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SK2 AND SK3 EXPRESSION DIFFERENTIALLY AFFECT FIRING FREQUENCY AND PRECISION IN DOPAMINE NEURONS

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Abstract—The firing properties of dopamine (DA) neurons in the substantia nigra (SN) pars compacta are strongly influenced by the activity of apamin-sensitive small conductance Ca^{2+}-activated K^+ (SK) channels. Of the three SK channel genes expressed in central neurons, only SK3 expression has been identified in DA neurons. The present findings show that SK2 was also expressed in DA neurons. Immuno-electron microscopy (iEM) showed that SK2 was primarily expressed in the distal dendrites, while SK3 was heavily expressed in the soma and, to a lesser extent, throughout the dendritic arbor. Electrophysiological recordings of the effects of the SK channel blocker apamin on DA neurons from wild type and $SK^{-/-}$ mice show that SK2-containing channels contributed to the precision of action potential (AP) timing, while SK3-containing channels influenced AP frequency. The expression of SK2 in DA neurons may endow distinct signaling and subcellular localization to SK2-containing channels.

Key words: substantia nigra, dopamine, SK channels, spontaneous activity, pacemaker.

INTRODUCTION

The release of dopamine (DA) from midbrain dopaminergic neurons, such as substantia nigra (SN) neurons, influences voluntary movement, cognition, and motivational state (Bernheimer et al., 1973; Nieoullon, 2002). Perturbations in DA signaling are implicated in the pathologies of attention-deficit hyperactivity disorder, schizophrenia, and Parkinson's Disease (Bernheimer et al., 1973; Verhoeff, 1999; Goto and Grace, 2007; Tripp and Wickens, 2008). The consequences of DA signaling depend on the timing and amount of DA released, which reflect the different activity patterns exhibited by DA neurons (Gonon, 1988; Chergui et al., 1994; Fiorillo et al., 2008). *In vivo*, a tonic, low frequency firing of APs maintains basal DA levels in DA neuron projection areas. Brief transitions to a burst firing mode result in barrages of high frequency APs that transiently elevate DA levels to trigger DA-mediated behaviors (Gonon and Buda, 1985; Chergui et al., 1994; Schultz, 2002). In freshly prepared brain slices with neurotransmission blocked, DA neurons exhibit an intrinsic, and regular pattern of AP firing, between 1–8 Hz (Grace and Onn, 1989; Lacey et al., 1989; Yung et al., 1991).

The Ca^{2+}-dependent K^+ channel, SK, contributes a sub-threshold outward current during pacemaking of SN neurons as well as the prominent afterhyperpolarization (AHP) following each AP that together regulates the regularity and frequency of AP firing (Shepard and Bunney, 1991; Nedergaard et al., 1993; Ping and Shepard, 1996). Selectively blocking SK channel activity with apamin increases the frequency and decreases the precision of AP firing, which can induce spontaneous transitions to burst firing (Shepard and Bunney, 1988, 1991; Ping and Shepard, 1996).

Previous studies have shown that primarily SK3 is expressed in SN DA neurons and its activity regulates the mean AP frequency (Wolfart et al., 2001). To further investigate the roles of SK3 channels in DA neurons, SK3 null mice were generated. The presence of an apamin-sensitive current in the SK3 null mice suggested SK2 expression. In other neurons, SK2 channels are selectively targeted to dendrites where previous studies have concluded the DA neuron pacemaker resides, prompting us to further examine the roles of SK2 and SK3 using wild type and knockout mice. Using immuno-electron microscopy (iEM) and electrophysiology, the results from adult mice show that SK2 was expressed in DA neurons, and that SK2- and SK3-containing channels provided differential influences on DA neuron activity. This reflects the

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Abbreviations: AHP, afterhyperpolarization; AP, action potential; Ca_V, voltage-dependent calcium channel; CV, coefficient of variation; DA, dopamine; iEM, immuno-electron microscopy; ISI, inter-spike interval; ISI-CV, coefficient of variation of the inter-spike interval; I_{SK}, SK-mediated current; Na_V, voltage-dependent sodium channel; SK, K_{Ca} 2, small-conductance calcium-activated potassium channel; SN, substantia nigra; TH, tyrosine hydroxylase; TTX, tetrodotoxin (voltage-dependent sodium channel antagonist).

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selective expression of SK2 in the dendrites, while SK3 is expressed in both the dendrites and the soma.

EXPERIMENTAL PROCEDURES

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$^{-/8}$ *mice*. The generation of SK2 null mice has been previously described (Bond 04).

SK3$^{-/-}$ *mice*. A single loxP 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 has 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 only from 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, CCCTCTT AATCATGGCCTCA); for SK1, 118 bp (primers: GCTCTTTTGC TCTGAAATGCC, CAGTCGTCGGCACCATTGTCC); for SK2, 151 bp (primers: GTCGCTGTATTCTTTAGCTCTG, ACGCTCA-TAAGTCATGGC); for SK3, 148 bp (primers: GCTCTGATT TTTGGGATGTTTG, CGATGATCAAACCAAGCAGG ATGA). 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 10× buffer, Mg ($C_f = 4$ mM), dNTPs ($C_f = 200$ mM), Platinum taq polymerase (Invitrogen, Carlsbad, CA) (0.6 units/20 μl reaction) and SYBR Green (Molecular Probes) (0.5× 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 min 1×; 95 °C, 30 s/64 °C, 45 s, 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 $2^{-\Delta\Delta Ct}$ (ΔCt ($SK_{Ct} - 18S_{Ct}$) 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 ΔCt 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 tyrosine hydroxylase (TH) using double-labeling 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. Slices recovered at 34 °C 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 (33 °C) 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 60×, 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 experiments 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. Whole cell voltage clamp 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. Extracellular recordings were made with ACSF filled pipettes. Cells exhibiting spontaneous activity were selected for recording. Recordings were made using either an Axopatch 200A or Multiclamp 700A 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 interspike interval (ISI) measurements were