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Tuning the excitability of midbrain dopamine neurons by modulating the Ca²⁺ sensitivity of SK channels

Huifang Ji¹, Charlotte Hougaard², Kjartan Frisch Herrik^{2,3,*}, Dorte Strøbæk², Palle Christophersen², and Paul D. Shepard¹

¹Maryland Psychiatric Research Center and Department of Psychiatry, University of Maryland School of Medicine, P.O. Box 21247, Baltimore, MD 21228, USA

²NeuroSearch A/S, Ballerup, Denmark

³Department of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen, Denmark

Abstract

Small conductance Ca²⁺-activated K⁺ (SK) channels play a prominent role in modulating the spontaneous activity of dopamine (DA) neurons as well as their response to synaptically-released glutamate. SK channel gating is dependent on Ca²⁺ binding to constitutively bound calmodulin, which itself is subject to endogenous and exogenous modulation. In the present study, patch-clamp recording techniques were used to examine the relationship between the apparent Ca²⁺ affinity of cloned SK3 channels expressed in cultured human embryonic kidney 293 cells and the excitability of DA neurons in slices from rat substantia nigra using the positive and negative SK channel modulators, 6,7-dichloro-1*H*-indole-2,3-dione-3-oxime and *R-N*-(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphthylamine. Increasing the apparent Ca²⁺ affinity of SK channels decreased the responsiveness of DA neurons to depolarizing current pulses, enhanced spike frequency adaptation and slowed spontaneous firing, effects attributable to an increase in the amplitude and duration of an apamin-sensitive afterhyperpolarization. In contrast, decreasing the apparent Ca²⁺ affinity of SK channels enhanced DA neuronal excitability and changed the firing pattern from a pacemaker to an irregular or bursting discharge. Both the reduction in apparent Ca²⁺ affinity and the bursting associated with negative SK channel modulation were gradually surmounted by co-application of the positive SK channel modulator. These results underscore the importance of SK channels in ‘tuning’ the excitability of DA neurons and demonstrate that gating modulation, in a manner analogous to physiological regulation of SK channels *in vivo*, represents a means of altering the response of DA neurons to membrane depolarization.

Keywords

6,7-dichloro-1*H*-indole-2,3-dione-3-oxime; burst firing; K_{Ca}2.3; rat; *R-N*-(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphthylamine; substantia nigra

Introduction

Midbrain dopamine (DA)-containing neurons have been implicated in a variety of basic brain functions and a number of neurological and psychiatric disorders. The scope of the actions of DA in the brain has been attributed to the ability of these neurons to encode and influence the encoding of information across different temporal domains (Grace, 1991; Schultz, 2007). Basal DA cell activity, consisting of stochastic firing in the range of 1–6 Hz, maintains tonic extracellular DA at levels optimal for motor control (Venton *et al.*, 2003; Schultz, 2007). Increases or decreases in tonic DA levels result in well-characterized changes in motor behavior. By contrast, phasic changes in neuronal activity, including episodic high-frequency firing (e.g. bursting), result in transient increases in DA release that have been implicated in reward learning and decision making in ways that are redefining the role of this neurotransmitter in substance abuse, addictive behaviour and the affective changes associated with schizophrenia and Parkinson's disease (Heien & Wightman, 2006).

Bursting activity elicited by salient environmental stimuli is triggered extrinsically by synaptic inputs that convey changes in an animal's sensorium. However, intrinsic mechanisms, in the form of specific ion channels, are also involved (Overton & Clark, 1997). Small conductance Ca^{2+} -activated K^{+} (SK) channels, particularly the SK3 subtype, play a prominent role in regulating DA cell firing pattern (Shepard & Bunney, 1991; Wolfart *et al.*, 2001). Apamin and other SK channel pore blockers increase spontaneous bursting *in vivo* (Waroux *et al.*, 2005; Ji & Shepard, 2006). Although 'natural' bursting is lost in brain slices, attenuation of SK currents restores phasic firing and enables N-methyl-D-aspartate to elicit bursting (Seutin *et al.*, 1993; Johnson & Seutin, 1997; Hopf *et al.*, 2007). SK channel pore block also enhances synaptic plasticity by interrupting the negative feedback exerted by Mg^{2+} on N-methyl-D-aspartate receptors (Ngo-Anh *et al.*, 2005). Collectively, these data suggest that modulation of SK3 currents in DA neurons could represent a mechanism for modifying their response to excitatory input.

The SK channels are gated solely by the binding of Ca^{2+} to calmodulin (CaM), which is constitutively linked to the channel (Xia *et al.*, 1998). Phosphorylation of SK2- and conceivably SK3-bound CaM by protein kinase CK2 changes the affinity of CaM for Ca^{2+} , influencing SK channel activity (Bildl *et al.*, 2004; Allen *et al.*, 2007). Importantly, neurotransmitter-induced activation of CK2 kinase and the subsequent reduction of SK2 channel Ca^{2+} sensitivity occur in response to stimulation of α_1 - and β -adrenergic as well as somatostatin receptors (Maingret *et al.*, 2008). Modulation of SK channel activity can also be induced by compounds that alter apparent Ca^{2+} affinity through phosphorylation-independent mechanisms (Pedarzani *et al.*, 2001; Strøbæk *et al.*, 2006). In the present study, voltage- and current-clamp recordings were conducted to characterize the pharmacological and functional interactions between positive and negative modulators on cloned SK3 channels and substantia nigra (SN) DA neurons in brain slices. Our results underscore the importance of these channels in 'tuning' the excitability of DA neurons and demonstrate the feasibility of pharmacologically modulating the apparent Ca^{2+} affinity of SK channels as a means of altering the response of these neurons to membrane depolarization.

Materials and methods

Cell culture

The human embryonic kidney 293 (HEK293) cells expressing human SK3 channels (Strøbæk *et al.*, 2004) were cultured in Dulbecco's modified Eagle's medium (Cambrex BioScience, Verviers, Belgium) fortified with 10% fetal calf serum (Sigma-Aldrich, St Louis, MO, USA) at 37°C and in 5% CO₂. At 60–80% confluence, cells were washed with phosphate-buffered saline, harvested by trypsin / ethylenediaminetetra-acetic acid (Cambrex BioScience) treatment and transferred to petri dishes containing cover-slips (diameter 3.5 mm, custom made at VWR International APS, Albertslund, Denmark).

Membrane currents were recorded using the inside-out mode of the patch-clamp technique. HEK293 cells, seeded on cover-slips, were transferred to a 15 µL recording chamber continuously superfused at 1 mL / min at room temperature (21–23°C). Patch pipettes (1.8–2.2 MX) were pulled from borosilicate glass tubing (O.D. 1.32 mm; Modulohm, Copenhagen, Denmark) using a horizontal electrode puller (Zeitz Instruments, Augsburg, Germany) and filled with a solution containing a high [K⁺] (in mM): 154 KCl, 2 CaCl₂, 1 MgCl₂ and 10 HEPES with pH adjusted to 7.4 with KOH. The bath / intracellular solution (pH 7.2) contained (in mM): 154 KCl, 10 HEPES and 10 ethylene glycol tetra-acetic acid or a combination of ethylene glycol tetra-acetic acid and nitrilotriacetic acid (10 mM in total). The concentrations of CaCl₂ and MgCl₂ needed to obtain the desired free concentration of Ca²⁺ (0.01–10 µM) and Mg²⁺ (1 mM) were calculated using *EQCAL* software (Cambridge, UK) and then added. Free Ca²⁺ concentrations were controlled by a Ca²⁺-sensitive electrode (WPI, Sarasota, FL, USA). An electronically-controlled micromanipulator (Eppendorf, Radiometer, Copenhagen, Denmark) was used to position patch pipettes and a Ag / AgCl pellet affixed to the chamber served as a reference. Experiments were performed using an EPC-9 amplifier (HEKA, Lambrecht, Germany) connected to a Macintosh computer using an ITC-16 interface. Voltage ramps from –80 to +80 mV (200 ms duration) were applied every 5 s from a holding potential of 0 mV. Data were filtered at 3 kHz.

Brain slice preparation

Experiments were performed using 70 male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA; post-natal day 13–21) and were conducted with prior approval from the University of Maryland School of Medicine Institutional Animal Care and Use Committee and in strict accordance with the procedures described in the *Guide for the Care and Use of Laboratory Animals*. Rats were anesthetized with chloral hydrate (400 mg / kg, i.p.) and decapitated. The brain was removed and placed in ice-cold artificial cerebrospinal fluid consisting of (in mM): 124 NaCl, 4.0 KCl, 1.25 NaH₂PO₄, 1.2 MgSO₄, 25.7 NaHCO₃, 2.45 CaCl₂, 0.15 ascorbate and 11 glucose (pH 7.35, 295–305 mOsm). Coronal sections (300 µm) containing the substantia nigra (bilateral) were prepared using a vibrating tissue slicer (WPI), submerged in oxygenated artificial cerebrospinal fluid and maintained at room temperature for a minimum of 1 h.

Individual slices were transferred to a 'submersion'-style recording chamber (RC-22C, Warner Instruments, Hamden, CT, USA) and perfused (1.5 mL / min) with oxygenated

artificial cerebrospinal fluid maintained at 30°C using a feedback-controlled bath heater (Warner Instruments). Whole-cell patch-clamp recordings were made from DA neurons in the SN and visualized with infrared differential interference contrast video microscopy at 40× using an Olympus BX51WI microscope equipped with a CCD camera (OLY-150IR). Cells were selected on the basis of their position in the slice and general appearance. Patch pipettes were pulled from standard wall borosilicate tubing (1.5 mm OD, WPI) using a horizontal micropipette puller (Sutter Instruments, Novato, CA, USA) and filled with a solution containing (in mM): 131 K-gluconate, 9 KCl, 20 HEPES, 0.1 ethylene glycol tetraacetic acid, 5 Mg-ATP and 0.5 GTP TRIS (pH 7.2; osmolarity 280–290 mOsm; 8–18 MΩ). Electrodes were advanced under positive pressure using a robotic micromanipulator (Sutter Instruments) and, on contact with a putative DA neuron, high resistance seals formed during application of continuous negative pressure. The membrane was ruptured by suction and the membrane potential monitored using an Axoclamp 2B amplifier. Voltage recordings were digitized at 10 kHz using a laboratory interface and acquired with the PCLAMP software package (Molecular Devices, Sunnyvale, CA, USA). Timed current pulses were generated using a digital pulse generator and applied to the cell through a balanced bridge circuit of the amplifier.

Test compounds were applied to the cells by exchanging the relevant intracellular salt solution (inside-out experiments) or the normal artificial cerebrospinal fluid (slice experiments) for solutions that differed only by the addition of a known concentration of agent. 6,7-dichloro-1*H*-indole-2,3-dione-3-oxime (NS309) and *R-N*-(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphthylamine (NS8593) were synthesized at NeuroSearch A / S (Ballerup, Denmark), dissolved in dimethylsulfoxide and diluted at least 1000-fold in the appropriate experimental solution.

Calculations and statistics

The EC_{50} values (for Ca^{2+} and NS309) derived from the voltage-clamp experiments were estimated from equilibrium concentration–response relationships fit to the Hill equation

$$I(C) = \frac{[I(\max) - I(0)] \times C^n}{EC_{50}^n + C^n} + I(0)$$

where $I(C)$ is the current at the concentration C , $I(\max)$ is the maximal current fitted at saturating concentrations, $I(0)$ is the current at zero concentration and n is the Hill coefficient.

Spike frequency adaptation was characterized by the percentage of adaptation of firing frequency where

$$F_{adap} = 100 \times (f_0 - f_{ss}) / f_0$$

f_0 and f_{ss} are the reciprocal of the interspike interval between the first and last pair of spikes in the train, respectively.

Unless otherwise indicated, omnibus testing was conducted using a one- or two-way repeated measures analysis of variance (r_{MANOVA}). Post-hoc comparisons between control and individual treatment groups were made using a Bonferroni t -test (one-way) or Hochberg's modification of the Bonferroni correction (two-way) to determine a threshold for significance that would maintain a family-wise type I error rate of 0.05 (Hochberg, 1988). Two-way comparisons were conducted using SAS PROC Mixed (SAS Institute, Cary, NC, USA) to run the generalized estimating equations method for unbalanced r_{MANOVA} (Liang & Zeger, 1986). The specific models used are provided in the text. All P values are two-tailed at $\alpha = 0.05$.

Results

Positive modulation of SK channel gating in SN dopamine neurons

The effects of increasing the apparent affinity of SK channels to $[Ca^{2+}]_i$ were investigated using the positive SK channel modulator, NS309. Whole-cell current-clamp recordings were obtained from SN neurons identified as dopaminergic on the basis of their unique electrophysiological properties including long duration action potential preceded by a slow depolarization, medium duration after-hyperpolarization (mAHP), pacemaker firing pattern and prominent sag conductance (Grace & Onn, 1989; Yung *et al.*, 1991). Bath application of NS309 significantly altered the spontaneous firing rate of SN DA neurons (r_{MANOVA} , $F_{5,21} = 25.2$, $P < 0.001$; Fig. 1A). Although the two lowest concentrations of the compound appeared to increase firing rate (0.1–0.3 μM , $n = 6$), these changes did not reach statistical significance (Bonferroni t -test, $P > 0.1$). Higher concentrations of NS309 (0.6–3 μM) decreased spontaneous firing of DA neurons in a concentration-dependent manner. Concentrations above 3 μM resulted in a complete cessation of spontaneous activity. The inhibitory effects of NS309 could not be attributed to a change in membrane potential and none of the concentrations tested altered input resistance (control: $435.4 \pm 26.6 M\Omega$; NS309_{3 μM} : $394.3 \pm 35.1 M\Omega$, $n = 6$ / group). Rather, the effects of the compound appeared to result from an increase in the duration of the mAHP following individual spikes (Fig. 1B). At high concentrations, a single action potential was typically followed by an afterhyperpolarization of sufficient amplitude and duration to oppose the slow depolarization that would ordinarily have brought the membrane potential to spike threshold.

Brief depolarizing current pulses, surrogates for excitatory inputs of varying strengths, were used to assess the effects of NS309 on the mAHP following repetitive spiking. Under control conditions, the mAHPs evoked in response to 0.25 or 0.5 nA current pulses averaged $11.5 \pm 1.3 mV$ ($n = 6$) and $15.4 \pm 0.44 mV$ ($n = 6$), and relaxed to 50% of their peak amplitude within 134.0 ± 10.4 and $152 \pm 8.4 ms$ ($n = 6$), respectively. Repeated application of NS309 (0.1–3 μM) increased the amplitude of mAHP at both current intensities (r_{MANOVA} , 0.25 nA: $F_{5,20} = 4.0$, $P < 0.05$; 0.5 nA: $F_{5,20} = 7.0$, $P < 0.001$) in a concentration-dependent manner (Fig. 1C). Addition of NS309 in concentrations of up to 0.6 μM had no effect on mAHP amplitude at the lower of the two current intensities. However, at 1 μM , the compound significantly increased mAHP compared with control (Fig. 1C, squares). Higher concentrations produced no further effect. A similar 'all-or-none' response pattern was

observed when a stronger stimulus current was used (0.5 nA). Notably, however, the precipitous increase in mAHP amplitude occurred at a lower concentration of NS309 (0.6 μ M, Fig. 1C, circles). In addition to increasing its amplitude, NS309 prolonged the duration of the mAHP as demonstrated by a significant increase in the time required for the potential to relax to 50% of its peak amplitude (Fig. 1D) (r_{MANOVA} , 0.25 nA: $F_{5,20} = 42.3$, $P < 0.001$; 0.5 nA: $F_{5,20} = 63.5$, $P < 0.001$). Again, the apparent potency of the compound was enhanced when stronger stimulus currents were used. At the highest concentration tested (3 μ M), NS309 produced a 21- and 24-fold increase in the half-life of the mAHP elicited by 0.25 and 0.5 nA current pulses, respectively. Consistent with its ability to increase the mAHP, NS309 reduced the number of spikes elicited by depolarizing current pulses in a concentration-dependent manner (r_{MANOVA} , 0.25 nA: $F_{5,20} = 6.2$, $P < 0.001$; 0.5 nA: $F_{5,20} = 5.5$, $P < 0.003$; Fig. 1C, numbers in brackets).

The established role of the mAHP in regulating the extent of membrane repolarization between spikes suggested that increases in the conductance could also alter spike frequency adaptation. Accordingly, a separate group of experiments was conducted to assess the effects of NS309 on spiking elicited by long duration (800 ms) rectangular current pulses of varying amplitudes (0.05–0.2 nA). Figure 2A illustrates the response of a representative DA neuron to depolarizing current injection in the presence of increasing concentrations of NS309 (0.1–3.0 μ M). As previously reported (Shepard & Bunney, 1991; Nedergaard, 2004), DA neurons showed little evidence of frequency adaptation in response to low stimulus amplitudes (Fig. 2, A₁, bottom trace). Higher currents reduced the interval between the first and second spike after which the firing frequency rapidly adapted to a new steady-state level that was maintained for the duration of the stimulus pulse (Fig. 2, A_{2–3}, bottom traces). Addition of NS309 reduced the number of spikes elicited by depolarizing pulses in a concentration- and current-dependent manner. Two-way r_{MANOVA} (model: spike number = concentration + current + concentration $\times$ current) revealed significant main effects for concentration ($F_{4,16} = 82.8$, $P < 0.0001$), current intensity ($F_{2,10} = 124.9$, $P < 0.0001$) and their interaction term ($F_{5,26} = 97.5$, $P < 0.0001$). This is further illustrated in Fig. 2B, where the effects of NS309 are clearly dependent on the number of spikes elicited. Low concentrations of the compound potently suppressed high frequency spiking elicited by higher intensity (0.1–0.2 nA) stimuli, whereas slower firing evoked in response to smaller stimulus currents (0.05 nA) was affected to a lesser degree. Current-dependent differences in the number of spikes elicited by depolarizing pulses were no longer evident at concentrations of NS309 $\geq 0.6 \mu$ M. Positive SK channel modulation also significantly increased spike frequency adaptation (Fig. 2C). Two-way r_{MANOVA} (model: F_{adap} = concentration + current + concentration $\times$ current) revealed significant main effects for concentration ($F_{4,16} = 75.21$, $P < 0.0001$), current intensity ($F_{2,10} = 41.6$, $P < 0.0001$) and their interaction ($F_{5,26} = 14.2$, $P < 0.0001$). The inhibitory effects of NS309 on current-evoked spiking precluded a representative assessment of spike frequency adaptation at concentrations above 0.6 μ M. However, as illustrated in Fig. 2, A_{1–3}, higher concentrations of the compound progressively increased the mAHP following individual spikes in the train.

Negative modulation of SK channel gating in SN dopamine neurons

The effects of reducing the apparent Ca^{2+} affinity of SK channels on the spontaneous activity of DA neurons were characterized in a sample of 29 SN DA neurons tested with various concentrations of NS8593. Basal firing rates, which did not differ between groups of cells tested with one or more of the four selected concentrations of NS8593 (1, 3, 6 and 10 μM ; ANOVA , $P > 0.1$) were pooled and compared with firing rates obtained 30–45 min after continuous bath application of NS8593. Although the negative modulator had an excitatory effect on several DA neurons, omnibus testing revealed no evidence of a concentration-dependent change in integrated firing rate (ANOVA , $F_{4,60} = 2.1$, $P > 0.5$; Fig. 3A). An identical approach was used to compare the effects of NS8593 on the coefficient of variation of interspike intervals, an index of the precision of firing. Under control conditions, the interspike interval coefficient of variation averaged $11.5 \pm 1.5\%$ and did not differ between groups of cells tested with different concentrations of NS8593 (ANOVA , $P > 0.1$). By contrast, the interspike interval coefficient of variation associated with spike trains collected during application of NS8593 differed significantly from pooled control values (ANOVA , $F_{4,60} = 13.0$, $P < 0.001$), with concentrations above 1 μM resulting in a significant increase in variability of discharge (Fig. 3B). The increase in interspike interval coefficient of variation was associated with the appearance of bursting activity characterized by a cluster of spikes riding on a depolarizing envelope that often resulted in a temporary cessation in firing (Fig. 3C). Although NS8593-induced bursting activity generally resembled that seen with apamin, spiking was occasionally observed during the plateau phase of the oscillation (Fig. 3C). As illustrated in the example shown in Fig. 3D, NS8593 also attenuated or blocked the mAHP following single spikes.

Pharmacological interactions between positive and negative SK channel modulators

Previous studies have shown that NS309 and NS8593 interact functionally at cloned SK3 channels, the principal channel subtype expressed by midbrain DA neurons (Wolfart *et al.*, 2001; Sarpal *et al.*, 2004). Unlike the SK channel pore blocker apamin, whose effects are essentially irreversible, the effects of NS8593 in cultured cells are surmounted by co-application of the positive SK channel modulator NS309 (Strøbæk *et al.*, 2006). We aimed at using the ‘competitive’ nature of positive vs. negative gating modulation *in vitro* as evidence of the molecular specificity of the effects observed in DA neurons supporting or falsifying the contention that modulation of the apparent Ca^{2+} affinity of SK channels is responsible for the observed changes in DA cell excitability and firing pattern in brain slices. In order to make this connection, we mapped out how the interaction depends on NS309 / NS8593 concentrations as well as on $[\text{Ca}^{2+}]_i$ using excised inside-out voltage-clamp experiments performed on human SK3 channels expressed in HEK293 cells (neither compound discriminates between rat and human isoforms of SK3). This configuration allows assessment of channel activity under controlled $[\text{Ca}^{2+}]_i$ and in the absence of any influence from metabolic or cytoplasmic second messenger cascades.

In the first series of experiments, we focused on the contribution of $[\text{Ca}^{2+}]_i$ to the NS309-mediated reversal of the inhibitory effects of NS8593 on human SK3 currents. Figure 4A shows selected I / V curves (left panel) as well as the time-course of the experiment (right panel) obtained from a multichannel isolated patch, bathed in symmetric 154 mM K^+

solutions with various highly buffered Ca^{2+} concentrations on the intracellular side. The patch was initially exposed to a low Ca^{2+} concentration (10 nM), a condition where the channel is not activated, and then to a higher concentration (10 μM) to obtain full activation. After once again deactivating the channel (Fig. 4, Aa and Ba), NS8593 (1 μM) was added and the Ca^{2+} concentration cumulatively increased from 10 nM to 30 μM (Fig. 4, Ab, Ac and Bb, Bc). Finally, NS8593 was washed out and $[\text{Ca}^{2+}]_i$ adjusted to 10 μM (Fig. 4, Ad and Bd) to define the current corresponding to the maximal open state probability, before again deactivating with 10 nM Ca^{2+} to assure that the current corresponding to a zero open state probability had not drifted during the experiment. Stationary currents reached at each Ca^{2+} concentration were normalized to the current level at 10 μM and used to construct the activation curve depicted in Fig. 4C. The dashed line shows the unmodified SK3 channel activation curve for Ca^{2+} [$\text{EC}_{50}(\text{Ca}^{2+}) = 0.42 \mu\text{M}$, individual data points omitted for clarity]. In accordance with previous observations (Strøbæk *et al.*, 2006), NS8593 potently shifts the activation curve to the right [apparent decrease in Ca^{2+} affinity; $\text{EC}_{50}(\text{Ca}^{2+}) = 1.11 \mu\text{M}$] concomitantly with a decrease in the steepness of the curve. As illustrated in Fig. 4B and C, addition of 10 μM NS309 shifted the activation curve to the left, surpassing the activation curve for the unmodified channel and yielding a significantly different apparent $\text{EC}_{50}(\text{Ca}^{2+})$ of 0.07 μM ($t_7 = 3.52$; $P < 0.01$), a value that did not differ from the $\text{EC}_{50}(\text{Ca}^{2+})$ obtained with NS309 alone. Importantly, however, the effect of NS309 is not strictly a complete reversal of the effects of NS8593 as demonstrated by the persistent albeit small reduction in maximal relative current.

In order to more accurately determine the concentration of NS309 needed to oppose the effects of NS8593, experiments were conducted to assess the effects of varying the concentrations of the positive and negative modulators at a fixed concentration of $[\text{Ca}^{2+}]_i$ (Fig. 5). After identifying its lower (10 nM Ca^{2+}) and upper (10 μM Ca^{2+}) limits (Fig. 5A), the SK3 current was adjusted to intermediate open state probability (~ 0.25) by continuous exposure to 0.3 μM Ca^{2+} . Application of 30 μM NS8593 reduced the current to a level indistinguishable from the background leak, indicating complete inhibition of the SK3 channel. Subsequent addition of NS309, in the continued presence of 30 μM NS8593, reactivated SK3 channels in a concentration-dependent fashion. The inhibitory effect of NS8593 was ‘reversed’ by approximately 1 μM NS309 and, as predicted by earlier results (Fig. 4C), the SK current superseded control levels reaching about 75% of the maximal current level obtained in the presence of 10 μM Ca^{2+} . Identical experiments performed using lower concentrations of NS8593 (1 and 10 μM) yielded similar results. The entire series of experiments is illustrated in Fig. 5B, in which normalized currents are plotted against the concentration of NS309. In the absence of the negative modulator, NS309 increased the inside-out SK current in a concentration-dependent manner with an EC_{50} value of 0.28 μM , Hill coefficient of 1.1 and an intercept of 0.21. Addition of NS8593 (1–30 μM) shifted the concentration–response relationship for NS309 to the right (r_{MANOVA} ; $F_{3,84} = 53.6$; $P < 0.001$), resulting in an increase in the estimated EC_{50} (NS8593_{1 μM} , 0.56 μM ; NS8593_{10 μM} , 1.80 μM ; NS8593_{30 μM} , 2.04 μM) and corresponding reduction in maximal SK current (NS8593_{1 μM} , 0.88; NS8593_{10 μM} , 0.79; NS8593_{30 μM} , 0.67). Consequently, the complete inhibition of SK current by NS8593 can be ‘reversed’ (indicated by the dashed line in Fig.

5B) by relatively low concentrations (~1 μ M) of NS309. In contrast, fully NS309-activated SK3 channels can only be inhibited by 30% in response to 30 μ M NS8593.

Functional interactions between SK channel modulators and dopamine cell excitability

The ability of NS309 to surmount the effects of the negative SK channel modulator on human SK3 current in HEK293 cells prompted us to ask whether similar concentrations of the positive modulator could also prevail over the effects of NS8593 in brain slices. As a 'negative control', we first demonstrated that the classical effects of apamin on DA firing were not reversible with NS309 (data not shown). As illustrated in Fig. 6, 10 μ M NS8593 disrupted the characteristic pacemaker activity observed under control conditions and led to the emergence of regenerative bursting oscillations. In this example, typical of higher concentrations of the negative modulator, spiking was clustered in bursts on the rising phase of the oscillation and terminated by depolarization inactivation. Addition of increasing concentrations of NS309 reduced the duration and increased the frequency of the oscillations (Fig. 6A), partially restoring the mAHP following individual spikes (Fig. 6B). The number of spikes per burst was also reduced, eventually resulting in the re-emergence of single spike firing with the occasional doublet. Spontaneous activity was completely suppressed in the presence of equimolar concentrations of NS8593 and NS309 (10 μ M; Fig. 6A, bottom trace).

NS8593 (10 μ M), alone or in combination with NS309 (0.6–10 μ M), significantly altered both the amplitude ($_{\text{RMANOVA}}$, 0.25 nA: $F_{5,23} = 8.6$, $P < 0.001$; 0.50 nA: $F_{5,23} = 23.7$, $P < 0.001$) and duration ($_{\text{RMANOVA}}$, 0.25 nA: $F_{5,23} = 17.1$, $P < 0.001$; 0.50 nA: $F_{5,23} = 34.0$, $P < 0.001$) of the mAHP elicited by 200 ms current pulses (Fig. 7). When applied alone, the negative SK channel modulator reduced the amplitude of mAHP from 11.6 ± 1.7 to 5.0 ± 0.55 mV at 0.25 nA and from 16.8 ± 0.5 to 8.7 ± 0.6 mV at 0.5 nA ($n = 6$) without altering its initial time-course. The addition of NS309 to perfusates containing 10 μ M NS8593 increased the duration and amplitude of mAHP in a concentration-dependent fashion. Of the two indices, the duration of the mAHP proved to be more sensitive to the effects of NS309 and was increased beyond control values (at 0.5 nA) by a lower concentration of NS309 (1 μ M) than that required to reverse the effects of NS8593 on mAHP amplitude (3 μ M; Fig. 7B). Although co-application of equimolar concentrations of the positive and negative modulator failed to increase mAHP amplitude beyond control (10 μ M, Fig. 7A), the duration of the potential was increased at concentrations of NS309 above 3 μ M.

Discussion

The results of the present study demonstrate that pharmacological modulation of the apparent Ca^{2+} affinity of SK channels alters the excitability of SN DA neurons. In addition to changing the spontaneous firing pattern, positive and negative SK channel modulation altered the response of DA neurons to brief current pulses reflecting a change in neuronal excitability that is likely to modify the cell's response to excitatory synaptic input. Although not explicitly competitive, the effects of SK channel modulators are mutually reversible, implying that these compounds could be useful as a means of titrating neuronal excitability in a manner likely to impact the various functions that have been attributed to these neurons.

Of the three SK channel subtypes identified within the central nervous system (SK1, 2 and 3), SK3 channels represent the predominant isoform in SN DA neurons (Kohler *et al.*, 1996; Stocker & Pedarzani, 2000; Sarpal *et al.*, 2004). SK3 mRNA is expressed at levels fourfold higher than either SK1 or SK2 mRNA and neither of the latter two isoforms appear to contribute to macroscopic SK currents in DA neurons (Wolfart *et al.*, 2001). Although the compounds used in the present study do not show preferential affinity for a particular SK channel subtype, it seems likely that their effects on DA neurons are mediated by an interaction with SK channels comprised of SK3 subunits.

Positive SK channel modulation

The positive SK channel modulator NS309 decreased the spontaneous firing rate of SN DA neurons in a concentration-dependent manner eventually resulting in a complete cessation of activity. The reduction in firing was accompanied by an increase in the mAHP following individual spikes and trains of action potentials elicited by depolarizing current pulses. Similar effects have been observed in the hippocampus and in DA neurons during application of the prototypical SK channel positive modulator, 1-ethyl-2-benzimidazolinone (1-EBIO) (Wolfart *et al.*, 2001; Pedarzani *et al.*, 2005). Although NS309 increased the amplitude and time-course of mAHP, its effects on the duration of the response were particularly prominent. Intermediate concentrations (3 μ M) increased mAHP amplitude by an average of 3.8 mV while prolonging the time required for the potential to relax to 50% of its peak value by over 20-fold. These data are consistent with a threefold increase in the decay time constant of I_{AHP} in DA neurons following 200 μ M 1-EBIO and probably reflect the ability of both compounds to slow the rate of channel deactivation by reducing the off-rate of SK-CaM-bound Ca^{2+} (Pedarzani *et al.*, 2001). They also serve to illustrate the marked differences in potency between 1-EBIO and NS309, the latter having been estimated to be approximately 700 times more potent at SK channels than 1-EBIO (Pedarzani *et al.*, 2005).

The results of several experiments suggested that the effects of the positive modulator on DA cell excitability were partially frequency-dependent. Thus, low concentrations of NS309 ($< 0.3 \mu$ M) were more effective in reducing high-frequency spiking than slower firing elicited by lower stimulus currents. Current-dependent differences in the number of spikes evoked by long duration current pulses diminished with increasing concentrations of NS309. At the highest concentrations tested, successive spikes showed a progressive increase in the amplitude and duration of mAHP. Similar 'use-dependent' effects were described by Wolfart *et al.* (2001) who noted that 1-EBIO had a greater inhibitory effect on fast firing DA neurons than on slower firing cells. As both compounds increase the apparent affinity of SK channels for $[Ca^{2+}]_i$, their effects would be expected to be enhanced by neuronal spiking. The median $[Ca^{2+}]_i$ in quiescent DA neurons has been estimated at 130 nM with levels rising two- to threefold at the end of the slow depolarization preceding spike generation (Wilson & Callaway, 2000). These levels are in excess of those needed to maximally activate human SK3 channels in the presence of 3–10 μ M NS309 (Hougaard *et al.*, 2007) and could account for the fact that, at 10 μ M, spontaneous firing was suppressed after a single action potential.

Negative SK channel modulation

Previous studies have established the ability of apamin, a potent and highly selective SK channel pore blocker, to eliminate mAHP in DA neurons (Shepard & Bunney, 1991; Wolfart *et al.*, 2001). The effects of the peptide, which binds to the outer pore vestibule of SK channels (Ishii *et al.*, 1997), are essentially irreversible in tissue slices. The present results suggest that a similar degree of functional SK channel inhibition can be achieved by negative modulation of SK channel gating. In a manner analogous to the positive modulators, NS8593 exhibits a strong Ca^{2+} -dependence, potentially shifting the SK channel Ca^{2+} response curve, albeit in the opposite (rightward) direction. NS8593 inhibits cloned SK3 channel activity with an estimated K_d of ~100 nM and, at 10 μM , completely blocked the SK-dependent I_{AHP} in hippocampal pyramidal neurons (Strøbæk *et al.*, 2006). In agreement with these findings, NS8593 completely suppressed mAHP in DA neurons. Like apamin, the reduction in mAHP was associated with a disruption in pacemaker firing and the emergence of bursting activity driven by a plateau oscillation in membrane potential (Ping & Shepard, 1996, 1999). At 10 μM NS8593, spikes rarely occurred during the plateau, presumably as a result of depolarization inactivation of spike-generating mechanisms. However, lower concentrations produced a type of bursting activity in which spiking was not only maintained during the plateau but showed evidence of frequency adaptation. Unlike apamin and other SK pore blockers, the functional effects of NS8593 are Ca^{2+} -dependent. Accordingly, increases in $[\text{Ca}^{2+}]_i$ resulting from activation of L-type Ca^{2+} channels during a burst (Nedergaard *et al.*, 1993; Ping & Shepard, 1999; Shepard & Stump, 1999) could reach levels, particularly in the smaller volume of the dendrites, that would negate the actions of NS8593 such that the negative modulation of SK channels becomes partly self-limiting. This might explain why spikes were observed during the asymptote of the plateaus elicited in response to NS8593.

Although the precise nature of the interaction between SK channels, CaM, and positive and negative modulators has yet to be elucidated, these compounds share the ability to modify the effect of Ca^{2+} binding and it was therefore of interest to determine the extent to which these compounds interact at the molecular and cellular level. Due to their lipophilic nature, NS309 and NS8593 are equally effective in modulating SK channel activity when applied to either side of the cell membrane (Strøbæk *et al.*, 2004, 2006). Results obtained in excised inside-out patches showed that, in addition to fully reversing the effects of 3 μM NS8593, threefold lower concentrations of NS309 surmounted the effects of the negative modulator, increasing the apparent Ca^{2+} affinity of the channel by an order of magnitude above control values. Similar effects were observed in nigral brain slices where the emergence of bursting activity, increased neuronal excitability and the reduction in mAHP induced by NS8593 was surmounted by comparatively low concentrations of the positive SK channel modulator. Given that the interaction between positive and negative modulators persists in isolated patches, it seems unlikely that either compound alters the second messenger cascades regulating channel activity through phosphorylation-dependent mechanisms. Rather, it seems more probable that these compounds alter apparent Ca^{2+} affinity by interacting with unique binding sites located on the channel itself or a closely associated subunit. Positive SK channel modulators appear to act at the C-terminal CaM binding domain directly affecting Ca^{2+} binding (Pedarzani *et al.*, 2001; Hougaard *et al.*, 2008), whereas the negative SK

channel modulator interacts with a transmembrane region to inhibit gating somewhere downstream from the Ca^{2+} binding site (Strøbæk *et al.*, 2008). Although these data suggest that the actions of positive and negative modulators are not strictly competitive, their ability to reversibly alter the apparent Ca^{2+} affinity of cloned SK3 channels was paralleled by changes in DA cell excitability, highlighting the importance of SK channel Ca^{2+} sensitivity *per se* as a pivotal mechanism for regulation of firing patterns.

Functional implications: synaptic plasticity and reward signaling

Positive and negative SK channel modulators ‘reversibly’ altered the response of DA neurons to depolarizing current injection suggesting, by analogy to the recently demonstrated physiological modulation of the Ca^{2+} sensitivity of SK2 channel gating (Allen *et al.*, 2007; Maingret *et al.*, 2008), that these channels (probably the SK3 subtype) may also play a role in the development of synaptic plasticity in these neurons. Increases in the excitability of CA1 neurons following apamin-induced blockade of SK2 channels are accompanied by a decrease in the threshold for induction of long-term potentiation (Behnisch & Reymann, 1998; Stackman *et al.*, 2002) and paralleled by enhanced spatial and object memory encoding in mice administered a low systemic dose of the SK channel blocker (Deschaux *et al.*, 1997; Fournier *et al.*, 2001; Stackman *et al.*, 2002). By contrast, mice overexpressing SK2 channels, the principal subtype expressed in hippocampal pyramidal cells, showed impaired induction of long-term potentiation and diminished performance in spatial learning and memory tasks (Hammond *et al.*, 2006). As in the hippocampus, activation of excitatory afferents to midbrain DA neurons results in N-methyl-D-aspartate receptor-dependent long-term potentiation (Bonci & Malenka, 1999; Nugent *et al.*, 2008). Long-term potentiation of excitatory synapses on DA neurons is also induced by stress or acute administration of many commonly abused drugs including amphetamine, morphine, cocaine, ethanol and nicotine (Saal *et al.*, 2003). Increasing the strength of excitatory synaptic input would be expected to enhance the phasic increases in DA cell firing elicited by environmental cues that predict reward. By amplifying the temporal difference error signal encoded by DA neurons, these changes could lead to a predisposition toward inappropriate action selection (Saal *et al.*, 2003; Wolf, 2003). Under these circumstances, positive SK3 channel modulators could function to oppose the increase in synaptic strength induced by drugs of abuse by shunting glutamate-mediated synaptic currents and / or facilitating Mg^{2+} block of the N-methyl-D-aspartate ionophore. However, negative SK3 channel modulation could be useful in amplifying reward signaling under conditions of diminished DA neurotransmission and associated anhedonia (e.g. Parkinson’s disease). As such, SK3 channels could represent a novel therapeutic target for the treatment of a variety of addictive and affective disorders.

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Abbreviations

1-EBIO	1-ethyl-2-benzimidazolinone
CaM	calmodulin
DA	dopamine
HEK293	human embryonic kidney 293
mAHP	medium duration after hyperpolarization
NS309	6,7-dichloro-1 <i>H</i> -indole-2,3-dione-3-oxime
NS8593	R- <i>N</i> -(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphthylamine
RMANOVA	repeated measures analysis of variance
SK	small conductance Ca^{2+} -activated K^{+}
SN	substantia nigra

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Fig. 1.

Positive SK channel modulation inhibits spontaneous firing and enhances the mAHP in SN DA neurons. (A) Cumulative concentration–response curve compiled from the integrated firing rate (mean $\pm$ SEM) of four to six DA neurons tested with increasing concentrations of NS309. Asterisks denote a significant difference from baseline. (B) NS309 (0.1–3 μM) increases the mAHP following single spikes in a concentration-dependent manner. Concentrations of NS309 that inhibited spontaneous firing ($> 0.3 \mu\text{M}$) did not alter spike threshold or the trajectory of the membrane potential preceding spike initiation. (C and D) Effects of NS309 on the mAHP following a train of spikes elicited by 200 ms constant current pulses. Top panel: Representative traces illustrating the amplitude and time-course of the mAHP elicited by 0.25 nA (left panel) and 0.5 nA (right panel) current pulses under control conditions (black) and in the presence of increasing concentrations of NS309 (0.1–3 μM , colored traces). Spikes have been omitted for clarity. Lower panel: Semi-log concentration–response curve illustrating the effect of NS309 on mAHP amplitude. Values in parentheses are the average number of spikes ($\pm$ SEM) elicited per current pulse. Note that the NS309-induced increase in mAHP amplitude was accompanied by a reduction in the

number of spikes evoked by depolarizing current pulses. (D) Effect of NS309 on the duration of the mAHP expressed as the time required for the potential to decay to 50% of its peak value. Values corresponding to 'C' on the horizontal axis denote control (pre-NS309) measurements. Each point represents the average response of three to six DA cells. Daggers and asterisks indicate values significantly different from corresponding controls ($\dagger$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Bonferroni *t*-test).

Fig. 2. Positive SK channel modulation decreases neuronal excitability and enhances spike frequency adaptation. (A) Examples of spike trains evoked by constant current depolarizing pulses (A_1 , 0.05 nA; A_2 , 0.1 nA; A_3 , 0.2 nA) under control conditions (lower traces) and during bath application of increasing concentrations of NS309 (stacked vertically from 0.1 to 3.0 μ M). Note the progressive increase in the mAHP following successive spikes in the train. All recordings were obtained from the same cell. (B) Relationship between the concentration of NS309 (abscissa) and stimulus intensity (symbols) on the number of spikes elicited by an 800 ms depolarizing current pulse (ordinate). (C) Concentration- and current-dependent effects of NS309 on spike frequency adaptation (F_{adap} ; see Materials and methods for details). Asterisks denote a significant difference from control values using a modification of the Bonferroni t -test for unbalanced two-way R_{MANOVA} (** $P < 0.01$, *** $P < 0.001$, see Materials and methods for details).

Fig. 3.

Negative SK channel modulation promotes bursting activity and blocks the post-spike afterhyperpolarization. Bar charts illustrating the effects of NS8593 (1–10 μ M) on (A) integrated firing rate and (B) interspike interval coefficient of variation. Individual DA cells ($n = 29$) were tested with a single ($n = 23$) or multiple ($n = 6$) concentrations of NS8593 (1 μ M, $n = 5$; 3 μ M, $n = 13$; 6 μ M, $n = 7$; 10 μ M, $n = 11$). Pre-drug (control) values ($n = 29$) were pooled for comparison with activity measured after a 20–30 min perfusion with NS8593. Asterisks indicate significant differences from control (** $P < 0.01$, *** $P < 0.001$). (C) Example of NS8593-induced bursting (6 μ M). Note the progressive decrease in spike amplitude and presence of small spikes during the plateau phase of the burst. (D) Single spikes recorded from a representative DA neuron immediately before (black trace) and 20 min after (grey trace) bath application of NS8593 (10 μ M).

Fig. 4.

Reduced sensitivity of human SK3 (hSK3) channels for Ca²⁺ in the presence of a negative SK channel modulator (NS8593) is reversed by co-application of NS309. hSK3 current measured from inside-out patches obtained from HEK293 cells stably expressing hSK3 upon application of 200 ms voltage ramps (−80 to +80 mV) elicited every 5 s from a holding potential of 0 mV. (A) I / V relationships were measured in the presence of 1 μM NS8593 at a free Ca²⁺ concentration of 0.01 μM (a), 0.1 μM (b) or 10 μM (c) and in the absence of NS8593 at 10 μM free Ca²⁺ (d) at the time-points indicated in the right panel. In the right panel, SK3 current (measured at −75 mV) is depicted as a function of time. The Ca²⁺ concentration in the bath / intracellular solution was varied from 0.01 to 30 μM as indicated and NS8593 (1 μM) applied as shown by the bar. (B) Similar experiments as in A but with a combination of 1 μM NS8593 and 10 μM NS309 applied to the bath / intracellular solution.

(C) Ca^{2+} concentration–response relationship of hSK3 in the presence of 1 μM NS8593, 10 μM NS309 alone or a combination of NS8593 and NS309. Currents from individual patches were normalized with respect to the effect of 10 μM Ca^{2+} (in the absence of compound). The curves are the fit of data to the Hill equation, yielding the following EC_{50} and Hill coefficients: 1.11 μM and 1.9 (NS8593, efficacy 89%), 0.07 μM and 2.7 (NS309, efficacy 100%), and 0.07 μM and 2.9 (NS8593 and NS309, efficacy 94%) ($n = 4-5$). The dashed line symbolizes the control Ca^{2+} concentration–response relationship in the absence of any compound (0.42 μM and 5.6, efficacy 100%; data published in Strøbæk *et al.*, 2006).

Fig. 5.

NS309 potentiates the human SK3 (hSK3) current even in the presence of NS8593. Current measured from inside-out patches obtained from HEK293 cells stably expressing hSK3. (A) Current as a function of time. Ca^{2+} in the intracellular / bath solution was at 0.01, 0.3 or 10 μM as indicated. NS8593 (30 μM) was applied to the solution as indicated after obtaining a stable current level at 0.3 μM Ca^{2+} resulting in a complete current inhibition. In the continuous presence of NS8593, increasing concentrations of NS309 were applied as shown by bars. (B) Concentration–response relationships for NS309 obtained from experiments

conducted as in A but with different concentrations of NS8593 present in the intracellular / bath solution. Currents were normalized with respect to the effect of 10 μM Ca^{2+} , which induces maximal SK channel activity, and data points represent mean $\pm$ SEM of four to five experiments. The solid lines are the fit of data to the Hill equation yielding the following EC_{50} , Hill coefficients and efficacies, respectively: control: 0.28 μM , 1.1 and 100%; 1 μM NS8593: 0.56 μM , 1.1 and 88%; 10 μM NS8593: 1.80 μM , 1.4 and 78%; 30 μM : 2.04 μM , 1.2 and 67%. * P < 0.05, ** P < 0.01, *** P < 0.001, compared with controls (NS309 alone); Bonferroni t -test.

Fig. 6.

The positive modulator, NS309, reverses the effects of negative SK channel modulation on the spontaneous activity of SN DA neurons. (A) Representative 20 s samples of spontaneous activity recorded under control conditions (upper trace), following continuous perfusion with NS8593 (10 μ M, second trace) and during co-application of NS8593 (10 μ M) plus increasing concentrations of NS309 (0.6–10 μ M). Addition of NS309 decreases the number of spikes / burst, reduces the duration of the accompanying plateau potential and increases the precision of firing. (B) Single spikes illustrating the ability of NS309 to reverse

suppression of the post-spike mAHP by NS8593 (10 μ M). mAHPs recorded under control conditions and in the presence of NS8593 alone were truncated here for clarity. Both images are illustrated in Fig. 3B in their entirety.

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Fig. 7.

Negative modulation of SK channels inhibits the mAHP in SN DA neurons; reversal by NS309. Log concentration-response curves illustrating the effects of increasing concentrations of NS309 on changes in the amplitude (A) and duration (B) of the mAHP induced by prior application of NS8593 (10 μ M). The mAHP was evoked using constant current 200 ms rectangular current pulses (dashed lines, 0.25 nA; solid lines, 0.50 nA). Solid symbols ('C' on the horizontal axis) denote control values, whereas cross-hatched symbols represent the effects of NS8593 alone. Each point is the average response of three to six

cells. Asterisks indicate significant differences from control values ($***P < 0.001$, Bonferroni *t*-test). Inset: Representative traces illustrating the effects of NS8593 (10 μ M) alone and in combination with NS309 (3 μ M) on mAHP elicited by a 200 ms depolarizing current pulse. Spikes have been truncated for clarity.