Temperature Sensitivity of Dopaminergic Neurons of the Substantia Nigra Pars Compacta: Involvement of Transient Receptor Potential Potential Channels

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Guateo, Ezia, Kenny K. H. Chung, Tharushini K. Bowala, Giorgio Bernardi, Nicola B. Mercuri, and Janusz Lipski. Temperature sensitivity of dopaminergic neurons of the substantia nigra pars compacta: involvement of Transient Receptor Potential channels. J Neurophysiol 94: 3069–3080, 2005. First published July 13, 2005; doi:10.1152/jn.00066.2005. Changes in temperature of up to several degrees have been reported in different brain regions during various behaviors or in response to environmental stimuli. We investigated temperature sensitivity of dopaminergic neurons of the rat substantia nigra pars compacta (SNc), an area important for motor and emotional control, using a combination of electrophysiological techniques, microfluorometry, and RT-PCR in brain slices. Spontaneous neuron firing, cell membrane potential/currents, and intracellular Ca2+ level ([Ca2+]i) were measured during cooling by ±10° and warming by ±5° from 34°C. Cooling evoked slowing of firing, cell membrane hyperpolarization, increase in cell input resistance, an outward current under voltage clamp, and a decrease of [Ca2+]i. Warming induced an increase in firing frequency, a decrease in input resistance, an inward current, and a rise in [Ca2+]i. The cooling-induced current, which reversed in polarity between −5 and −17 mV, was dependent on extracellular Na+. Cooling-induced whole cell currents and changes in [Ca2+]i were attenuated by 79% in the presence of 2-aminoethoxydiphenylborane (2-APB; 200 μM), and the outward current was reduced by 20% with ruthenium red (100 μM). RT-PCR conducted with tissue punches containing the SNc revealed mRNA expression for TRPV3 and TRPV4 channels, known to be activated in expression systems by temperature changes within the physiological range. 2-APB, a TRPV3 modulator, increased baseline [Ca2+]i, whereas 4αPDD, a TRPV4 agonist, increased spontaneous firing in 7 of 14 neurons tested. We conclude that temperature-gated TRPV3 and TRPV4 cationic channels are expressed in nigral dopaminergic neurons and are constitutively active in brain slices at physiological temperatures, where they affect the excitability and calcium homeostasis of these neurons.

INTRODUCTION

Although it is generally believed that brain temperature is tightly regulated, changes of up to several degrees centigrade have been observed not only during fever or heat stroke, but also during different behavioral states (e.g., Abrams and Hammel 1964; Kiyatkin and Mitchum 2003; Moser et al. 1993) or after administration of certain addictive drugs such as heroin, cocaine, or methamphetamine (e.g., Brown et al. 2003; Kiyatkin and Brown 2003; Kiyatkin and Wise 2002). Hyperthermia can itself be damaging or can potentiate other insults such as cerebral ischemia (e.g., Kim et al. 1996). On the other hand, moderate brain hypothermia may be neuroprotective (e.g., Garnier et al. 2001; Laptook and Corbett 2002; Ovbiagele et al. 2003). Interestingly, the protective effect of low temperature is greater than that predicted from reduction of metabolic rate alone (Busto et al. 1987).

The hypothalamus contains neurons that are highly temperature sensitive (Q10 of firing frequency >2.0) and plays a role in thermoregulation (Hori et al. 1999; Kobayashi and Takehashi 1993). Neurons in other brain regions have also been reported to show high temperature sensitivity (Boulant and Dean 1986). However, the pathophysiological significance of temperature-induced responses in such neurons remains unclear. Furthermore, the cellular mechanisms involved in these responses are not well determined. They could, at least in part, depend on temperature-gated ion channels. This possibility is supported by the discovery of temperature-sensitive transient receptor potential (TRP) channels. TRP channels are a large (>20) of related plasmalemmal proteins, classified in three main subfamilies: TRPC, TRPV, and TRPM (Clapham 2003; Montell 2001; Moran et al. 2004; Patapoutian et al. 2003; Vennekens et al. 2002). Several members of the TRP channel family are sensitive only to very high or very low temperatures and have been implicated in thermoreception at the periphery (for review see Patapoutian et al. 2003). TRPV3 and TRPV4 channels, both expressed in the CNS (Guler et al. 2002; Liedtke et al. 2000; Smith et al. 2002; Wissenbach et al. 2001; Xu et al. 2002) are sensitive to temperature changes within the physiological range (TRPV3: around 37°C, Peier et al. 2002b; Xu et al. 2002; TRPV4: between 25 and 43°C, Guler et al. 2002; Watanabe et al. 2002b). Because these cation channels are also permeable to Ca2+, they may play a role in the regulation of intracellular Ca2+ homeostasis.

The present study focuses on the effect of changes in temperature on dopaminergic neurons in the substantia nigra pars compacta (SNc). This region is an important element of the basal ganglia and plays a role in motor and emotional control (e.g., Bonci et al. 2003; Diana and Tepper 2002). Degeneration of SNc neurons, and to a smaller degree of adjacent dopaminergic cells in the ventral tegmental area (VTA), is associated with Ca2+ overload and leads to the motor symptoms of Parkinson’s disease (e.g., Hirsch et al. 1997) and to anhedonia (Isella et al. 2003). It has been recently suggested that the activity of the nigro-striatal system may be affected by changes in local temperature (see DISCUSSION).

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Therefore we characterized the temperature sensitivity of SNc neurons using a combination of electrophysiological, Ca\textsuperscript{2+} imaging, and RT-PCR techniques.

**METHODS**

**Tissue preparation**

All procedures were approved by the Animal Ethics Committees of the University of Tor Vergata and University of Auckland. After halothane or CO\textsubscript{2} anesthesia, the brain was rapidly removed from 2- to 4-wk-old Wistar rats and slices (thickness, 200–250 μm) cut with a vibratome (VT 1000s, Leica). In most experiments, horizontal slices containing the substantia nigra and the subthalamic nucleus (STN) were cut. After preincubation at 34°C in artificial cerebrospinal fluid (ASCF, containing (in mM) NaCl 126, KCl 2.5, MgCl\textsubscript{2} 1.2, NaH\textsubscript{2}PO\textsubscript{4} 1.2, CaCl\textsubscript{2} 2.4, glucose 10, and NaHCO\textsubscript{3} 24; 290 mM NaCl 1–2) and transfer to a recording chamber (volume, 0.6 ml), slices were submerged in ASCF (flow, 2.5 ml min\textsuperscript{-1}) and gassed with 95% O\textsubscript{2}-5% CO\textsubscript{2} (pH 7.4). In some experiments, extracellular sodium concentration ([Na\textsuperscript{+}]) was reduced (by 83%) by substituting NaCl with choline chloride (126 mM, with 3 mM choline) to prevent cholinergic stimulation, or extracellular Ca\textsuperscript{2+} was removed and Mg\textsuperscript{2+} concentration increased to 7.3 mM (with 1 mM EGTA).

**Extracellular and whole cell patch-clamp recordings**

Conventional extracellular recordings were performed using an AC amplifier (NL104, NeuroLog; bandwidth, 70 Hz to 3 kHz) and glass microelectrodes (3–5 MΩ) filled with a solution that contained (in mM): NaCl 145, KCl 3, CaCl\textsubscript{2}, 1, MgCl\textsubscript{2}, 10, and Hepes 10, and glucose 15. Firing frequency was measured with a digital frequency meter using 5-s bins and the data (action potentials and firing frequency) were acquired with AxoScope (v.8, Axon Instruments).

For whole cell patch clamping, the recording chamber was mounted on the stage of an upright microscope (Axioskop FS, Zeiss, or E600FN, Nikon) and individual neurons were visualized using an infrared differential interference contrast (IR-DIC) system and ×40 water immersion objective (Olympus or Nikon). Patch pipettes (2–5 MΩ) were filled with a solution containing (in mM): K-glucuronate 145, CaCl\textsubscript{2} 0.1, MgCl\textsubscript{2} 2, Heps 10, EGTA 0.75, ATP(Mg\textsuperscript{2+})\textsubscript{2} 2, and GTP(Na\textsuperscript{+})\textsubscript{3} 0.3 (pH 7.3). For microfluorometry, 0.25 mM fura-2 (pentapotassium salt, Molecular Probes) was added to the pipette solution. For recording of the reversal potential, K-glucuronate was substituted with CsCl. Whole cell recordings were performed with Axopatch 1D or Multiclamp 700A amplifiers (Axon Instruments). In voltage clamp, V\textsubscript{hold} was −60 mV. Hyperpolarizing holding current was used in current-clamp recordings (cf. Griffin and Boultant 1995). Data were acquired using Clampex/AxonScope software (v.9, Axon Instruments). Continuous measurements (34–50 Hz) of cell membrane resistance (R\textsubscript{m}) and capacitance (C\textsubscript{m}), as well as electrode access resistance (R\textsubscript{e}; ≤20 MΩ), were made in voltage clamp using 5-mV command pulses (20–30 ms) and the on-line “membrane test function” of Clampex 9. All data were analyzed with Origin (v.6, OriginLab). In both voltage- and current-clamp recordings the grounding electrode was connected to the recording bath through a bridge made of either agar or filter paper and thus was not directly exposed to temperature changes.

**Microfluorometry**

Neurons were filled with a Ca\textsuperscript{2+}-sensitive ratiometric dye, fura-2, by diffusion from the patch pipette. UV excitation was provided by a 75-W xenon lamp. Excitation light was filtered alternately at 340 and 380 nm. Emitted light passed a barrier filter (510 nm) and was detected by a CCD camera (Photonic Science). Images were acquired at 6- or 12-s intervals using IonVision software (ImproVision). The time course of fluorescence changes, corresponding to changes in [Ca\textsuperscript{2+}], was calculated for the cell soma. Values were corrected for background fluorescence measured from a region >100 μm away from the soma. Calcium levels were expressed as the ratio, R = (F340soma – F380soma)/(F340soma – F380bg), where F340 and F380 are the fluorescence emitted at the excitation wavelengths 340 and 380 nm, respectively, for the soma and background (bg) (Gryniewicz et al. 1985; Tozzi et al. 2003). It has previously been established that, in contrast to single wavelength dyes, the ratiometric fura-2 indicator shows only a very slight change in 340/380 nm fluorescence ratio arising from temperature (Oliver et al. 2000).

**Control of temperature**

Temperature in the recording chamber was changed by warming or cooling ACSF near the inflow port of the chamber. The temperature was monitored with a miniature probe immersed near the slice (BAT-12 thermometer with IT-18 probe, Physitsem Instruments). Baseline temperature was 34°C. Cooling stimuli were applied by reducing ACSF temperature in the recording bath by 2, 5, or 10°C transiently (1–2 min), or using more sustained temperature changes (10–15 min). Warming stimuli were delivered by increasing ACSF temperature by 2 or 5°C for approximately 2 or 10 min.

**Drug application**

Drugs were applied by switching the standard ACSF to one containing a known concentration of the drug(s). Full exchange of the solution in the recording chamber occurred over about 1 min. 1-Sulpiride was obtained from Ravizza; N-(2,6-dimethylphenyl carbamoylmethyl)-triethylammonium bromide (QX-314) and tetrodotoxin (TTX) from Alomone Labs; 2-aminoethoxydiphenylborane (2-APB), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), [d-(-)]-2-amino-5-phosphonopentanoic acid (D-AP5), (S)-α-methyl-4-carboxyphenylglycine ([S]-MCPG), and 4-ethyleneamphetamine-1,2-dimethyl-6-methylaminopyrimidinium chloride (ZD 7288) from Tocris; and dopamine, BaCl\textsubscript{2}, CdCl\textsubscript{2}, ruthenium red, scopalamine, tetraethylammonium bromide (TEA) chloride, tolbutamide, and 4-α-phorbol 12,13-didecanoate (4αPDD) from Sigma.

**RT-PCR analysis**

 Messenger RNA content was determined in tissue punches taken from the SNc region, using a technique similar to one described previously (Comer et al. 1997). In brief, punches were taken from 200- to 250-μm-thick slices with a cut hypodermic needle. Total RNA was extracted using TRIZOL (Gibco BRL) and reverse-transcription (RT) performed using SuperScript II (Life Technologies). Reactions were performed in a 20-μl volume containing 0.4 U AmpliTag Gold DNA Polymerase (Applied Biosystems) and specific primers, either for the “positive” control tyrosine hydroxylase (TH) or for TRPV3/4. A single round of PCR (35 cycles) was performed after heat activation of the enzyme (90°C, 10 min). The primer sequences and conditions for the amplification of TH mRNA were described previously (Comer et al. 1998). TRPV3: sense primers 5‘CGA CCC GGT CCT GGA GCT CTG CAA, antisense primers 5‘CCA TTC CGT CCA ACC CTT CTG CTG; TRPV4: sense primers 5‘CGT CCA AAC Ctg Cgt ATG AAG Ttc, antisense primers 5‘CCT CCA TCT GTT GCT ACG. Each cycle involved denaturation at 94°C for 45 s, annealing at 58°C (TRPV3) or 55°C (TRPV4) for 30 s, and elongation at 72°C for 90 s (2 mM MgCl\textsubscript{2}). Negative controls contained either no starting RNA or no RT enzyme. Positive controls for TRPV3 and TRPV4 were obtained by amplifying RNA extracted from dorsal root ganglia. PCR products were run on a 2% agarose gel stained with ethidium bromide. Amplified PCR products were purified using a QIAquick
PCR purification kit (Qiagen) for DNA sequencing performed with an ABI prism TM 377 sequencer.

Statistical analysis

Data are presented as means ± SE. Statistical difference was determined by standard or paired Student’s t-test, or by ANOVA, with significance levels ≤0.05.

RESULTS

Effects of temperature on dopaminergic neuron excitability

In extracellular recordings, all tested SNc neurons (n = 14) met the following criteria (Diana and Tepper 2002): 1) slow and regular firing; 2) biphasic or triphasic spike waveform with an inflection on the rising phase; 3) spike duration ≥1.5 ms; and 4) inhibition of firing after exposure to 30 μM dopamine (Fig. 1A). Changing the temperature from 34 to 39°C (for 10 min) increased the firing frequency from 2.51 ± 0.22 to 1.12 ± 0.36 Hz (Fig. 1C1), whereas reduction of temperature from 34 to 29°C slowed firing by 1.66 ± 0.44 Hz (Fig. 1D1). During both warming and cooling, the initial change in firing was followed by partial adaptation, and the responses were reversible on returning the temperature to baseline (Fig. 1, C2 and D2). The temperature coefficient (Q10) of the initial changes in firing rate was calculated using an Arrhenius plot. The Q10 was 2.7 and 7.7 for warming and cooling, respectively (Fig. 1B).

In whole cell patch-clamp recordings, SNc neurons fulfilled the following two criteria in voltage clamp (e.g., Guatteo et al. 1999; Lin et al. 2003): 1) a time- and voltage-dependent inward current (Ih) evoked by hyperpolarizing voltage pulses (from −60 to −120 mV in 20-mV increments); and 2) an outward current following bath application of 30 μM dopamine (Fig. 2, A1 and A3). Neurons were considered “Ih positive” if the inward current increased by ≥200 pA over 600 ms with a command from −60 to −100 mV (mean, 740 pA; range, 200–1,290 pA; n = 21). In current clamp, depolarizing pulses caused a regular firing with a frequency generally not exceeding 10 Hz, whereas hyperpolarizing current pulses evoked a typical “sag” potential as a result of activation of Ih current (Fig. 2A2). In whole cell voltage-clamp mode, fast temperature stimuli above or below 34°C (2 or 5° warming; 2, 5, or 10° cooling; ~2 min) evoked reversible changes of the holding current (Ihold), with warming inducing an inward and cooling an outward current (Fig. 2B, top). The amplitude of the current was dependent on the magnitude of the temperature stimulus. The mean Q10 value for the whole cell current measured during temperature ramps from 24 to 39°C in 16 neurons was 2.75 (Fig. 3D). No temperature threshold was observed.

Repeated stimuli (5°C warming or cooling; n = 7 and 8, respectively) did not change the magnitude of subsequent responses (five stimuli within 10 min; P > 0.05; data not shown). Longer temperature stimuli (10–15 min), similar to those used in extracellular recording experiments, were also tested. These evoked larger currents: for example, a 63.9 ± 13.0 pA (n = 8) peak outward current with cooling by 5° for 10 min, compared with 37.2 ± 7.7 pA (n = 7) with 5° cooling for 2 min (Fig. 2, B and C). This difference indicates slow kinetics of temperature-induced currents. Temperature stimuli

![FIG. 1. Temperature sensitivity of substantia nigra pars compacta (SNc) neurons studied with extracellular recordings. A: identification of SNc neurons during extracellular recording (A1, slow and regular firing; A2, long-lasting action potentials; and A3, inhibitory response to dopamine). B: Arrhenius plot of the firing frequency during 5°C warming and cooling (n = 7) based on data presented in C and D. Q10 was 2.7 for warming (34–39°C) and 7.7 for cooling (34–29°C). C: increase in firing frequency of SNc neurons with 5°C warming. Single-unit recording (C2) and group data (C1) showing the control firing frequency and peak response. D: decrease in firing frequency in response to 5°C cooling from a single neuron (D2) and group data (D1). **P < 0.01.](http://jn.physiology.org/)

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also evoked reversible changes in input resistance ($R_{in}$), with warming decreasing and cooling increasing $R_{in}$ (Fig. 2 middle). In addition, small changes in cell membrane capacitance were observed ($C_{m}$; Fig. 2 bottom), suggesting temperature-induced changes in cell volume.

Because increasing temperature often caused a deterioration in whole cell recordings, most of the subsequent tests were conducted with cooling stimuli, using 10° temperature drops (from 34 to 24°C), which evoked large and reproducible responses.

**Properties of cooling-induced outward current**

Transient (=2 min) lowering of temperature by 10° evoked an outward current in voltage clamp (45 ± 3.4 pA; $n = 23$; Fig. 3A), or cell membrane hyperpolarization in current clamp (from $-48.4 \pm 2.6$ to $-57.9 \pm 3.9$ mV; $n = 10$, $P < 0.001$; Fig. 3B). The outward current was insensitive to K$^+$ channel blockers (tolbutamide 100 μM, $P = 0.87$; BaCl$_2$ 300 μM, $P = 0.12$; both $n = 4$, paired $t$-test) and blockers of voltage-gated Na$^+$ (TTX 1 μM, $P = 0.29$; QX-314 300 μM, $P = 0.06$; both $n = 4$) and Ca$^{2+}$ (CdCl$_2$ 50 μM, $P = 0.79$; $n = 4$) channels.

**FIG. 2. Temperature-induced changes in membrane current, membrane resistance, and membrane capacitance in whole cell recordings. A:** identification of SNc neurons during whole cell patch-clamp recording. In voltage clamp (A1), negative voltage steps (from $V_{hold} = -60$ to $-120$ mV, 20-mV increments) elicited $I_h$ current. In current clamp, a depolarizing current step evoked regular firing, whereas the 2 hyperpolarizing steps produced a “sag” potential (A2). In voltage clamp, bath application of dopamine (DA) evoked an outward current (A3). **B:** changes in holding current ($I_{hold}$), membrane resistance ($R_{in}$), and cell capacitance ($C_{m}$) in response to transient (=2 min) temperature stimuli above and below 34°C. **C:** changes in $I_{hold}$ in response to sustained (10–15 min) temperature changes.

**FIG. 3. Temperature-induced changes in membrane current, membrane potential, and intracellular Ca$^{2+}$ level ([Ca$^{2+}$]$_i$) measured from SNc neurons in whole cell patch-clamp recordings. A1: group data ($n = 23$) and individual recording showing the outward current ($V_{hold} = -60$ mV) induced by 10° cooling. B1: group data ($n = 10$) and individual recording of the membrane potential during 10° cooling. A2 and B2: group data ($n = 23$ and $n = 5$, respectively) and individual recordings of changes in [Ca$^{2+}$]$_i$ measured with the fura-2 technique as 340/380 ratios concurrent with the voltage- and current-clamp recordings. C1: whole cell voltage-clamp recording showing inward current in response to 5°C warming. C2: increase in [Ca$^{2+}$]$_i$ recorded concurrently. Pooled data show inward current ($n = 5$) and 340/380 ratios ($n = 5$) at 34 and 39°C. D1: averaged changes in holding current during whole cell voltage-clamp recordings in response to warming from 24 to 39°C ($n = 16$). D2: data extracted from D1 during the warming period (inward current, about 1 min of data) with holding current plotted against temperature. $Q_{10}$ was calculated from the exponential growth curve fitted to the data. *$P < 0.05$; **$P < 0.01$.**
It was also insensitive to the $I_h$ current blocker ZD 7288 (10 μM, $P = 0.7$; $n = 4$), to ionotropic and metabotropic glutamate receptor antagonists (CNQX 10 μM, d-AP5 50 μM, and (S)-MCPG 500 μM; all $n = 5$; $P = 0.31$), and to the dopamine D2 antagonist, sulpiride (5 μM; $n = 3$; $P = 0.42$, not shown). Finally, the outward current was also observed when recordings were made with a KCl-based pipette solution.

The reversal potential of the current induced by 10°C cooling was measured using Cs+–based patch pipettes and applying the temperature stimulus at different holding potentials. The current reversed polarity at $-4.8 \pm 4.6$ mV ($n = 4$, Fig. 4, B1 and C). The reversal potential was also calculated by applying voltage steps from $-100$ to $-10$ mV (10-mV increments from a holding potential of $-60$ mV) at 34 and at 24°C. Under these conditions, the cooling-induced current reversed at $-17.3 \pm 6.4$ mV ($n = 3$, Fig. 4B2). All current reversal experiments were conducted in the presence of 1 μM TTX, 50 μM ZD 7288, 100 μM CdCl$_2$, and 20 mM TEA chloride.

To establish whether the cooling-induced current could also be evoked in cells in another part of the basal ganglia, whole cell recordings were made in parasagittal sections from both SNc neurons ($n = 7$) and STN neurons ($n = 7$). STN, identified by anatomical landmarks, contained neurons that were smaller in size, showed no clear $I_h$ current in response to hyperpolarizing commands and in current clamp responded to depolarizing pulses with fast (>10 Hz) regular firing (Shen and Johnson 2000). Changing the temperature from 34 to 24°C (10 min) induced outward currents in both type of cell. However, the current was significantly smaller in STN neurons, both in absolute amplitude and when normalized for cell capacitance.

At 10 min, the capacitance-corrected current was $0.228 \pm 0.030$ pA/pF in STN and $0.325 \pm 0.029$ pA/pF in SNc neurons ($P < 0.05$; not illustrated).

**Temperature-induced changes of $[Ca^{2+}]_i$**

Because measurements of the reversal potential of the cooling-induced current suggested the involvement of nonselective cationic channels, and because the temperature-sensitive TRPV3 and TRPV4 channels thought to be involved in these responses (see following text and the DISCUSSION) are Ca$^{2+}$ permeable (TRPV3: $P_{Ca}/P_{Na} \approx 10$, Clapham 2003; Xu et al. 2002; TRPV4: $P_{Ca}/P_{Na} \approx 6$, Vriens et al. 2004; Watanabe et al. 2002b), we performed microfluorometry using the ratiometric dye fura-2 combined with whole cell patch-clamp recording to assess temperature-induced changes in $[Ca^{2+}]_i$. The outward current evoked by cooling from 34 to 24°C, or the corresponding cell membrane hyperpolarization, was accompanied by a decrease in $[Ca^{2+}]_i$, as indicated by a decrease in the 340/380 nm fluorescence ratio (Fig. 3, A2 and B2). The decrease in calcium signal observed in current clamp (0.19 ± 0.05, $n = 5$) was greater than that in voltage clamp (0.09 ± 0.02, $n = 23$, $P < 0.01$). On the other hand, the inward current evoked by 5°C...
warming (74.0 ± 7.8 pA, n = 5; Fig. 3C1) was associated with an increase in [Ca$^{2+}$]$_i$ (0.06 ± 0.01, n = 5; Fig. 3C2).

The role of extracellular Na$^+$ and Ca$^{2+}$ in cooling-evoked responses

The contribution of Na$^+$ to cooling-induced currents was assessed by reducing [Na$^+$] in the standard ACSF (see Methods). After bath perfusion with ACSF containing low Na$^+$ (7–15 min), which by itself produced an outward current, the outward current induced by 10°C cooling was strongly attenuated (P < 0.01, n = 10; Fig. 5, A1 and B1), indicating that the cooling-induced current is mainly sodium dependent. Under the same conditions the decrease in [Ca$^{2+}$]$_i$ was also significantly diminished (P < 0.05, n = 8, Fig. 5, A2 and B2). However, this does not argue against the independence of changes in the [Ca$^{2+}$]$_i$ from changes in membrane current because [Na$^+$]$_o$ reduction also has other effects on Ca$^{2+}$ homeostasis, as is evident from the change in baseline [Ca$^{2+}$]$_i$ (e.g., inhibition or reversal of the Na$^+$/Ca$^{2+}$ exchanger).

In a separate set of experiments, the contribution of [Ca$^{2+}$]$_o$ to the cooling-induced current and [Ca$^{2+}$]$_i$ was assessed by removing calcium from the ACSF. Decrease in [Ca$^{2+}$]$_i$ was monitored by measuring [Ca$^{2+}$]$_i$ during activation of voltage-gated Ca$^{2+}$ channels with depolarizing steps from −60 to −10 mV (30 s, applied every 2 min), and 10°C cooling stimuli were applied when the test response disappeared (±10 min after perfusion with this “zero” Ca$^{2+}$ solution; not illustrated). In the absence of extracellular Ca$^{2+}$ the cooling-induced outward current was not changed (P = 0.86, n = 11; Fig. 5, C and D). However, the calcium response was reduced (P < 0.05, n = 11). This demonstrates the independence of changes in the whole cell current and [Ca$^{2+}$]$_i$, and suggests that the reduced Ca$^{2+}$ response during cooling results from a decreased calcium entry from the extracellular space.

The effects of 2-APB and ruthenium red on temperature-induced responses

The effects of a nonselective TRPV3 channel modulator, 2-APB, and of a TRPV3/4 channel blocker, ruthenium red (Clapham et al. 2002; Tozzi et al. 2003; Watanabe et al. 2002b; Xue et al. 1992), were tested on the temperature-evoked responses. Both the 10°C cooling-induced outward current and decrease in [Ca$^{2+}$]$_i$ were strongly inhibited by 2-APB (200 μM; P < 0.01 and P < 0.05, respectively; Fig. 6A). Application of 2-APB consistently increased baseline [Ca$^{2+}$]$_i$, whereas the effects of the drug on $I_{\text{hold}}$ were variable. The warming-induced inward current was also significantly inhibited in the presence of 2-APB (to 20.9 ± 7.6% of control; n = 3, not illustrated). Warming-evoked changes in [Ca$^{2+}$]$_i$ could not be reliably measured because application of 2-APB itself evoked an increase in calcium signal, possibly arising from an agonist effect on TRPV3 channels (see following text).

Ruthenium red (100 μM) had a small but significant effect in reducing the cooling-induced outward current (P < 0.05, n = 5).

![FIG. 5. Dependency of cooling-induced outward current on extracellular Na$^+$ and Ca$^{2+}$](http://jn.physiology.org/)

*P < 0.05.
n = 5; Fig. 6B). The inward current induced by 5°C warming was not significantly reduced by this drug (n = 6; not illustrated).

**TRPV3 and TRPV4 mRNA expression in the SNc**

Of the six members of the TRP channel family that are temperature gated (TRPV1-4, TRPM8, TRPA1; for review see Patapoutian et al. 2003), TRPV3 and TRPV4 are expressed in the CNS and are sensitive to temperature changes within the range 22–40°C (Watanabe et al. 2002b; Xu et al. 2002), similar to that tested in the present study. To determine whether mRNA coding for these channels is present in the SNc, we performed RT-PCR with tissue punches taken from this midbrain region in slices obtained from two different rats (Fig. 7A).

All samples showed expression of TH mRNA (positive control) as well as of TRPV3 and TRPV4 (Fig. 7B). Controls that did not contain RNA template or RT enzymes did not show expression of specific products. Sequencing revealed that the amplified TRPV3 segment had 97 and 90% homology with the mouse (accession number NM145099) and human (accession number NM145068) TRPV3 cDNA, respectively. The amplified TRPV4 sequence exactly matched that of the rat TRPV4 cDNA (accession number AF263521).

**Pharmacological activation of TRPV3 and TRPV4 channels**

TRPV3 and TRPV4 channels expressed in cell lines have been shown to be activated not only by an increase in temper-
ature but also by the specific ligands 2-APB (Chung et al. 2004; Hu et al. 2004) and 4αPDD (Nilius et al. 2004; Watanabe et al. 2002a), respectively. The effects of these agonists on the responses of dopaminergic SNc neurons were tested while the temperature was maintained at 34°C. In current-clamp recordings, 4αPDD (10 μM) increased firing frequency from 0.95 ± 0.13 to 1.43 ± 0.24 Hz in seven of 14 neurons tested (Fig. 8, A and B; \( P < 0.05 \), \( n = 7 \), paired \( t \)-test), but did not affect firing of the remaining seven cells. \([\text{Ca}^{2+}]_i\) was not measured in these experiments. The effect of 4αPDD on \( R_{\text{in}} \) was tested in seven additional neurons by injecting 30-pA hyperpolarizing current pulses (200 ms, every 10 s), while the baseline potential was kept at approximately −70 mV using a continuous hyperpolarizing current. Although the drug caused a small depolarization (2.0 ± 0.3 mV), no significant changes of \( R_{\text{in}} \) were detected (from 137 ± 13 to 145 ± 12 MΩ; \( P = 0.36 \), paired \( t \)-test; not shown), suggesting that the drug-activated conductance is located in distal dendrites.

The effects of 2-APB (200 μM) were tested in voltage clamp. Application of the drug consistently increased intracellular calcium signal from 0.87 ± 0.04 to 1.08 ± 0.06 (\( P < 0.01 \), paired \( t \)-test, \( n = 14 \); not illustrated). However, 2-APB had no significant effects on membrane current (\( P = 0.69 \), paired \( t \)-test).

**DISCUSSION**

We investigated the temperature sensitivity of single neurons of the substantia nigra pars compacta in rat brain slices. Here we show that both the excitability and intracellular \( \text{Ca}^{2+} \) homeostasis of principal dopaminergic neurons in this region are significantly affected by changes in temperature above and below 34°C, and present the first evidence that these responses are, at least in part, mediated by channels that have some features of temperature-sensitive TRPV3 and TRPV4 channels.

Various environmental stimuli or certain behaviors have been reported to change the temperature of the whole brain or specific nuclei, including the regions containing mesencephalic dopaminergic cells of the SNc and VTA, and their projections. For example, temperature increases ≤3°C were observed in the nucleus accumbens (within the ventral striatum) during goal-directed behavior (Kiyatkin and Mitchum 2003) and in the VTA during cocaine administration (Kiyatkin and Brown 2003). The VTA region, which shares many properties with the SNc, is believed to play a crucial role in a wide range of rewarding behaviors (Holstege et al. 2003). Interestingly, activity-related brain hyperthermia occurs before and to a greater extent than the change observed in the temperature of blood supplying the brain (Kiyatkin et al. 2002), suggesting that local temperature changes result from increased cellular activity. In addition, it has been argued that neuronal activity-related changes in temperature should be regarded as a factor controlling neuronal function, rather than as a by-product of altered metabolic activity (Kiyatkin 2004; Kiyatkin and Mitchum 2003). These findings warrant the study of the mechanisms underlying the temperature sensitivity of all neurons, not only of the hypothalamic neurons that are involved in temperature homeostasis.

Changes of temperature evoked consistent changes in firing frequency of SNc neurons. A 5° temperature increase above 34°C augmented their firing frequency with \( Q_{10} = 2.7 \), whereas a drop below 34°C decreased their firing with \( Q_{10} = 7.7 \). The lower temperature coefficient during warming may be explained by the fact that SNc neurons are unable to fire at frequencies higher than about 10 Hz because of depolarization block (Grace et al. 1997). Similar changes of firing were observed in extracellular and whole cell current-clamp recordings, indicating that temperature-induced responses were not dependent on intracellular dialysis associated with patch-clamp recording. However, temperature is likely to also have nonspecific effects on channels involved in the generation of action potentials. Thus more specific effects were investigated using voltage-clamp recordings where membrane currents were monitored directly.

In whole cell voltage-clamp recordings with a 34°C baseline temperature, cooling of SNc neurons evoked an outward current and warming stimuli an inward current. Because the reversal potential of the temperature-induced currents was between −5 and −17 mV (depending on the measuring technique), and because \( R_{\text{in}} \) increased during cooling and decreased during warming, our data are consistent with the hypothesis that cooling leads to a closure of a nonselective cationic conductance that is constitutively active at 34°C, whereas warming leads to further activation of this conductance. Thus the observed “inward” and “outward” currents are likely to be a result, respectively, of further activation or closure of the

![FIG. 8. Effect of pharmacological activation of TRPV4 channels in SNc neurons by 4α-phorbol 12,13-didecanoate (4αPDD). A: increase in firing frequency evoked by bath application of 4αPDD (10 μM). A1: neuron firing. A2: histogram showing firing frequency (bin size 15 s) of the neuron. B: group data (\( n = 7 \)) showing mean firing frequency during control, 4αPDD application, and washout. \(* P < 0.01\).](http://jn.physiology.org/)

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channels mediating the cationic current. The temperature sensitivity of the whole cell current ($Q_{10} = 2.75$) was found to be higher than the membrane conductance of relatively temperature-insensitive channels ($Q_{10} = 1.5$; Xu et al. 2002), although no apparent threshold was identified. Temperature-induced changes in $R_{in}$ have been reported during recordings from neurons located in other parts of the brain (e.g., CA1 hippocampal, hypothalamic, and neocortical neurons; Griffin and Boulant 1995; Lee et al. 2005; Thompson et al. 1985; Volgushev et al. 2000). These data may indicate that temperature-sensitive conductances are also present outside the SNc.

Changes in temperature affected the intrinsic properties of dopaminergic neurons, rather than their synaptic inputs. Indeed, cooling-induced outward currents were unchanged in the presence of TTX or in the absence of extracellular Ca$^{2+}$, suggesting that action potentials and Ca$^{2+}$-dependent synaptic release were not involved. Moreover, the responses were not prevented by antagonists of ionotropic and metabotropic glutamate and dopamine D$_2$ receptors. Further, GABAergic transmission was not involved, as shown by the reversal potential of the cooling-induced responses that differed from the equilibrium potential that Cl$^-$ ions and GABA$_A$ receptor–mediated responses, and the equilibrium potential for Cl$^-$ ($-92$ mV) for GABAergic acid type A (GABA$_A$) receptor–mediated currents. The argument that Cl$^-$ ions and GABA$_A$ transmission was not involved is supported by the finding that cooling-induced currents did not reverse in polarity during recordings made with KCl-filled pipettes. Cooling-induced outward current was not significantly blocked by antagonists of voltage-gated K$^+$, Na$^+$, or Ca$^{2+}$ channels, by the $I_h$ current blocker ZD 7288, or by tolbutamide, a blocker of an ATP-dependent K$^+$ channel. These findings support the idea that a temperature-sensitive cell membrane conductance(s) unrelated to these channels was involved.

Previous studies have demonstrated that various types of neurons respond to changes in temperature in different ways. For example, some molluscan neurons show responses similar to those observed in the present study; warming stimuli cause depolarization and an increase in firing rate, and cooling stimuli cause hyperpolarization and a decrease in firing (Carpenter 1981). However, CA1 pyramidal cells in the hippocampus and neocortical neurons respond to cooling with depolarization and hyperpolarize during warming (Fuji et al. 2002; Volgushev et al. 2000), which are effects opposite to the responses observed in SNc neurons. In the hypothalamus and medial thalamus, some neurons respond with depolarization or increase in firing frequency to temperature increases, others are activated during cooling, and still others are temperature insensitive (Hori et al. 1999; Kobayashi and Takahashi 1993; Travis et al. 1995; but see Griffin and Boulant 1995). Interestingly, some trigeminal ganglion neurons respond to cooling with an increase rather than a decrease of [Ca$^{2+}$]; (Thut et al. 2003). In our experiments, cooling-induced outward current (both absolute and relative to membrane capacitance) was significantly lower in STN than in SNc cells, again showing that under identical experimental conditions different types of neurons respond differently to changes in temperature. Although electrophysiological and calcium responses may depend on a number of factors (e.g., effects of temperature on the activity of Na$^+$/K$^+$ ATPase, intracellular and extracellular pH changes, etc.), they may also arise from differences in the level of expression and activation (or inactivation) of temperature-gated TRP channels.

Six members of the TRP channel family are highly temperature sensitive: TRPV1–TRPV4, TRPM8, and TRPA1 (Bennett et al. 2003; Patapoutian et al. 2003; Peier et al. 2002a; Story et al. 2003). TRPA1 and TRPM8 channels were probably not involved in the responses of SNc neurons because these cold-sensitive channels are normally activated at temperatures well below the range used in our experiments ($<22^\circ$C). Moreover, icilin, a known TRPA1 and TRPM8 receptor agonist (e.g., Behrendt et al. 2004), does not affect cooling-induced responses in SNc neurons (E Giaid et al., unpublished observation). TRPV1 and TRPV2 channels are also unlikely to be involved because both are heat sensitive, with activation thresholds higher than the temperature used in our experiments ($>43$ and $52^\circ$C, respectively; Caterina et al. 1997, 1999). In contrast, heterologously expressed TRPV3 and TRPV4 channels have been reported to have some properties similar to those of the conductance involved in the temperature sensitivity of SNc neurons. TRPV3 channels mediate cell depolarization and an increase in [Ca$^{2+}$], in response to temperature increases in the physiological range (activation threshold $22$–$33^\circ$C; Peier et al. 2002b; Xu et al. 2002) and are widely expressed in the CNS (Smith et al. 2002; Xu et al. 2002). TRPV4 channels are also activated at low temperatures in the physiological range ($>25^\circ$C), mediate calcium accumulation in cell lines and in native aortic endothelial cells (Watanabe et al. 2002b), and are expressed in the hypothalamus and other brain regions (Guler et al. 2002; Liedtke et al. 2000; Wissenbach et al. 2000). In light of these results as well as our own, we hypothesize that TRPV3 and/or TRPV4 channels confer temperature sensitivity on SNc neurons and that temperature-induced currents in these neurons are evoked by modulation of the activity of these channels, which are constitutively active in slices kept at $34^\circ$C.

Similarly to the results obtained by Xu et al. (2002) in CHO cells expressing TRPV3, and by Guler et al. (2002) and Watanabe et al. (2002b) in HEK293 cells expressing TRPV4, our data indicate that the temperature-sensitive channels of SNc neurons are permeable to Ca$^{2+}$ and Na$^+$. Calcium permeability was suggested by a rise in [Ca$^{2+}$] during the temperature increase from 34 to $39^\circ$C and a fall during cooling from 34 to $24^\circ$C. On the other hand, cooling from 34 to $24^\circ$C led to a decrease of [Ca$^{2+}$]. Changes in [Ca$^{2+}$] are difficult to interpret because they reflect a balance among the influx, release from intracellular stores, extrusion, and reuptake of this ion (Rizzuto and Pozzan 2003; Shuttleworth and Thompson 1991). However, the changes were compatible with the hypothesis that warming increases and cooling decreases a tonic Ca$^{2+}$ influx. In current-clamp recordings, [Ca$^{2+}$], decreased to a greater extent than in voltage-clamp recordings, presumably as the result of reduction of an additional Ca$^{2+}$ inflow through voltage-dependent Ca$^{2+}$ channels that are active when the cell is spontaneously firing and inactive when firing stops as a result of cooling. However, Ca$^{2+}$ ions were not the main carriers of cooling-induced outward current, as demonstrated by experiments that showed an insignificant reduction of this current in Ca$^{2+}$-free ACSF. The major carrier of the temperature-sensitive current in SNc neurons was Na$^+$ ions. Indeed, lowering [Na$^+$] abolished the cooling-induced outward cur-
rent almost completely. A decrease in resting Na\textsuperscript{+} permeability during cooling has been also reported in other types of cells such as molluscan neurons (Marmor 1971). Like TRPV3 channels expressed in cell lines (Xu et al. 2002), the temperature-sensitive conductance in SNc neurons appears to be permeable to Cs\textsuperscript{+}, as indicated by a cooling-induced inward current in Cs\textsuperscript{+}-filled neurons at $V_{\text{hold}} = +30$ mV, presumably the result of inhibition of Cs\textsuperscript{+} outflow. Cesium permeability of heterologously expressed TRPV4 channels has not yet been reported.

Other findings are compatible with the notion that TRP channels are involved in the responses observed in this study. We found that the nonselective TRP channel modulator 2-APB (Clapham et al. 2002; Tozzi et al. 2003) almost completely abolished cooling-evoked outward current and [Ca\textsuperscript{2+}], drop, as well as warming-induced inward current. Ruthenium red, a nonselective TRPV channel blocker (Clapham et al. 2002; Guler et al. 2002; Watanabe et al. 2002b; Xu et al. 2002) was less effective and evoked only a small reduction in the cooling-induced outward current. The reasons that the cooling- and warming-induced responses were reduced by 2-APB are unclear, but the effects may be a consequence of the complex actions of this drug on TRP channels (Chung et al. 2004; Clapham et al. 2002; Hu et al. 2004; Tozzi et al. 2003), as well as on IP3 receptors (Bootman et al. 2002). It is also unclear why application of 2-APB on its own (i.e., without application of cooling or warming stimuli) produced an increase of [Ca\textsuperscript{2+}], in all neurons and variable changes of membrane current. On the other hand, the effect of the selective TRPV4 channel agonist 4aPDD resembled the warming-induced response, increasing the firing frequency of 50% of tested SNc neurons. Recent data from Nilius’ laboratory (Vriens et al. 2004; Watanabe et al. 2002b) demonstrated that 4aPDD and temperature control the opening state of TRPV4 channels by different mechanisms. Therefore our results obtained using the drug and with temperature changes may not be directly comparable.

In addition, our RT-PCR analysis revealed the expression of TRPV3 and TRPV4 mRNAs in the SNc region. Both were detected together with mRNA for tyrosine hydroxylase, confirming that RNA was extracted from the ventral midbrain area containing dopaminergic neurons. Previous studies have identified expression, at the protein or mRNA level, of several other channels expressed in cell lines (Guler et al. 2002; Smith et al. 2002; Watanabe et al. 2002b). In addition, the Q10 value of [Ca\textsuperscript{2+}], and was decreased by cooling, leading to an outward current or inhibition of firing, an increase of $R_{\text{m}}$, and a decrease of [Ca\textsuperscript{2+}]. Further experiments are necessary to determine why some of the properties of this temperature-sensitive native conductance differ from those of TRPV3/4 channels expressed in cell lines (Guler et al. 2002; Smith et al. 2002; Watanabe et al. 2002b; Xu et al. 2002). These differences include a high contribution of Na\textsuperscript{+} relative to Ca\textsuperscript{2+} ions, low sensitivity to blockade by ruthenium red, and the absence of current sensitization with successive temperature stimuli in SNc neurons. Current sensitization in TRPV3 channels expressed in cell lines has been reported by Xu et al. (2002), but gradual desensitization to repeated stimuli was seen in heterologous TRPV4 channels (Watanabe et al. 2002b). In addition, the Q10 value that we established for the temperature-induced whole cell current (2.75) was considerably lower than that reported for heterologously expressed TRPV3 channels (Xu et al. 2002). It is likely that the properties of native channels differ from those of channels expressed in cell lines as homomers as the result of heteromerization of TRPV3 with TRPV4 or with other members of the TRP channel family, as has been described for TRPV3 coexpressed with TRPV1 (Smith et al. 2002). Additionally, or alternatively, channel properties may change as a result of coexpression of channel-associated proteins, such as the adaptor protein Homer-3, which regulates TRPV3 channels (Kang et al. 2004). It also remains to be determined whether overactivity of TRPV3/4 channels in SNc neurons is involved in the pathophysiological processes leading to Ca\textsuperscript{2+} overload and cell death and whether inactivation of these channels could have neuroprotective effects.

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