same representative wild type neuron. Panel C represent ensemble data. (A) Top panel shows examples of spontaneous APs recorded in the loose patch configuration from a wild type DA neuron in control solution (black) and after apamin application (red). Below is a diary plot of the ISIs for the same cell. Apamin was added at time 0. The shaded areas denote the control (black) and apamin (red) ISIs used for graphs and statistical comparisons. (B) Histogram of ISIs recorded in control solution (black) and after apamin application (red) showing that apamin decreased the average ISI in wild type DA neurons. (C) Cumulative probability histograms of ISIs from a representative cell ($C_i$) and for all wild type cells ($C_{ii}$) in control solution (black) after apamin application (red). The black and red points denote the mean ISIs. ($D_i$) Diary plot of the effect of apamin (added at time 0) on the CV of ISIs. Each point represents the CV calculated from 30 s epochs of ISIs. Error bars are ± S.E.M.

**Figure 7. Effects of SK channel activity on SK2$^{-/-}$ DA neurons.**

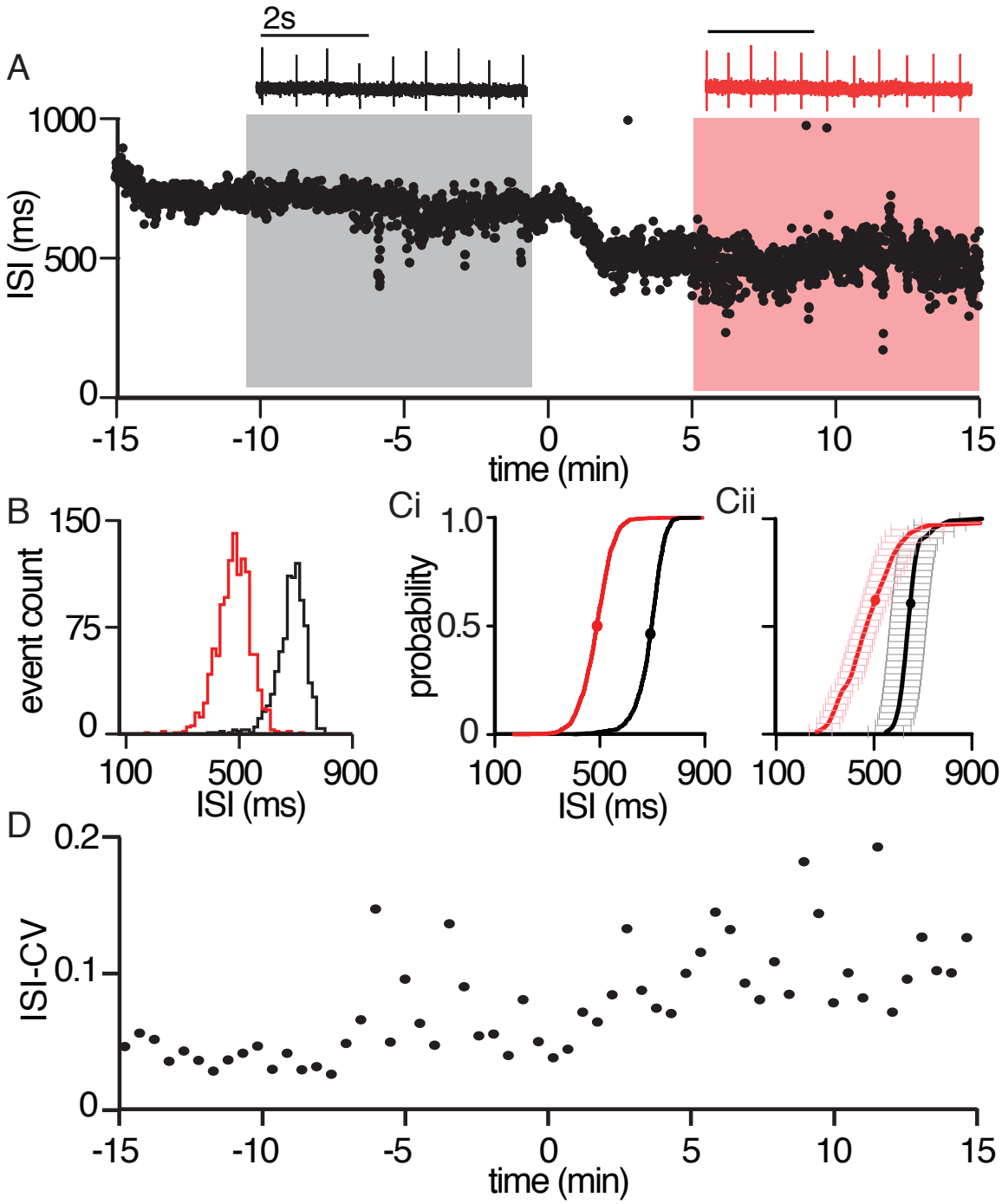

Figure 7. Panels A,B,Ci, and Di are derived from the same representative $SK2^{-/-}$ neuron. Top panels show examples of spontaneous APs recorded in the loose patch configuration from an $SK2^{-/-}$ DA neuron in control solution (black) and after apamin application (red). Below is a diary plot of the ISIs for the same cell. Apamin was added at time 0. The shaded areas denote the control (black) and apamin (red) ISIs used for graphs and statistical comparisons. (B) Histogram of ISIs recorded in control solution (black) and after apamin application (red). (C) Cumulative probability histograms of ISIs from a representative $SK2^{-/-}$ DA neuron ($C_i$) and for all $SK2^{-/-}$ DA neurons ($C_{ii}$) in control solution (black) after apamin application (red). The black and red points denote the mean ISIs. (D) Diary plot of the effect of apamin (added at time 0) on the CV of ISIs. Each point represents the CV calculated from 30 s epochs of ISIs. Error bars are ± S.E.M.

**Figure 8. SK2-containing channels influence AP timing in SK3$^{-/-}$ DA neurons.**

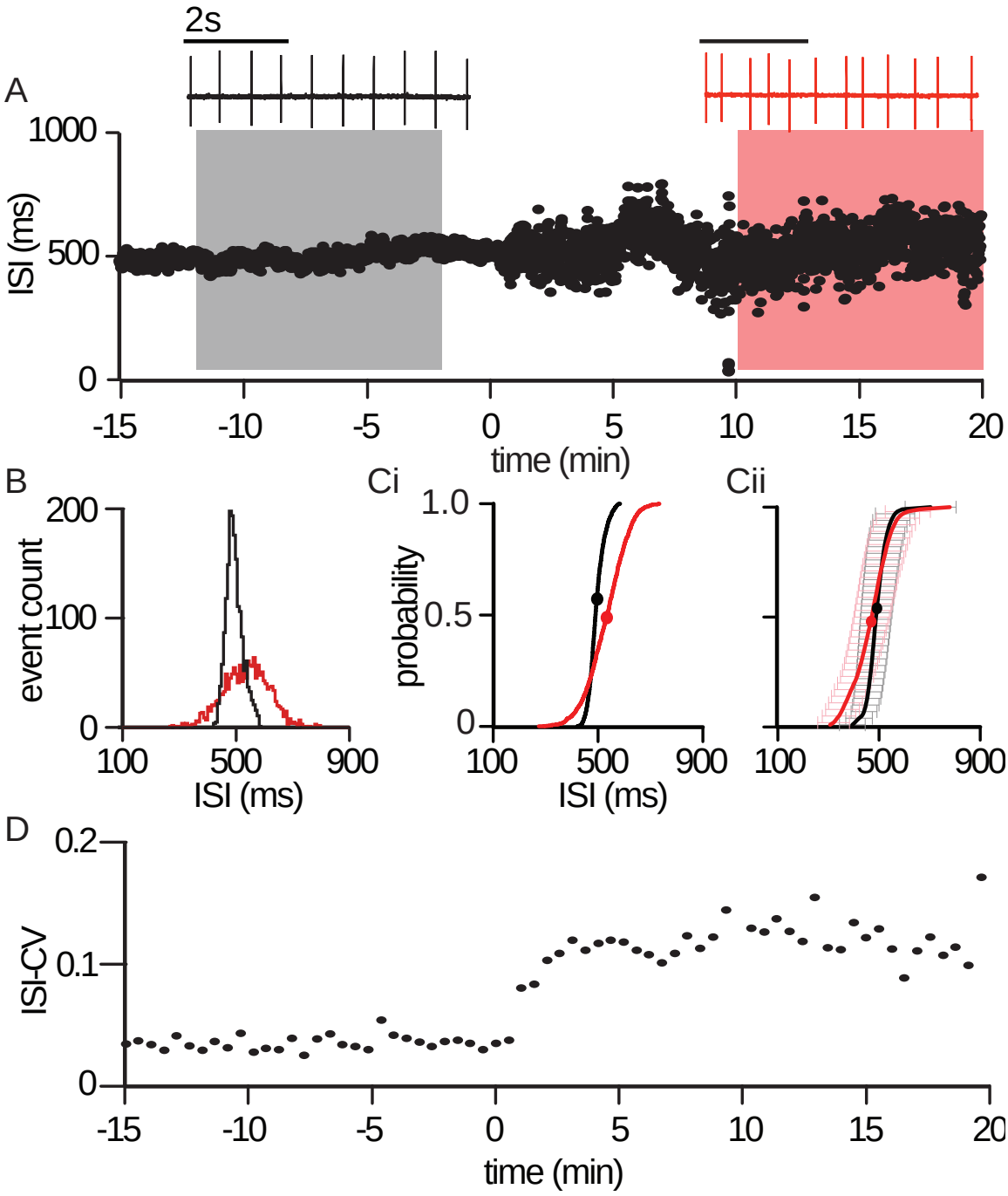

Figure 8. Panels A,B,Ci, and Di are derived from the same representative $SK3^{-/-}$ neuron. Top panel shows example traces of spontaneous APs recorded in the loose patch configuration from an $SK3^{-/-}$ DA neuron in control solution (black) and after apamin application (red). Below is a diary plot of the ISIs for the same cell. Apamin was added at time 0. The shaded areas denote the control (black) and apamin (red) ISIs used for graphs and statistical comparisons. (B) Histogram of ISIs recorded in control solution (black) and after apamin application (red). (C) Cumulative probability histograms of ISIs from a representative $SK3^{-/-}$ DA neuron ($C_i$), and for all $SK2^{-/-}$ DA neurons ($C_{ii}$) shown in control solution (black) after apamin application (red). The black and red points denote the mean ISIs. (D) Diary plot of the effect of apamin (added at time 0) on the CV of ISIs. Each point represents the CV calculated from 30 s epochs of ISIs. Error bars are ± S.E.M

**Figure 9. Summary bar graphs**

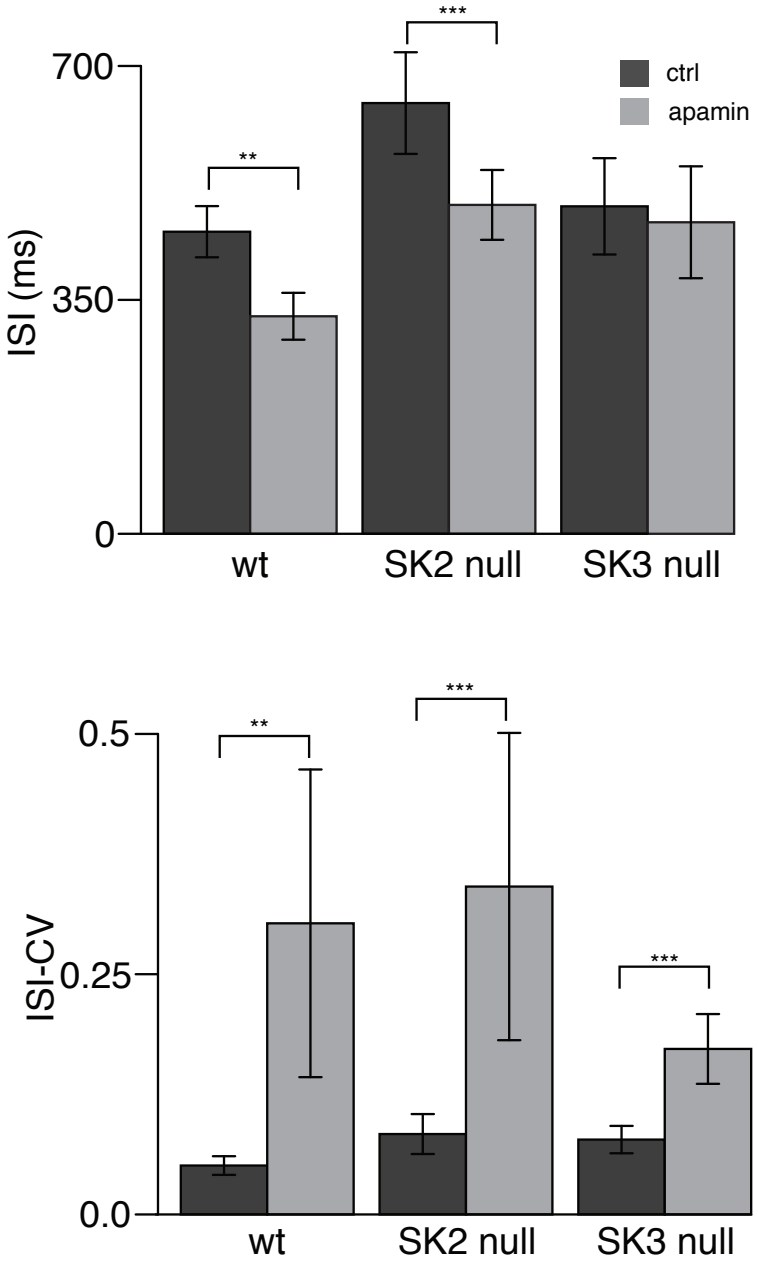


Figure 9. Summary bar graphs showing the effects of apamin on the mean ISI (top) and ISI-CV (bottom) in the different genotypes examined.

**Figure 10. The selective blockade of SK2-containing channels affects the ISI but not the CV of wild type dopamine neurons**

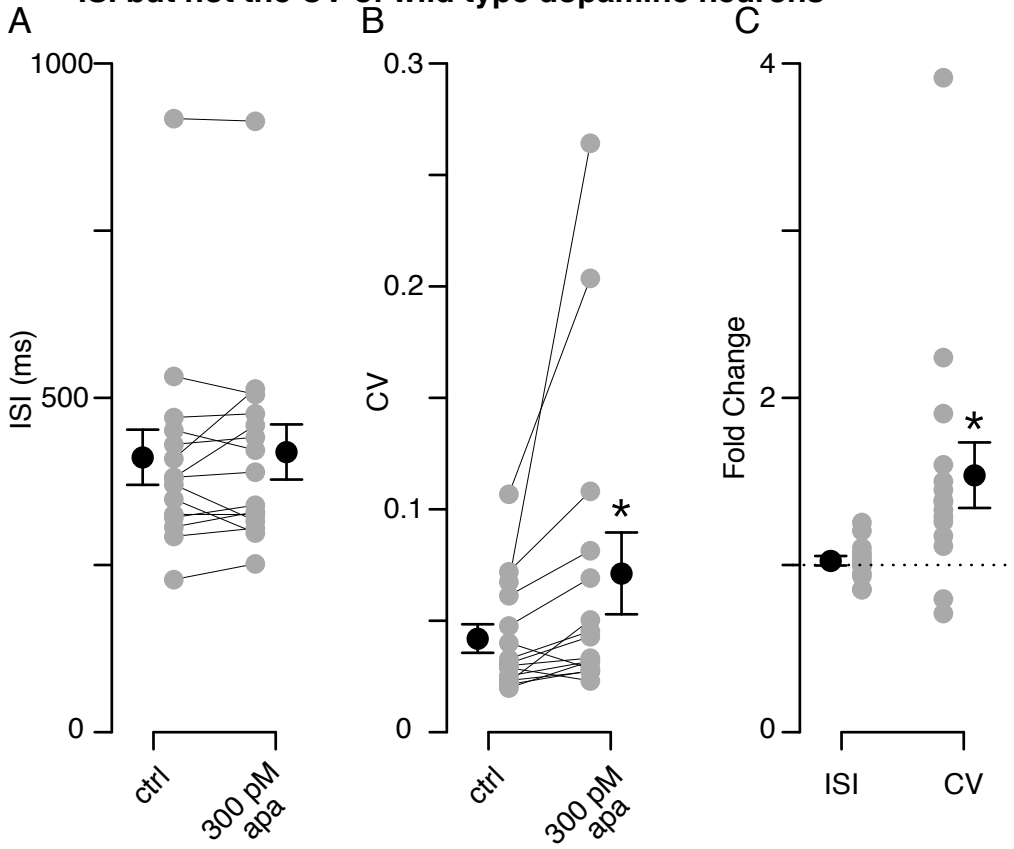


Figure 10. Summary of the effect of 300 pM apamin on the mean AP ISI (A) and CV (B) in wild type DA neurons.The fold change of both measurements is plotted in C. At the concentration used, apamin had no affect on the mean ISI, but significantly increased the ISI-CV. Grey circles connected by lines represent the responses of individual cells. Population means are plotted in black and significant differences from control values are indicated by asterisks. Error bars are ± S.E.M.

**Table 1. Effect of 200 nM apamin on the firing properties of wild type, $SK2^{-/-}$ and $SK3^{-/-}$ dopamine neurons.**

| g | n | Control | Apamin | Effect | % Change | p value |
|---|---|---|---|---|---|---|
| **ISI** | | | | | | |
| wt | 8 | 452 ± 38 | 325 ± 35 | -127 ± 31 | -31% | 0.006 |
| $SK2^{-/-}$ | 9 | 644 ± 76 | 492 ± 52 | -152 ± 44 | -24% | 0.009 |
| $SK3^{-/-}$ | 8 | 490 ± 72 | 466 ± 84 | -24 ± 25 | -5% | 0.37 |
| **CV** | | | | | | |
| wt | 8 | 0.05 ± 0.01 | 0.30 ± 0.16 | 0.25 | 595% | 0.006 |
| $SK2^{-/-}$ | 9 | 0.08 ± 0.02 | 0.34 ± 0.16 | 0.26 | 408% | 0.001 |
| $SK3^{-/-}$ | 8 | 0.08 ± 0.01 | 0.17 ± 0.04 | 0.09 | 221% | 0.003 |

Table 1. The firing properties of nigral DA neurons were monitored using a loose-attached patch configuration. The mean ISI and CV were calculated in control ACSF and following the addition of apamin (200 nM). ISI values for control, apamin, and effect are given in milliseconds.

**Table 2. Effect of 300 pM apamin on the firing properties of wild type and SK3$^{-/-}$ dopamine neurons.**

| g | n | Control | Apamin | Effect | % Change | p value |
|---|---|---|---|---|---|---|
| **ISI** | | | | | | |
| wt | 15 | 411 ± 41 | 419 ± 41 | 5 ± 47 | 3% | 0.48 |
| SK3$^{-/-}$ | 10 | 304 ± 28 | 250 ± 26 | -44 ± 10 | 24% | 0.009 |
| **CV** | | | | | | |
| wt | 15 | 0.04 ± 0.01 | 0.07 ± 0.02 | 0.03 ± 0.01 | 154% | 0.005 |
| SK3$^{-/-}$ | 10 | 0.07 ± 0.01 | 0.19 ± 0.06 | 0.12 ±0.05 | 255% | 0.004 |

Table 2. Summary of the effects of 300 pM apamin on the ISI and CV of DA neurons from wild type and SK3$^{-/-}$ mice.

# *Chapter IV.*

# *The effect of 300 pM apamin on SK3$^{-/-}$ dopamine neurons*

## Introduction

Following the publication of the results presented above, an additional series of experiments was performed using SK3$^{-/-}$ mice to further examine the roles of SK channels. In wild type DA neurons, the preferential blockade of SK2 containing channels with 300 pM apamin increased the CV, but did not affect the mean ISI. Application of 300 pM apamin will block greater than 90% of homomeric SK2 channels while blocking less than 10% of homomeric SK3 channels; heteromeric SK channels are predicted to have an intermediate sensitivity (Strassmaier: 2005eb; Lamy et al., 2010). However, even under optimal circumstances, there is some overlap of the apamin dose-response curves that precludes a completely selective block of homomeric SK2 channels while leaving all SK3 channels unblocked. The application of 300 pm apamin to wild type DA neurons increased the CV by 154% while the complete blockade of SK channels with 200 nM apamin increased the CV by almost 600%. The discrepancy raises the possibility that the initial effect was due to the blockade of 10% of the SK3 channels and blocking the remaining 90% increased the effect to 600%. Thus it is possible that the effects of 300 pM apamin on wild type DA neurons reflects the block of a small, but important, fraction of SK3 channels and was sufficient to cause the observed change in CV. This possibility was tested by applying 300 pM apamin to DA neurons from SK3$^{-/-}$ mice.

## Results

In this series of experiments 300 pM apamin decreased the ISI of $SK3^{-/-}$ DA neurons by 18% ± 2.6% (control: 304 ± 28; 300 pM apamin: 250 ± 26, $p<0.05$, n=10; Figure 11A). AP timing was also changed by 300 pM apamin. The CV was increased by 255% ± 49% (control: 0.07 ± .01; 300 pM apamin: 0.19 ± 0.06; $p < 0.05$; n = 10; Figure 11B). Surprisingly, in control conditions, the ISI of this second cohort of $SK3^{-/-}$ DA neurons was significantly shorter than observed in the first cohort (cohort 2: 304 ± 28 ms; n = 10; cohort 1: 490 ± 72 ms; n = 8; $p < 0.05$; Figure 12A).

Cumulative distribution histograms reveal the genotypic differences of the ISI between wild type and $SK3^{-/-}$ DA neurons. Wild type DA neurons were slower in control conditions, than those from $SK3^{-/-}$ mice, and unaffected by apamin (Figure 12B), where 300 pM apamin increased the frequency of $SK3^{-/-}$ DA neurons. Additionally, 300 pM apamin had a larger affect on the CV of $SK3^{-/-}$ DA neurons, which, in the presence of apamin, was significantly greater than observed in wild type ($SK3^{-/-}$: 0.19 ± 0.06, n = 10; wild type: 0.07 ± 0.02, n=15; $p<0.05$; Figure 12C) Together, the sensitivity of the ISI to apamin and the decreased ISI relative to that found in $SK3^{-/-}$ DA neurons from the first series of experiments suggest differences between the two experimental populations.

## Discussion

One principle result to emerge from these studies is that SK2 containing channels regulate the timing of APs in nigral DA neurons. The purpose of

applying a relatively low concentration of apamin, 300 pM was to determine if the preferential blockade of SK2 containing channels in wild type DA neurons was sufficient to alter AP timing. Given the mechanism of apamin block and the relative sensitivities of SK channels (Lamy et al., 2010; Weatherall et al., 2011), the experiment tests whether the blockade of homomeric SK2 channels by 300 pM apamin is sufficient to increase the CV to a similar extent to that observed in wild type DA neurons. The results showed that the CV of both wild type and $SK3^{-/-}$ DA neurons was increased by 300 pM apamin showing that the blockade of SK2 channels is sufficient to alter AP timing.

The results also suggest that SK2 and SK3 may work synergistically to regulate the CV. In $SK3^{-/-}$ DA neurons the CV in 300 pM apamin was greater than that of wild type counterparts. This could indicate that wild type DA neurons express heteromeric SK channels that are less completely blocked by 300 pM apamin. Alternatively, the increased CV observed in $SK3^{-/-}$ DA neurons may be a function of the additional affect on the ISI. In wild type DA neurons the low pass filter function of homomeric SK3 channels preferentially lengthens short intervals. In $SK3^{-/-}$, lacking this filter, the ISIs are unconstrained by SK3 channel activity and may vary in both directions, potentially accounting, at least in part, for the increased CV in 300 pM apamin. The decreased affect of 300 pM apamin on the CV of wild type DA neurons suggests that SK3 containing channels may also regulate the CV.

A surprising result of these experiments was the difference observed between

the two cohorts of $SK3^{-/-}$ mice. In the first cohort, the mean ISI of $SK3^{-/-}$ DA neurons was not different than that of wild type DA neurons and was unaffected by apamin (200 nM). However, in a subsequent set of experiments, using younger mice, the ISI in control solution was significantly shorter than that of wild type mice in control solution ($SK3^{-/-}$: 304 ± 28 ms, n = 10; pooled wild type: 424 ± 30 ms, n = 22,; $p < 0.05$) and 300 pM apamin decreased the mean ISI (control: 304 ± 28; 300 pM apamin: 250 ± 26, $p<0.05$, n=10). The initial $SK3^{-/-}$ mice ranged in age from 93 to 179 days, while those the second cohort, used in the low apamin experiments were 53 to 58 days old. The affect of apamin on the ISI of DA neurons from younger, but not older, mice may reflect some combination of the loss, or masking of SK2-mediated influence on the ISI.

A likely possibility is that compensation for the deletion of SK3 develops over time to mask the effect of apamin on the ISI. This hypothesis is consistent with the short ISI observed in young $SK3^{-/-}$ DA neurons compared to that of wild types, and the absence of that difference in their older counterparts. In wild type DA neurons, the homomeric SK3 channels were relatively unaffected by 300 pM apamin and continue to regulate the ISI, acting as a low pass filter. The overlap of the ISI distributions from wt and old $SK3^{-/-}$ mice recorded in control solution (Figure 12D) suggests the presence of a low pass filter in both cases, and the insensitivity of the $SK3^{-/-}$ ISIs to apamin shows that SK2 does not contribute to that filter. Therefore, the decreased ISI of the younger mice suggests the effective lack of the low pass filter and may indicate that SK2 channels influence AP frequency via a distinct mechanism, one obscured in wild type DA neurons by

the influence of SK3 activity and by compensation in the DA neurons from older SK3$^{-/-}$ mice.

Several results show that SK2 containing channels regulate the timing of nigral DA neurons. The CV of wild type and SK3$^{-/-}$ DA neurons was increased by 300 pM apamin indicating that the blockade of SK2 channels is sufficient to alter AP timing. However, if the DA neurons from younger SK3$^{-/-}$ mice in fact represent the uncompensated loss of SK3 channels then it suggests that homomeric SK3 channels and SK2 containing channels employ distinct mechanisms to achieve functional overlap.

**Figure 11. Both the ISI and CV of SK3$^{-/-}$ dopamine neurons are altered by 300 pM apamin**

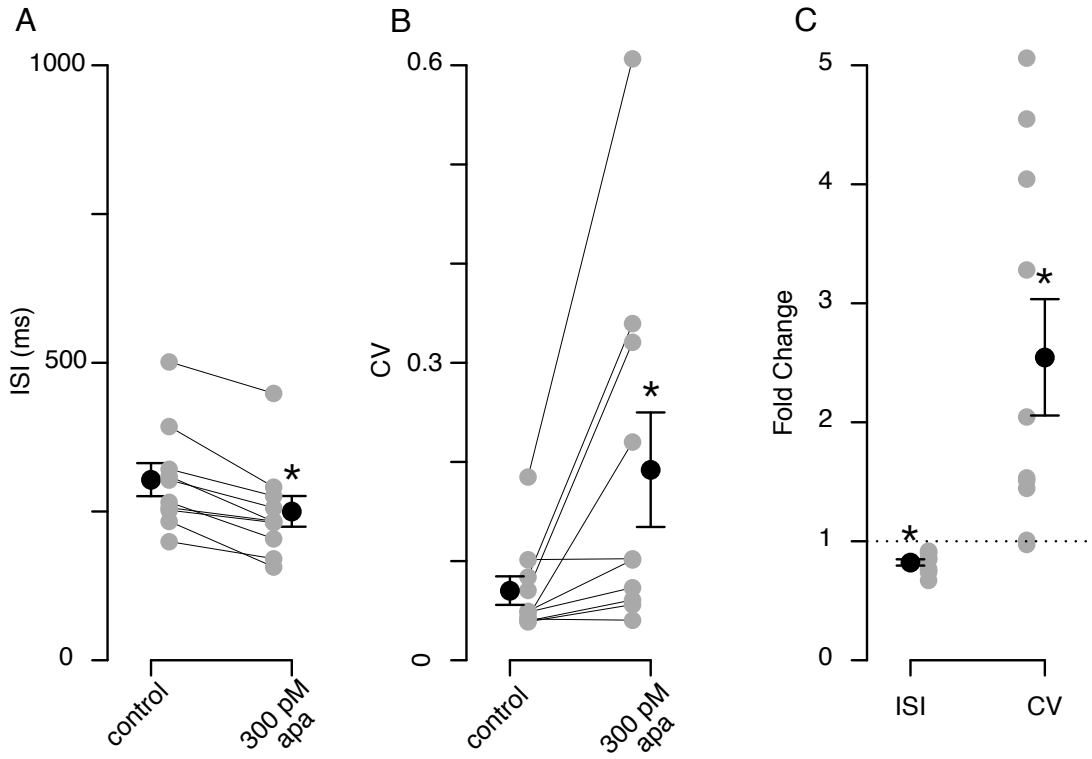


Figure 11. Summary of the effect of 300 pM apamin on the mean AP ISI (A) and CV (B) in SK3$^{-/-}$DA neurons. (A) Apamin (300 pM) reduced the mean ISI of SK3$^{-/-}$ DA neurons. (B) AP timing was also affected as the CV was significantly increased. The fold change of both effects are plotted in panel C. Grey circles connected by lines represent the responses of individual cells. Population means are plotted in black and significant differences from control values are indicated by asterisks. Error bars are ± S.E.M.

**Figure 12. Genotypic comparisons of SK3$^{-/-}$ DA neurons**

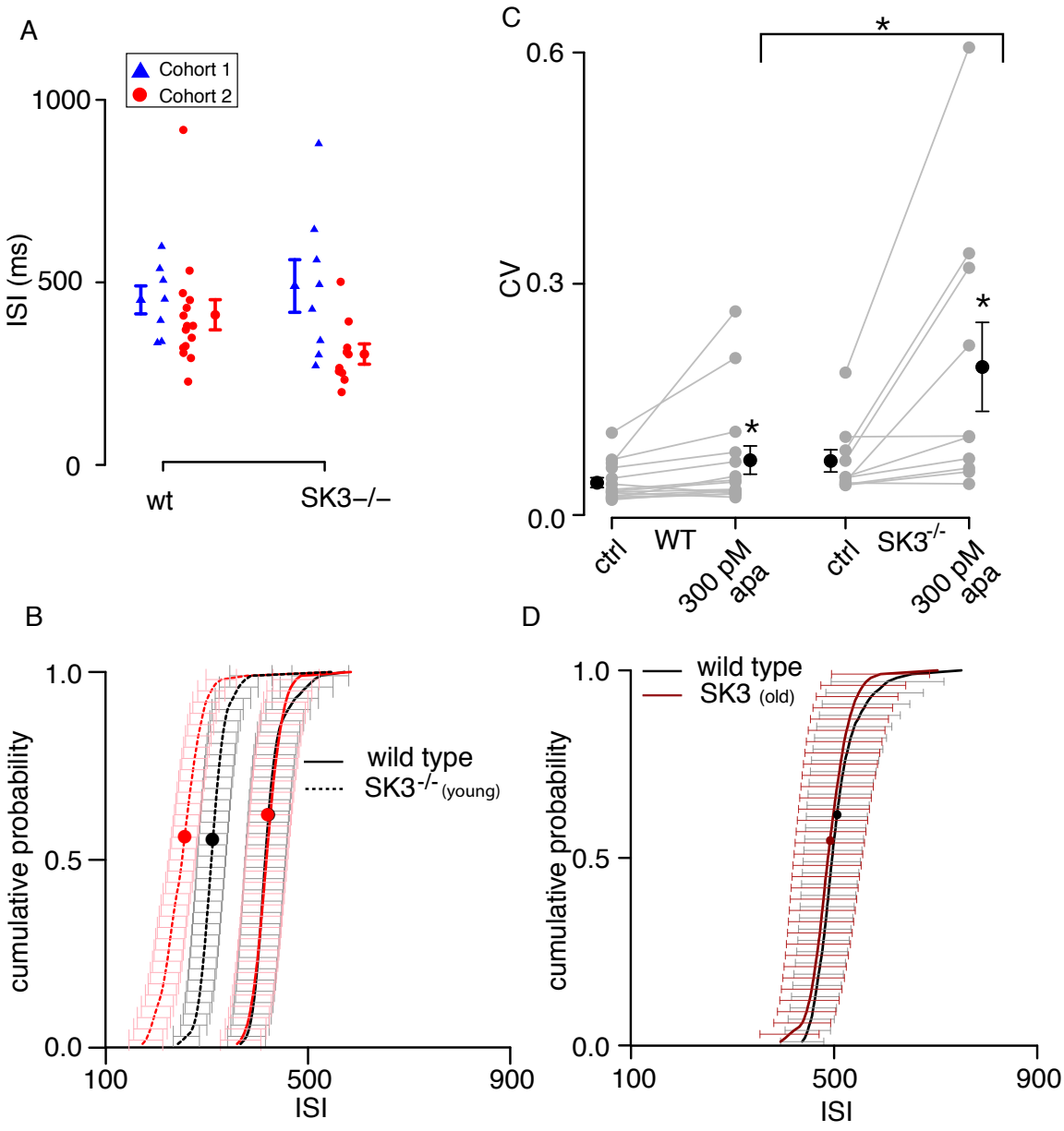

Figure 12. SK3$^{-/-}$ DA neurons exhibited several cross-group differences. (A) A comparison of the ISI of wild type and SK3$^{-/-}$ DA neurons in control ACSF. There was no difference in the ISI of wild type DA neurons from cohort 1 (blue triangle) and cohort 2 (red circles). DA neurons from the first cohort of SK3$^{-/-}$ mice fired significantly slower than those of the second, younger, cohort. (B) The cumulative probability distributions of the wild type (solid lines) and SK3$^{-/-}$ (dashed lines) DA neurons in control and 300 pM apamin. Apamin had no affect on the distribution of wild type ISIs, but significantly shifted the distribution of intervals in SK3$^{-/-}$ DA neurons. The mean ISIs are indicated by the black (control) and red (apamin) points. (C) 300 pM apamin increased the CV of wild type and SK3$^{-/-}$ DA neurons. The effect on SK3$^{-/-}$ DA neurons was significantly larger (asterisk, top). Individual cells are indicated by connected grey circles. Black markers are population averages, with significant differences of paired comparisons are indicated by asterisks over the average markers. (D) Cumulative distributions of intervals recorded in control solution from wild type and old SK3$^{-/-}$ mice (cohort 1). The similarity suggests other ion channels are compensating for the loss of SK3. Error bars are ± S.E.M.

# *Chapter V*

## *The role of SK channels in regulating synaptic transmission in dopamine neurons*

### Introduction

SK channel activity shapes the EPSPs of several types of neurons (Faber et al., 2005; Ngo-Anh et al., 2005; Faber, 2010). In CA1 neurons this is mediated by SK2 that is selectively trafficked to the dendrites, and requires the $SK2_L$ isoform for the targeting of SK channels into the PSD. This raises the question of whether, in addition to influencing intrinsic properties as described above, SK channels may influence the EPSPs of DA neurons. Before assessing a potential role for SK channels in shaping postsynaptic responses, as demonstrated at the Schaffer collateral - CA1 synapse, it must first be determined if SK channels act presynaptically to alter transmitter release onto DA neurons.

Postsynaptic responses reflect the amount of transmitter released from presynaptic terminals and the postsynaptic sensitivity to that transmitter. This can be expressed with the relation $E = NP_Rqr$, where $E$ is the postsynaptic response, often described as the EPSC amplitude measured under ideal conditions, $N$ is the number of vesicles available for release, $P_R$ is the probability of release, $q$ is the amount of transmitter per vesicle and $r$ is the sensitivity of the postsynaptic receptors (Johnson and Wernig, 1971; Bennett and Florin, 1974; Zucker, 1989). EPSC changes due to presynaptic affects will alter $N$, $p$ or $q$, while postsynaptic affects are assumed to be due to changes in $r$. To achieve the spatial and

temporal resolution required to assess synaptic transmitter release the indirect measurement of the postsynaptic responses is substituted for the direct measurement of release. Two measures have been devised to detect changes in transmitter relies on statistical arguments assuming that transmitter release follows a binomial distribution (Martin and Pilar, 1964; Faber and Korn, 1982), and the second, the paired-pulse ratio (PPR) assumes that *r* is invariant over the short time scales between pulses (Katz and Miledi, 1968; Zucker, 1973).

The binomial distribution describes the probability of obtaining *k* successes in *n* independent pass/fail trials, given a probability *p* of success for each trial. When applied to synaptic transmission, *n* becomes *N,* the number of presynaptic release sites and *p* the probability of success is equivalent to $P_R$, the probability that a vesicle is released. Therefor, each stimulation represents *n* simultaneous trials resulting in *k* vesicle fusions that determine *E* the EPSC amplitude. If $E = NP_Rqr$ and the assumptions underlying the binomial distribution hold, then $1/CV^2 = Np(1-p)^{-1}$. Importantly, this means that $1/CV^2$ ($CV^{-2}$) is sensitive to changes in presynaptic parameters N and $P_R$, but not the postsynaptic parameter, r (Malinow and Tsien, 1990). EPSCs evoked with paired pulse stimuli are also used to probe for changes in the probability of release.

The release probability of a synapse can be expressed as a relation that is dependent on the ratio of the EPSC amplitudes ($EPSC_2/EPSC_1$), referred to as the paired pulse ratio (PPR). A PPR greater then 1 is referred to as paired pulse facilitation (PPF) while a PPR less then one is paired pulse depression (PPD).

According to the facilitation model of synaptic transmission, PPF is due to the accumulation of presynaptic $Ca^{2+}$, which increases the probability of release by successive stimulations (Katz and Miledi, 1968), while PPD reflects the depletion of the releasable pool of vesicles (del Castillo and Katz, 1954). These two processes occur simultaneously; a train of 5 EPSCs, may grow and then diminish, or shrink throughout the train due vesicle depletion despite accumulating $Ca^{2+}$.

## Results

Voltage clamp recordings of EPSCs were made from DA neurons to determine if SK channels influence neurotransmitter release. The $Cs^{+}$ based internal solution used for these experiments was supplemented with 5 mM BAPTA and QX-314 and D-600 to block $Na_V$ and $Ca_V$ channels respectively. The goal was to eliminate calcium and voltage-dependent activity in the postsynaptic cell, converting it, as much as possible into a passive detector of glutamate release. To this end, receptor antagonists were included in the ACSF to block $GABA_A$, $GABA_B$, D2, and NMDARs. Thus, the synaptically evoked EPSC was due predominately to AMPAR activity. Given the $Ca^{2+}$-dependence of SK channels, paired stimuli were delivered at a range of frequencies to induce $Ca^{2+}$ accumulation in the presynaptic terminal and reveal a possible SK mediated effect on synaptic release, as seen with apamin. Paired pulse intervals of 20 and 50 ms were interleaved throughout experiments with an inter-trial interval of 20 s. Statistical analysis showed that $EPSC_1$ amplitude was significantly affected by time, or

rundown, ($p < 0.05$) but there was no additional affect of apamin ($p = 0.5$), indicating that SK channels do not influence the amplitude of single EPSPs. This result is corroborated by the variability of $EPSC_1$ amplitude as measured by $CV^{-2}$. Analysis of EPSC1 $CV^{-2}$ in control and apamin revealed no significant differences (Figure 14A). Another measure of presynaptic function, the PPR was calculated for each trial. The results for each inter pulse interval were averaged to determine the PPR in control ACSF and apamin (200 nM); a minimum of 9 responses were averaged for each group. ANOVA revealed no significant effects of apamin on the PPR (20 ms ISI: ctrl- 0.92 ± 0.17, apa- 0.98 ± 0.12; $p > 0.05$; $n = 7$; 50 ms ISI: ctrl- 0.85 ± 0.08, apa- 0.81 ± 0.07).

These results show that SK channels do not influence presynaptic glutamate release onto DA neurons. Therefore, synaptic stimulation was used to examine whether SK channel activity influenced the postsynaptic response. Mixed AMPAR/NMDAR mediated EPSPs were elicited every 30 seconds in the presence of $GABA_A$ and $GABA_B$ antagonists. Constant current injection maintained the somatic membrane potential at -65 mV. Following ten minutes of stable baseline recording in control ACSF, apamin was added. Across all cells, the EPSP amplitude in apamin was 95% ± 3% that observed in control ACSF ($p > 0.05$).

## Discussion

SK channels do not appear to regulate AMPAR/NMDAR mediated EPSP onto DA neurons through either pre- or postsynaptic mechanisms. Presynaptic

function was assessed indirectly by recording AMPAR mediated EPSCs. A caveat to this approach is that any effect of apamin on the postsynaptic cell might be interpreted as a presynaptic effect on transmitter release. Postsynaptic affects were minimized by recording in voltage clamp mode to minimize the contribution of voltage activated channels to the EPSC. The internal solution was $Cs^+$ based to minimize $K^+$ channel currents, and contained QX314 and D600 to block $Na_V$ and $Ca_V$ channels respectively. As further insurance that SK channels in particular would not be active prior to apamin treatment, 5mM BAPTA, a rapid chelator of $Ca^{2+}$, was also included in the internal solution. Activity-Dependent glutamate release depends on the activation of $Ca_V$ channels that act as a $Ca^{2+}$ source for the transmitter release machinery (del Castillo and STARK, 1952; Katz and Miledi, 1967). SK channels often act as activity-dependent regulators of membrane potential or $Ca^{2+}$ signaling. If SK channels provide feedback onto $Ca_V$ channels activated by single APs, apamin would be predicted to increase $Ca^{2+}$ influx and $P_R$, resulting in an increased EPSC amplitude and an altered $CV^{-2}$. It is possible that the time dependent rundown of EPSC amplitude masked an affect of apamin on $EPSC_1$, however an analysis of the effect of apamin on the $CV^{-2}$ of $EPSC_1$ amplitudes indicates that presynaptic SK channels do not influence $P_R$ due to single APs.

The variability of EPSC amplitudes is a result of the stochastic processes of transmitter release rather than moment to moment variability in the number or availability of postsynaptic receptors (del Castillo and Katz, 1954; Boyd and Martin, 1955; Martin and Pilar, 1964; Bennett and Florin, 1974; Faber and Korn,

1982). The lack of an affect of apamin on $EPSC_1$-$CV^{-2}$, a measure of the variability of $EPSC_1$ amplitudes, indicates that SK channels do not affect the $P_R$ or the number of release sites. This result also suggests that the EPSC rundown observed was not due to the loss of presynaptic release sites or a change in $P_R$, but a result of the loss of postsynaptic receptors. The absence of rundown of EPSPs also supports this conclusion (Figure 14). With the exception of dAP-5 to block NMDARs, the presynaptic terminals were in the same ACSF in the EPSC and EPSP experiments, suggesting that the internal solution designed to isolate presynaptic function induced EPSC rundown in the postsynaptic cells. The insensitivity of two separate measures of presynaptic function to both rundown and apamin suggests that *n* and $P_R$ remained stable throughout the experiments. Comparison of $CV^{-2}$ of both $EPSC_1$ and $EPSC_2$ amplitudes is a possible positive control to ensure sensitivity to $P_R$.

The analysis of $EPSC_1$ amplitudes indicates that SK channels do not influence transmitter release due to single APs. However, at many synapses, paired APs given in rapid succession elicit a larger EPSC in response to the second AP than the first. This is thought to be the result of higher presynaptic $Ca^{2+}$ concentrations following $AP_2$ than $AP_1$. (Katz and Miledi, 1968; Miledi and Thies, 1971). $Ca^{2+}$ influx from $AP_2$ contributes to residual $Ca^{2+}$ from $AP_1$ resulting in higher $Ca^{2+}$ levels and a greater $P_R$ and EPSC. It is possible that SK channels could selectively influence $EPSC_2$ by several mechanisms. $Ca^{2+}$ influx from $AP_1$ may activate SK channels, too slowly to influence $EPSC_1$, but allow them to regulate $Ca^{2+}$ influx during a second AP, or that higher $Ca^{2+}$ levels associated with a

second AP would be sufficient to allow SK channel mediated feedback onto $Ca_V$ channels. The PPR was insensitive to apamin, indicating that SK channels do not influence transmitter release during paired pulse protocols. Together these data offer sufficient evidence to conclude that SK channels do not regulate glutamate release onto nigral DA neurons.

Because SK channels did not influence transmitter release, apamin was used to assess a postsynaptic role for SK channels in shaping evoked mixed NMDAR/ AMPAR EPSPs. EPSP amplitude was not affected by apamin, which indicates that SK channels do not influence NMDAR mediated responses, or CaV channels activated directly by receptor mediated depolarizations, as demonstrated in the hippocampus, amygdala and cortex (Faber et al., 2005; Ngo-Anh et al., 2005; Faber, 2010). It is possible that EPSPs may induces voltage-dependent $Ca^{2+}$ responses in the dendrites that activate SK channels. The voltage-dependenvoltage-dependence of CaV1.3 activation during the SOP supports such a possibility. Maintaining the soma at -65 mV potentially masked such a role by excluding the voltage-dependent activation of $Ca^{2+}$ sources.

**Figure 13. SK channel activity does not affect glutamate release onto dopamine neurons**

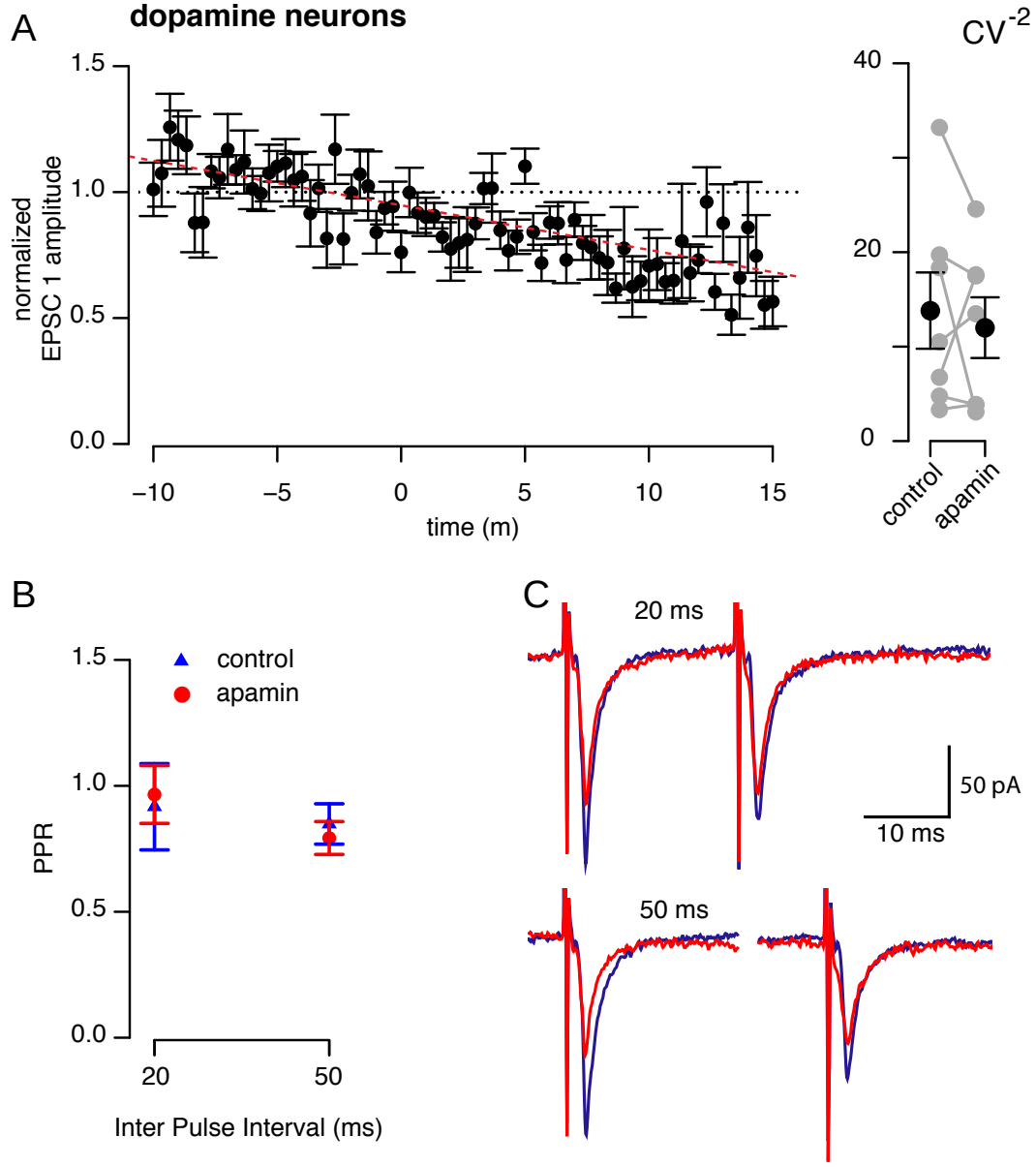

Figure 13. (A) The diary plot of EPSC1 amplitudes (left panel) shows significant rundown over time. The dashed red line is a linear fit of control data. Apamin was added at time 0. The $CV^{-2}$ of EPSC1 amplitudes (right panel) was unaffected by apamin. Individual cells are represented by the connected grey circles and the black points are the population means. (B) Summary data of PPR recorded at 20 ms and 50 ms intervals. Apamin had no effect on the PPR. (C) Averaged representative traces of 20 ms (top) and 50 ms (bottom) PPRs recorded in control (blue) and apamin (red). Error bars are ± S.E.M.

**Figure 14. EPSPs are not affected by apamin**

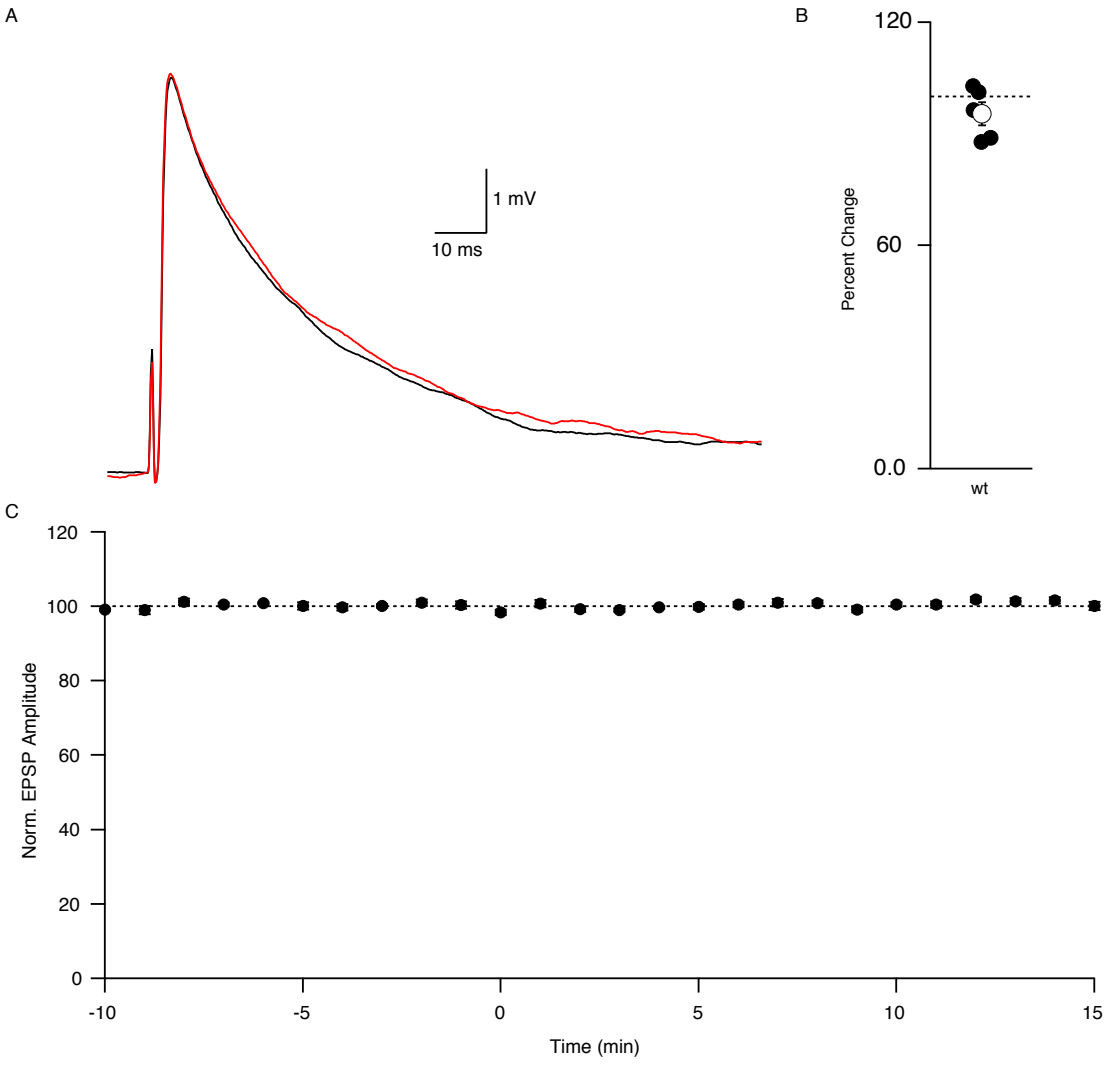

Figure 14. Mixed AMPAR/NMDAR EPSPs were evoked every every 30 s in control ACSF and following the addition of apamin. (A) Averages of representative traces in control (black) and apamin (red) showing that apamin did not alter the EPSP waveform. (B) Percent change of the mean EPSP amplitudes of individual cells (black) and summary data (white). (C) A summary diary plot of average EPSP amplitudes expressed as percent of control amplitude. Apamin was added at time 0. Error bars are ± S.E.M.

# *VI. Discussion*

## Results Summary

*Wild Type Dopamine neurons*

Dual labeling iEM experiments revealed that SK2 is expressed in wild type nigral DA neurons and is largely excluded from the soma, but concentrated in the distal dendrites. In contrast, labeling for SK3 was evenly distributed in the soma and dendrites. When 200 nM apamin was added to block all apamin-sensitive channels in synaptically isolated DA neurons from wild type mice, the AP frequency was increased and the regularity of AP timing was disrupted, as reflected by the increased ISI-CV. The preferential block of SK2-containing channels with 300 pM apamin reproduced only the effect on the CV, suggesting that SK2 channels selectively affect the regularity of AP firing.

In some other neurons, notably principal neurons in hippocampus, amygala, and prefrontal cortex, dendritic and synaptic SK channels regulate synaptic transmission and the induction of synaptic plasticity (NgoAnh:2005jp; Lin:2008ir; Faber:2005ie; Faber, 2010). To determine whether SK channels in DA neurons influence glutamate release, AMPAR-mediated EPSCs were recorded from nigral DA neurons, with or without bath-applied apamin. Neither the amplitude nor $CV^{-2}$, a measure of variability, of single EPSCs was affected by apamin. Similarly, apamin had no effect on the PPR of EPSCs recorded at 20 ms and 50 ms intervals. Taken together, these findings rule out an effect of SK channel activity on presynaptic glutamate release onto DA neurons. In the subsequent set of experiments, postsynaptic effects of SK channel activity were examined; EPSPs

were evoked in the absence and presence of apamin to determine if SK channels shape EPSPs. Mixed AMPAR/NMDAR responses were unaffected by apamin. Thus SK channel activity in nigral DA neurons does not influence fast glutamatergic EPSPs, and principally affects intrinsic excitability. Moreover, SK2 and SK3 channels serve different roles.

### *SK2$^{-/-}$ dopamine neurons*

To examine the novel finding that SK2 is expressed in wild type DA neurons, and to gain insight into their roles, the firing properties of wild type and SK2$^{-/-}$ or SK3$^{-/-}$ DA neurons were examined. The intrinsic firing properties of SK2$^{-/-}$ DA neurons clearly differed from their wild type counterparts. In control conditions, SK2$^{-/-}$ DA neurons fired significantly slower than those from wild type mice, and while apamin increased the firing rate and CV, firing remained significantly slower than for wild type DA neurons in apamin. Tail-currents evoked by brief depolarizing pulses are largely apamin-sensitive and thought to represent the currents underlying the AHP of nigral DA neurons. Apamin-sensitive tail-currents recorded from SK2$^{-/-}$ mice were significantly reduced from those observed in wild type mice. Finally, qPCR results from midbrain micro-dissections detected 42% more SK3 mRNA in tissue harvested from SK2$^{-/-}$ mice than from wild type mice. Taken together these results implicate a role for SK2-containing channels in the intrinsic firing properties of DA neurons.

### *SK3$^{-/-}$ dopamine neurons*

Tail-currents recorded from SK3$^{-/-}$ DA neurons were much smaller than those of

wild type or SK2$^{-/-}$ DA neurons, but still contained an apamin-sensitive component, revealing the contribution of SK2 channels. Apamin consistently increased the ISI-CV of SK3$^{-/-}$ DA neurons, but the effect on firing frequency varied. In initial experiments, the firing rate and CV in control conditions were no different from those of wild type DA neurons and apamin selectively increased the CV without affecting the firing frequency. In a second, younger, group of SK3$^{-/-}$ mice, DA neurons in control conditions fired faster than wild type DA neurons and treatment with 300 pM apamin, a dose that selectively blocks SK2 channels, increased both the firing rate and the CV.

## Interpretations

Results from wild type, SK2$^{-/-}$ and SK3$^{-/-}$ DA neurons provided strong evidence that SK2 containing channels selectively influence AP timing, whereas SK3 channels influence AP timing and frequency. 300 pm apamin increased the CV in wild type and SK3$^{-/-}$ DA neurons confirming that wild type DA neurons express functional SK2 containing channels. When the concentration of apamin was increased to 200 nM, in wild type DA neurons, the CV was further affected (300 pm apamin- 0.07 ± 0.02, 200 nM apamin- 0.30 ± 0.16, $p < 0.05$), but not in SK3$^{-/-}$ DA neurons (300 pm apamin- 0.19 ± 0.06, 200 nM apamin- 0.17 ± 0.04). This continued increase in the CV of wild type DA neurons suggests two possibilitieis. The low apamin concentration blocked some fraction of heteromeric channels that were then completely blocked by higher concentrations of apamin or that the homomeric SK3 channels influence both the frequency and timing of APs.

Our results are the first demonstration that nigral DA neurons express functional SK2 containing channels. Unlike pyramidal neurons, SK channels do not appear to influence glutamate release onto DA neurons, or the AMPAR/NMDAR mediated EPSPs. Possible postsynaptic roles were investigated within the relatively narrow confines of subthreshold, single EPSPs. In DA neurons more intense NMDAR activation is associated with bursting activity and the induction of LTP by $Ca^{2+}$ and PKA dependent mechanisms (Harnett et al., 2009). $Ca^{2+}$ transients mediated by IP3 coupled mGluR activation are also required. As mGluR mediated $Ca^{2+}$ transients in DA neurons activate SK channels (Fiorillo and Williams, 1998), it is possible that under these conditions SK channels could influence the induction of synaptic plasticity. Whether SK channel activity is able to influence this form of LTP remains unexplored.

Results from $SK3^{-/-}$ DA neurons provided the initial evidence that DA neurons express SK2 containing channels. In DA neurons, apamin-sensitive tail currents were thought to be generated by homomeric SK3 channels (Wolfart et al., 2001). Indeed, tail currents recorded from $SK3^{-/-}$ DA neurons reflect the loss of SK3 and were ~10% of the amplitude of those recorded from wild type counterparts. However, the apamin-sensitive currents detected reveal the functional expression of SK2 channels. Furthermore, apamin altered the firing properties of $SK3^{-/-}$ DA neurons, indicating that these channels served a functional role. The investigation of the firing properties of $SK3^{-/-}$ DA neurons found that cells from young mice fired faster than their wild type counterparts in control ACSF, and that 300 pM apamin increased the firing rate and increased the CV. DA neurons from

older mice exhibited evidence of compensation; the firing rate in control conditions was no different than that of wild type neurons. Firing properties should be the same regardless of whether SK channel activity is eliminated genetically or pharmacologically. Apamin (200 nM) had no effect on the ISI of DA neurons from older mice, but increased the CV. The disparity between the affects of apamin and the control-firing rates in the old and young cohorts suggests that the functional effects of SK3 deletion are compensated for as the mice age. The proposal that compensation develops over time should be explicitly tested, by recording from $SK3^{-/-}$ mice over a defined spectrum of ages. The finding that in both young and old cohorts, apamin increased the CV suggests that in $SK3^{-/-}$ DA neurons, SK2 channels regulate AP timing.

A principal contribution of this work is the finding that SK2 containing channels regulate the firing properties of nigral DA neurons. Results from $SK2^{-/-}$ DA neurons show that deleting SK2 channels increased levels of SK3 mRNA, decreased the apamin-sensitive tail current, and decreased the firing frequency, relative to wild type DA neurons.

Indeed, there is some reason to believe that the increased levels of SK3 mRNA detected by qPCR from $SK2^{-/-}$ mice reflect increased functional expression. While $SK2^{-/-}$ DA neurons fire more slowly than wild type DA neurons, apamin decreased the ISI in both genotypes by similar amounts (wt: -127 ms ± 31ms; $SK2^{-/-}$: -152 ms ± 44 ms). However, apamin is reported to have a greater effect on DA neurons that fire faster (Wolfart and Roeper, 2002). An equivalent effect on the

slower SK2$^{-/-}$ cells may reflect increased expression of SK3. Despite the evidence for the compensatory upregluation of SK3 expression, the persistence of the firing rate difference in apamin shows that SK3 is unable to substitute for SK2 and does not contribute to the slowed pacing.

The altered pacing of SK2 $^{-/-}$ DA neurons suggests that SK2 containing channels influence the PM mechanism of DA neurons. As in wild type DA neurons, apamin increased AP frequency and CV of SK2$^{-/-}$ DA neurons. The persistence of slowed firing despite the continued expression of SK3 channels indicates that SK2 subunits convey to SK channels unique properties, necessary for the normal functioning of DA neurons. SK2 is selectively trafficked to the dendrites of CA1 pyramidal neurons and SK2$_L$ undergoes the more targeted expression in the PSD, while SK3 appears to be uniformly distributed (Decimo et al., 2006; Lin et al., 2008; Allen et al., 2011). Pacemaking in wild type DA neurons may reflect the targeting of SK2-containing channels, not simply to the dendrites, but to specific $Ca^{2+}$ sources in the dendrites. This idea is supported by the fact that CaV1.3 channels, are coupled to SK channels, preferentially expressed in the dendrites, and a component of the PM mechanism (Nedergaard et al., 1993; Takada et al., 2001).

The AHP is dependent on AP mediated $Ca^{2+}$ influx (Abel et al., 2004). In DA neurons, the AHP regulates PM frequency and manipulations that diminish it i.e. the blockade of SK channels, speed DA neurons firing rates (Shepard and Bunney, 1988; Harris et al., 1989; Nedergaard et al., 1993). Tail currents are

thought to reflect the activity of the channels contributing to the AHP. In DA neurons, the apamin-sensitive tail-currents are due, almost exclusively, to the activity of SK3 channels (Wolfart et al., 2001). The currents reported here, from adult mice (>p30) were ~ 40 fold larger (1175 ± 117 pA), yet the firing properties of DA neurons from both studies were consistent. The correlation of tail currents and the AHP relies the degree to which the voltage-clamp protocol mimics the physiological activation of channels during APs. The small tail currents observed by Wolfart and Roeper faithfully represented the firing properties and the AHP of DA neurons. The large tail current amplitudes and their poor correlation with firing rate indicate that the apamin-sensitive tail currents obtained in this study rely in part on $Ca^{2+}$ sources that are not activated by APs. The reduced tail currents recorded from $SK2^{-/-}$ DA neurons support this conclusion and suggest a diminished contribution from a $Ca^{2+}$ source not associated with the AHP.

The apamin-sensitive tail currents recorded from $SK2^{-/-}$ DA neurons were smaller than those recorded from wild type DA neurons. This could indicate that fewer SK3 channels were present to be activated and/or there was less $Ca^{2+}$ influx to activate the channels. However, the similar effect of apamin on the ISI of wild type and $SK2^{-/-}$ DA neurons suggests that in $SK2^{-/-}$ DA neurons, $Ca_V3$-coupled SK3 channels function normally to regulate AP frequency via the AHP (Wolfart et al., 2001). This implies that diminished tail currents are not due to fewer SK channels, or diminished $Ca_V3$-mediated $Ca^{2+}$ influx. The functional coupling of SK channels to two $Ca_V$ channel $Ca^{2+}$ sources has been demonstrated in nigral DA

neurons, $Ca_V3$ and $Ca_V1.3$ $Ca^{2+}$ channels (Nedergaard et al., 1993; Wolfart and Roeper, 2002). The reduction of $Ca_V1.3$ mediated $Ca^{2+}$ influx is supported by the slowed pacing of $SK2^{-/-}$ DA neurons an effect mirrored in wild type DA neurons by low concentrations of the $Ca_V1.3$ antagonists (Mercuri et al., 1994; Liu et al., 2007).

It is interesting that $SK2^{-/-}$ DA neurons had dramatically decreased apamin-sensitive tail currents (even though mediated by SK3 channels), decreased firing rate, and upregulation of SK3 expression, all contrasted by the effect of apamin which simply altered the CV. This seeming disparity and more importantly, findings that PM firing is something that is largely absent in vivo, (Shepard and German, 1988; Tepper et al., 1995) suggest that the regularly timed APs are not relevant to DA neurons, leaving open the in vivo role of SK2 channels.

*SK2 containing channels regulate the SOP*

The data support a model in which homomeric SK3 channels expressed in the somatic and perisomatic plasma membrane contribute to the AHP, acting as a low pass filter. Further, SK2-containing channels which may be either homomeric or heteromeric are preferentially targeted to the distal dendrites where they feedback onto CaV1.3 channels of the pacemaker.

The SOP is mediated by the cyclic interplay of $Ca_V1.3$ and SK channels (Nedergaard et al., 1993). Many studies using various techniques place the origin of these currents in the dendrites of nigral DA neurons (Fujimura and Matsuda, 1989; Harris et al., 1989; Wilson and Callaway, 2000; Chan et al., 2007). The

change in $Ca^{2+}$ concentration over a single pacemaker cycle is predicted to be largest in the fine process of the distal dendrites where cytoplasmic volume is most limited (Wilson and Callaway, 2000). SK2 subunits are selectively targeted to these same fine dendrites, and in wild type DA neurons, the preferential blockade of SK2 containing channels increases the ISI-CV. The final experiment connecting the blockade of SK2 containing channels to a change in the SOP remains to be performed.

*The SOP drives pacemaker activity*

The idea that the SOP represents the pacemaker mechanism is still the subject of active debate. Many studies have found that blocking $Ca_V1.3$ channels silences nigral DA neurons in acute slice preparations (Nedergaard et al., 1993; Mercuri et al., 1994; Chan, 2005), while others have found that similar manipulations produce no effect or actually increase AP frequency (Puopolo et al., 2007; Guzman et al., 2009; Khaliq and Bean, 2010). Guzman et. al observed that in comparison to APs from the same cell, the SOP had a lower frequency and increased CV, and concluded that the slow and variable SOP could not drive PM firing. A recent modeling study found that the different experimental results are reproduced by changes in conductance values as modest 1% (Drion et al., 2011). According to the model, several ion channels are able to generate an SOP and maintain pacing activity, the critical feature being that the half-maximal activation potential is greater than -50 mV. The possibility that several ion channels can maintain PM firing is one explanation for inconsistent affects of $Ca_V1.3$ blockade.

One prediction of the model presented by Drion et. al is born out in the results of several studies and supports the idea that the continued activity of DA neurons is necessary for DA neuron survival. Thus, in mouse models of Parkinson's disease (MPTP, rotenone), DA neurons were rescued by genetic or pharmacological suppression of KATP channel activity that would otherwise silence firing (Liss et al., 2005). Two papers from another group found that abolishing pacing in DA neurons by blocking $Ca_V1.3$ channels induced the expression of an alternative, 'juvenile' PM mechanism relying on $I_h$ and $Na_V$ channels (Chan et al., 2007) and subsequent results showed that both PM mechanisms exist simultaneously in DA neurons (Guzman et al., 2009). These findings highlight an overriding necessity to maintain continued AP firing. Studies performed in cultured DA neurons found that AP mediated $Ca^{2+}$ influx was necessary to maintain intracellular $Ca^{2+}$ levels within a narrow range to support survival (Douhou et al., 2001; Toulorge et al., 2011). $Ca_V1.3$ channels are sources of significant $Ca^{2+}$ influx during the PM cycle (Puopolo et al., 2007). By varying the degree of feedback on $Ca_V1.3$ channels,the phosphorylation dependent $Ca^{2+}$ sensitivity of SK2 subunits may represent a mechanism to regulate intracellular $Ca^{2+}$ levels. However, a minimal model finds that any voltage-dependent channel with a half maximal activation > -50 mV can support pacing including most $Na_V$ and $Ca_V$ channels (Drion et al., 2011). Dynamic clamp experiments found amplifier-simulated $Ca_V1.3$ currents to support pacing despite the fact that dynamic clamp currents were not carried by $Ca^{2+}$ (Putzier et al., 2009). The robustness of pacing may be apparent in-vitro or in-silico, but the challenges to maintain spontaneous

activity in vivo may be more substantial.

Approximately 70% of afferents onto DA neurons originate from the spontaneously active GABAergic neurons of the basal ganglia (Smith and Bolam, 1989; Bolam and Smith, 1990). The dominance of inhibitory afferents and auto-inhibitory mechanisms suggests that basal PM activity exceeds that required to maintain DA tone in projection areas such as the striatum and tonic inhibition of PM activity regulates DA levels. Experiments in vitro find that PM activity is supported by a variety of conductances and fail to discuss this in the context of significant inhibitory tone (Chan et al., 2007; Guzman et al., 2009). If maintaining spontaneous activity is important, the PM mechanism must then be able to initiate firing following pauses in activity from GABAR activation, following AP failures, and following bursts where voltage-dependent channels may have begun to inactivate. The SOP has the required characteristics to fulfill these requirements.

An important aspect of a PM mediated by the SOP is that it allows APs to be uncoupled from the PM mechanism. $GABA_A$ receptor activation acts as shunt to regulate NMDA mediated bursts (Paladini et al., 1999). It is likely that the same mechanism when applied to the SOP allows $GABA_A$ activation to influence the ISI in a graded fashion. This is observed in vitro when $GABA_A$ antagonists speed pacing (Tepper and Lee, 2007). In DA neurons, the $Cl^-$ reversal potential is ~ -60 mV (Gulácsi et al., 2003), so even maximal $GABA_A$ activation leaves the membrane potential within the range of the SOP, allowing it to initiate firing when

the inhibition is removed.

## Future Experiments

This study opens the door to future investigations regarding the role of SK2 in DA neurons. The hypothesis that SK2-containing channels are coupled to CaV1.3 channels should be explicitly tested by assessing the effect of low and hight concentrations of apamin on the SOP. If the hypothesis is true, 300 pM apamin would would alter the ISI, indicating the functional coupling of CaV1.3 and SK2 containing channels. Given that TTX should also eliminate the contribution of AP mediated SK3 channel activity, a further effect of 200 nM apamin would suggest the presence of heteromeric SK channels. A second question to arise from this study is whether the phosphorylation dependence of SK2 channel $Ca^{2+}$ sensitivity influences PM in DA neurons. This possibility can be tested by assessing CV changes in the CK2 activator,4,5,6,7-Tetrabromo-2-azabenzimidazole (TBB), in wild type as compared to the $SK2^{-/-}$ mice, acting as negative controls.

# Conclusions

In the work presented here, wild type, $SK2^{-/-}$ and $SK3^{-/-}$ mice were used to demonstrate the expression of SK2 containing channels in nigral DA neurons. These channels are selectively trafficked to distal dendrites where they appear to interact with the pacemaking mechanism. The preferential blockade of SK2 containing channels with 300 pM apamin increases only the CV of wild type DA neurons.

SK channels did not influence glutamate release onto DA neurons of the substantia nigra. Apamin had no affect on AMPA mediated EPSCs, as indicated by two measures of presynaptic function, the PPR and $CV^{-2}$. Similarly, SK channels did not shape EPSPs. Apamin failed to affect mixed AMPAR/NMDAR responses.

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