Expression and functional properties of TRPM2 channels in dopaminergic neurons of the substantia nigra of the rat

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Chung KK, Freestone PS, Lipski J. Expression and functional properties of TRPM2 channels in dopaminergic neurons of the substantia nigra of the rat. J Neurophysiol 106: 2865–2875, 2011. First published September 7, 2011; doi:10.1152/jn.00994.2010.—Transient receptor potential melastatin 2 (TRPM2) channels are sensitive to oxidative stress, and their role in the brain, particularly in the substantia nigra pars compacta (SNc), remains unknown. In this study, we assessed the expression and functional properties of TRPM2 channels in rat dopaminergic SNc neurons, using acute brain slices. RT-PCR analysis revealed TRPM2 mRNA expression in the SNc region. Immunohistochemistry demonstrated expression of TRPM2 protein in tyrosine hydroxylase-positive neurons. Channel function was tested with whole cell patch-clamp recordings and calcium (fura-2) imaging. Intracellular application of ADP-ribose (50–400 μM) evoked a dose-dependent, desensitizing inward current and intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) rise. These responses were strongly inhibited by the nonselective TRPM2 channel blockers clotrimazole and flufenamic acid. Exogenous application of H\(_2\)O\(_2\) (1–5 mM) evoked a rise in [Ca\(^{2+}\)]\(_i\), and an outward current mainly due to activation of ATP-sensitive potassium (K\(_{ATP}\)) channels. Inhibition of K\(^+\) conductance with Cs\(^+\) and tetraethylammonium unmasked an inward current. The inward current and/or [Ca\(^{2+}\)]\(_i\) rise were partially blocked by clotrimazole and N-(p-amylcinnamoyl)anthranilic acid (ACA). The H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)]\(_i\) rise was abolished in “zero” extracellular Ca\(^{2+}\) concentration and was enhanced at higher baseline [Ca\(^{2+}\)]\(_i\), consistent with activation of TRPM2 channels in the cell membrane. These results provide evidence for the functional expression of TRPM2 channels in dopaminergic SNc neurons. Given the involvement of oxidative stress in degeneration of SNc neurons in Parkinson’s disease, further studies are needed to determine the pathophysiological role of these channels in the disease process.

Oxidative stress; hydrogen peroxide; ADP-ribose; calcium; whole cell patch-clamp recording

Oxidative stress is believed to be a major factor in the pathogenesis of a number of neurodegenerative disorders including Parkinson’s disease (PD), and it is considered to play a role in both the initiation of the disease process and the secondary neural damage (see, e.g., Chinta and Andersen 2008; Jenner 2007; Reynolds et al. 2007; Zhou et al. 2008). Oxidative stress results from a loss of redox balance within the cell, when the production of reactive oxygen species (ROS) overwhelms the detoxification capability of the cell.

Cell damage resulting from oxidative stress is believed to be caused mainly by the direct toxic reaction of free radicals with DNA, proteins, carbohydrates, and lipids (Davies 2000). In addition, recent studies have identified a number of ion channels belonging to the transient receptor potential (TRP) channel superfamily that are sensitive to oxidative stress and may contribute to ROS-induced cell death (Ramsey et al. 2006). These include TRP melastatin 2 (TRPM2) (Eisfeld and Luckhoff 2007; Jiang et al. 2010; Sumoza-Toledo and Penner 2011), TRPC3/4 (Poteser et al. 2006), TRPP2 (Montalbetti et al. 2008), TRPV1 (Crouzin et al. 2010), and TRPA1 (Andersson et al. 2008) channels. Of particular interest are TRPM2 channels, as they are permeable to calcium and are highly expressed in the brain, including the cerebral cortex, cerebellum, striatum, and hippocampus (see, e.g., Bai and Lipski 2010; Hara et al. 2002). In addition, expression of these channels was detected in the human substantia nigra with Northern blotting (Nagamine et al. 1998; Uemura et al. 2005). Functional expression of native TRPM2 channels has been demonstrated in striatal (Bao et al. 2005; Fonfria et al. 2005; Hill et al. 2006), hippocampal (Olah et al. 2009), and cortical (Kaneko et al. 2006) neurons.

TRPM2 channels contain an enzymatic domain located near the COOH terminus that shares significant homology to NUDT9 (ADP-ribose pyrophosphatase) containing the Nudix sequence motif (Perraud et al. 2001). These channels are strongly activated by H\(_2\)O\(_2\) and ADP-ribose (ADPR) and are modulated by other ROS, reactive nitrogen species, ADPR-related compounds (NAD and cyclic ADPR), TNF-α, arachidonic acid, and temperature changes (Hara et al. 2002; Perraud et al. 2001; Sano et al. 2001; Tagoshi et al. 2006; Toth and Csanyadi 2010; Wehage et al. 2002). TRPM2 protein forms a voltage-independent nonelective cationic channel (P\(_{Ca}/P_{Na} \sim 0.7\); Perraud et al. 2001; Sano et al. 2001; Xia et al. 2008). Intracellular calcium is required for channel activation and can markedly potentiate the effects of H\(_2\)O\(_2\) or ADPR (Csanyadi and Torocsik 2009; Du et al. 2009; McHugh et al. 2003; Starkus et al. 2007).

TRPM2 channels have been demonstrated to mediate cell death in heterologous expression systems and native neuronal cells by increasing susceptibility to ROS (including H\(_2\)O\(_2\)), TNF-α, and amyloid β-peptide (Fonfria et al. 2005; Hara et al. 2002; Ishii et al. 2007; Kaneko et al. 2006; Yang et al. 2006; Zhang et al. 2003, 2006). In contrast, reduction of channel expression or inhibition of its function increased cellular resistance to damage. However, it is unclear whether these channels affect the function of dopaminergic neurons of the substantia nigra pars compacta (SNc) and contribute to the...
damaging effects of oxidative stress observed in PD. Our recent study suggested the involvement of TRPM2 channels in the response of SNc neurons to rotenone, a neurotoxin used to model PD (Freestone et al. 2009). In the present study, we investigated the expression of these channels in SNc neurons at the molecular and functional levels. We hypothesized that TRPM2 channels mediate the specific effects of exogenously applied ADPR and H2O2, leading to changes in neuronal excitability and calcium homeostasis, and potentially to damage of these neurons.

**METHODS**

Brain tissue was obtained from Wistar rats (P15–P28). Live brain slices were used for mRNA extraction, patch-clamp recordings, and Ca2+ imaging, whereas fixed tissue was used for immunohistochemistry. All procedures were approved by the University of Auckland Animal Ethics Committee in accordance with the New Zealand Animal Welfare Act 1999.

**Brain slices.** Rats were anesthetized with 100% CO2 or chloral hydrate (450 mg/kg ip) and decapitated. The brain was quickly removed, and coronal midbrain slices (250 μm) containing the SNc were cut with a vibrotome in artificial cerebral spinal fluid (standard aCSF, in mM: 115 NaCl, 2.5 KCl, 2.4 CaCl2, 1.3 MgCl2, 1.2 NaH2PO4, 24 NaHCO3, 10 glucose; 4–8°C) equilibrated with carbogen (95% O2-5% CO2). After cutting, slices were allowed to recover in a holding chamber containing carbogenated aCSF for at least 40 min at 34°C.

For mRNA extraction, aCSF was substituted with sterile phosphate-buffered saline (PBS, in mM: 137 NaCl, 3 KCl, 10 Na2HPO4, 2 KH2PO4) and slices were processed immediately after cutting. The SNc region was cut from midbrain slices transfused under a dissecting microscope with a scalpel blade or a modified hypodermic needle as described by us previously (Comer et al. 1998).

**Reverse transcription-polymerase chain reaction.** Total RNA was extracted from the dissected tissue with the RNeasy Mini Kit (Qiagen). DNase digestion was performed on-column. Approximately 1 μg of total RNA was extracted per milligram of tissue. First-strand cDNA was synthesized with SuperScript III Reverse Transcriptase and oligo(dT) primers (Invitrogen). Platinum Taq DNA Polymerase (Invitrogen) was used for PCR amplification. Primers for tyrosine hydroxylase (TH; Comer et al. 1998) were used for positive controls. PCR products were amplified by electrophoresis on 1.5% agarose gels. PCR products were analyzed by electrophoresis on 1.5% agarose gels with aCSF, in mM: 135 K-methanesulfonate, 10 HEPES, 0.75 EGTA, 2 MgATP, 0.3 NaGTP, 2 MgCl2, 0.1 CaCl2 or a Cs+ -based [in mM: 118 Cs-methanesulfonate, 10 CsH2PO4, 2 CsCl, 0.1 CaCl2] solution. Neurons were patched after visualization by infrared differential interference contrast (IR-DIC) under a ×40 objective. Voltage-clamp recordings were performed with a Multiclamp 700A amplifier ( Molecular Devices) using a 10-kHz low-pass filter. Pipette and cell capacitance were compensated for each cell. Access resistance was measured routinely, and recordings were discarded if the resistance exceeded 25 MΩ. In all recordings, cells were clamped at −60 mV (after adjusting for junction potential).

**Calcium imaging.** Intracellular free calcium concentration ([Ca2+ ]i) was measured with fura-2 (pentapotassium salt, 0.25 mM; Invitrogen) included in the pipette solution concurrently with whole cell currents recorded in voltage clamp. Except for experiments in which cells were loaded with ADPR, [Ca2+ ]i measurements were delayed at least 10 min after entering the whole cell configuration to allow diffusion of the calcium indicator into the cell. Cells were illuminated with a xenon arc lamp (175 W, Lambda LS; Sutter Instrument) and a filter wheel (Optospec, Cairn Research) that allowed switching of the excitation between 340 ± 15 and 380 ± 5 nm. A band-pass emission filter (540 ± 20 nm) was used. Images were captured with a cooled CCD camera (12-bit, ORCA-ER; Hamamatsu) and 2 × 2 binnning and 200- to 400-ms exposure. A pair of images (340- and 380-nm excitation) was captured every 5 or 10 s. Images were acquired and analyzed with Imaging Workbench (v. 5.2; INDEC BioSystems). Fluorescence ratios were converted to concentrations according to the procedure described by Grynkiewicz et al. (1985).

**Drugs.** ADPR and 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isouquinolone (DPQ) (both from Sigma-Aldrich) were added to the pipette solution. H2O2 (Scharlau) was slowly infused (0.73 ml/h) at high concentration into the inflow port of the recording chamber with a syringe pump (SP100iZ; WPI) to achieve a final concentration of 1–5 mM in the recording chamber. The following drugs were dissolved in aCSF: 2-amino-5-phosphonopentanoic acid (AP5), clotrimazole, flufenamic acid (all from Sigma-Aldrich) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNOX; Tocris). Stock solutions of N-(p-amidinocinnamoyl)anthranilic acid (ACA; Calbiochem), glibenclamide (Tocris), and tolbutamide (Sigma-Aldrich) were prepared in DMSO; the final DMSO concentration in aCSF did not exceed 0.1%.

**Data analysis.** Electrophysiological and calcium imaging data were analyzed with Clampfit (v. 9; Molecular Devices), Excel (v. 11; Microsoft), and Origin (v. 7.5; OriginLab). Statistical tests were performed with Statistica (v. 8; StatSoft). Student’s t-test or split-plot ANOVA followed by planned comparisons was used to determine
statistical significance. Data are presented as means ± SE. Differences were considered statistically significant at $P < 0.05$.

**RESULTS**

**TRPM2 mRNA and protein expression.** To assess the expression of TRPM2 mRNA in the SNc region, RT-PCR was performed on freshly dissected SNc tissue (Fig. 1A). Two primer sets targeting different regions of the rat TRPM2 mRNA sequence were used (see METHODS). Gel electrophoresis detected PCR products of the expected size for each primer set [443 and 358 bp, $n$ (animals) = 2; Fig. 1B]. The amplicons were sequenced and found to be identical to the reported cDNA sequence. In addition, RT-PCR performed with previously described TRPM2 primers (Bai and Lipski 2010) resulted in amplicons of predicted size (not shown). Expression of TH mRNA was tested and confirmed that the collected tissue included dopaminergic neurons.

To examine TRPM2 protein expression in the SNc region, fluorescence immunohistochemistry was performed on midbrain sections. Dopaminergic neurons were immunolabeled with an anti-TH antibody that allowed visualization of both their cell bodies and proximal dendrites. As expected, TH-immunoreactive neurons were found in the SNc, with fewer TH-positive cells scattered in the substantia nigra pars reticulata (SNr; Fig. 2). TRPM2 protein was labeled with either the rabbit anti-rat TRPM2 antibody (Fig. 2) or the rabbit anti-human TRPM2 antibody (data not shown). The pattern of immunostaining was identical between sections incubated with the two antibodies. TRPM2 immunoreactivity was observed in cells throughout midbrain slices, including the SNc and SNr regions. At the cellular level, TRPM2 immunoreactivity was punctate, with staining in the cytoplasm of the soma and proximal dendrites. Double-labeling with anti-TH and anti-TRPM2 antibodies revealed TRPM2 protein expression in dopaminergic neurons (Fig. 2). In addition, TRPM2 immunoreactivity was present in non-TH-immunoreactive cells.

**Identification of dopaminergic SNc neurons.** The following criteria were used to identify dopaminergic SNc neurons during whole cell patch-clamp recording (Freestone et al. 2009; Grace and Bunney 2000; Guatteo et al. 2005):

1) location near the ventral border of the medial lemniscus; 2) large fusiform shaped soma ($>20 \mu m$); 3) in voltage clamp, the presence of $I_h$ in response to hyperpolarizing voltage commands ($-80$ and $-100 \text{ mV}$; $800 \text{ ms}$); 4) in current clamp, the presence of a depolarizing “sag” in response to hyperpolarizing current steps (up to $200 \text{ pA}$; $800 \text{ ms}$); 5) in current clamp, response to depolarizing current steps (up to $100 \text{ pA}$, $800 \text{ ms}$) in the form of a burst of action potentials exhibiting frequency adaptation; and 6) in current clamp, slow and regular firing ($<5 \text{ Hz}$) with zero current. With a $K^+$-based pipette solution, membrane resistance was $97 \pm 6 \text{ M\Omega}$, membrane capacitance was $150 \pm 7 \text{ pF}$, membrane potential was $-57 \pm 2 \text{ mV}$, and resting $[\text{Ca}^{2+}]_i$ was $93 \pm 11 \text{ nM (n = 13)}$. 

**Fig. 1.** Transient receptor potential melastatin 2 (TRPM2) mRNA expression in the substantia nigra pars compacta (SNc) region. A: midbrain slice showing the SNc region (arrow) used for tissue punch and RNA extraction. Calibration bar: 1 mm. B: RT-PCR products obtained with primers targeting tyrosine hydroxylase (TH; 220 bp) and TRPM2 (443 and 358 bp).

**Fig. 2.** TRPM2 immunoreactivity in the SNc region. A: confocal image showing immunoreactivity for TH (green) and TRPM2 (red) at low magnification ($\times10$ objective, NA 0.45, merged image). TH-immunoreactive cell bodies were found in the SNc (orientated diagonally), with some cells scattered in the substantia nigra pars reticulata (top right). B–D: high-magnification confocal images ($\times63$ objective, NA 1.4, oil immersion) showing TH (B) and TRPM2 (C) labeling separately and as a merged image (D). Rabbit anti-rat TRPM2 (NB110-81601; Novus Biologicals) was used. Calibration bars: 200 $\mu m$ (A), 25 $\mu m$ (B–D).
**Response to ADPR.** TRPM2 channels are gated by ADPR, and intracellular application of this compound was previously found to selectively activate these channels (see introduction). To examine functional expression of TRPM2 channels in dopaminergic SNc neurons, the effects of increased cytoplasmic levels of ADPR were tested. Whole cell patch-clamp recordings and fura-2 imaging were employed to monitor whole cell currents and $[\text{Ca}^{2+}]_i$ concurrently. Data acquisition commenced immediately upon entering the whole cell configuration. Recordings with patch pipettes containing 50–400 nM ADPR, or no drug (controls), were performed (Fig. 3, A and B). At 400 nM, ADPR evoked a desensitizing inward current and $[\text{Ca}^{2+}]_i$ rise [607 ± 235 pA, $P = 0.044$; $\Delta 213.4 \pm 82.5$ nM, $P = 0.048$; n(control) = 6, n(ADPR) = 8]. Smaller responses were evoked with 200 nM [228 ± 49 pA, $P < 0.01$; $\Delta 94 \pm 27$ nM, $P < 0.01$; n(control) = 10, n(ADPR) = 10], while no statistically significant effects were detected with 50 nM [62 ± 74 pA, $P = 0.846$; $\Delta 2 \pm 12$ nM, $P = 0.416$; n(control) = 10, n(ADPR) = 9]. The peak amplitudes of the ADPR-evoked current and calcium responses were dose dependent (Fig. 3, A and B). These data are consistent with the activation of TRPM2 channels.

The ADPR-induced changes in whole cell current and $[\text{Ca}^{2+}]_i$ were further examined by using two nonselective TRPM2 channel blockers, clotrimazole and flufenamic acid. Preincubation of slices with clotrimazole (5 μM) reduced both the ADPR (200 μM)-induced inward current (by 85%, $P = 0.037$) and $[\text{Ca}^{2+}]_i$ rise (by 65%, $P = 0.029$, n = 12) (Fig. 3, C and D). Similarly, flufenamic acid (50 μM; n = 12; Fig. 3, C and D) abolished the inward current (reduction by 101%, $P < 0.01$) and significantly reduced the $[\text{Ca}^{2+}]_i$ rise (by 80%, $P = 0.016$).

**Response to exogenous H$_2$O$_2$.** Exogenous H$_2$O$_2$, an agent commonly used for inducing oxidative stress because of its relative stability and membrane permeability, was applied to the slice. H$_2$O$_2$ is known to activate TRPM2 channels (see introduction) and, in SNc neurons, ATP-sensitive potassium (K$_{\text{ATP}}$) channels (Avshalumov et al. 2007). We assessed the effects of H$_2$O$_2$ on SNc neurons before and after blocking K$_{\text{ATP}}$ channels.

Bath application of 5 mM H$_2$O$_2$ induced an outward current (232 ± 36 pA, $P < 0.01$, n = 7; measured 10 min after onset of application; Fig. 4, A and B), consistent with previous studies (Avshalumov et al. 2005; Geracitano et al. 2005), and an increase in $[\text{Ca}^{2+}]_i$ (Δ 28.6 ± 7.9 nM, $P = 0.017$; Fig. 4, C and D). During prolonged exposure (>15 min), the integrity of the cell membrane and/or the seal often deteriorated, as indicated by the development of a sudden inward current (>1 nA) and a large rise in $[\text{Ca}^{2+}]_i$ (not shown). Therefore analysis was restricted to ≤15 min of H$_2$O$_2$ application. To eliminate the K$_{\text{ATP}}$ channel-mediated response, slices were preincubated with tolbutamide (100 μM) or glibenclamide (10 μM). In the presence of these blockers, the H$_2$O$_2$ (5 mM)-evoked current was significantly reduced (69 ± 13 pA, $P = 0.042$, n = 6;
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Effects of TRP channel inhibitors and DPQ on H₂O₂-evoked responses. To assess the contribution of TRPM2 channels to the H₂O₂-evoked responses, two nonselective blockers of TRPM2 channels were used: clotrimazole and ACA. Recordings were performed with the Cs⁺-based pipette solution to eliminate the effects of K<sub>ATP</sub> and other K⁺ channels. Slices were treated with the channel blockers for >10 min before and during H₂O₂ application. In the presence of clotrimazole (5 μM), the H₂O₂-evoked inward current was reduced by 50% and the [Ca<sup>2+</sup>]<sub>i</sub> rise by 64% (P = 0.013 and P < 0.01, n = 4; Fig. 7, A and B). ACA (20 μM) application also resulted in a smaller [Ca<sup>2+</sup>]<sub>i</sub> rise (48% reduction, P = 0.022, n = 6; Fig. 7, A and B), but its effect on the inward current was not statistically significant (P = 0.276). Baseline values of whole cell current and [Ca<sup>2+</sup>]<sub>i</sub> were not changed by clotrimazole or ACA preincubation.

It has been suggested that TRPM2 channel activation by oxidative stress is mediated through an increase in intracellular ADPR concentration (Buelow et al. 2008; Fonfria et al. 2004; Perraud et al. 2005). This process was demonstrated to be dependent on PARP-1 (polyADPR polymerase), an enzyme upstream of ADPR synthesis. To test whether PARP-1 activity is required for the H₂O₂-induced responses, DPQ (50 μM; PARP inhibitor), was added to the pipette solution. Baseline whole cell current was larger (i.e., more negative) in neurons loaded with DPQ. However, the effects of the drug on H₂O₂-

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Fig. 4. Activation of ATP-sensitive potassium (K<sub>ATP</sub>) channels in response to exogenous H₂O₂. A and C: averaged changes in whole cell current and [Ca<sup>2+</sup>]<sub>i</sub>, in response to H₂O₂, with and without pretreatment with K<sub>ATP</sub> channel blockers [control: n = 7; tolbutamide (Tol, 100 μM) or glibenclamide (Gli, 10 μM); n = 6]. B and D: averaged responses measured 10 min after the onset of H₂O₂ application showing a significant reduction of the outward current in the presence of the blockers. **P < 0.01.

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Fig. 5. Effects of blocking potassium conductance on H₂O₂-induced responses. A and C: averaged changes in whole cell current and [Ca<sup>2+</sup>]<sub>i</sub>, in response to H₂O₂, recorded with 1 mM, whereas a significant and reversible change in [Ca<sup>2+</sup>]<sub>i</sub> was observed with 3 mM (Δ 275.9 ± 50.3 nM, P < 0.01). The H₂O₂-induced [Ca<sup>2+</sup>]<sub>i</sub> rise clearly demonstrated a dose dependence (P < 0.01).
TRPM2 channel activation has been shown to be dependent on resting \([Ca^{2+}]_i\), and only a minimal response was elicited when \([Ca^{2+}]_i\) was buffered to zero (Csanady and Torocsik 2009; Starkus et al. 2007). To test the effect of baseline \([Ca^{2+}]_i\) on H\(_2\)O\(_2\)-induced responses, recordings were made with a “high Ca\(^{2+}\)” pipette solution (total \([Ca^{2+}]_i\) increased to 0.89 mM, calculated free \([Ca^{2+}]_i\) \(750 \text{nM; MAXC, http://maxchelator.stanford.edu}\) in the presence of tolbutamide (100 \(\mu\)M) or glibenclamide (10 \(\mu\)M). Baseline \([Ca^{2+}]_i\), was 71 \pm 10 nM in cells patched with the pipettes containing standard K\(^{+}\)-based pipette solution \((n = 6)\) and 115 \pm 18 nM in neurons patched with the “high Ca\(^{2+}\)” solution \((n = 7)\). The discrepancy between the measured and expected free \([Ca^{2+}]_i\) most likely resulted from endogenous buffering and active Ca\(^{2+}\) extrusion. The H\(_2\)O\(_2\)-induced \([Ca^{2+}]_i\) rise was significantly higher in cells patched with the “high Ca\(^{2+}\)” pipette solution \((\text{control: } \Delta 43.7 \pm 13.3 \text{nM}, n = 6; \text{high Ca}^{2+}\) pipette: \(\Delta 324.5 \pm 54.6 \text{nM}, n = 6; P < 0.01;\) Fig. 9, C and D). These results demonstrate a strong dependence of the H\(_2\)O\(_2\)-induced \([Ca^{2+}]_i\) rise on baseline \([Ca^{2+}]_i\). The effect of higher baseline \([Ca^{2+}]_i\) on the H\(_2\)O\(_2\)-evoked whole cell current was not statistically significant \((P = 0.641;\) Fig. 9, A and B).

Effects of ionotropic glutamate receptor blockers on H\(_2\)O\(_2\)-induced responses. Previous studies have shown that H\(_2\)O\(_2\) and other ROS can inhibit glutamate transporters (Trotti et al. 1998) and that the effects of exogenous H\(_2\)O\(_2\) are partially mediated by the activation of ionotropic glutamate receptors in hippocampal slices (Avshalumov and Rice 2002). To test the involvement of these receptors in the H\(_2\)O\(_2\)-induced responses, a combination of AP5 (100 \(\mu\)M) and CNQX (20 \(\mu\)M) was bath applied 5 min after the onset of H\(_2\)O\(_2\) application. Neither the H\(_2\)O\(_2\)-induced current nor the calcium response was affected in the presence of these blockers (data not shown).

DISCUSSION

Redox-sensitive channels are activated by oxidative stress, an important factor in the pathogenesis of PD and other neurodegenerative disorders (see, e.g., Barnham et al. 2004). Potentially, such channels can transduce a nonspecific pathological increase in ROS levels into specific cellular responses, including activation of cell death pathways. While a number of redox-sensitive TRP channels have been identified (TRPC3, TRPP2, TRPV1, and TRPA1; see introduction), TRPM2 are most likely to act as sensors of oxidative stress in dopaminergic SNc neurons. There are no data supporting the expression of TRPP2 and TRPA1 channels in ventral midbrain. TRPV1 channels have been detected in SNc neurons (Marinelli et al. 2007). However, at least in hippocampal neurons, TRPV1 channels appear to mediate the effect of oxidative stress by modulation of glutamate neurotransmission rather than a direct action on the neurons (Crouzin et al. 2010). TRPC3 channels are expressed in ventral midbrain mainly in oligodendrocytes (Fusco et al. 2004) and only in a small subset of SNc neurons \((<10%;\) Tozzi et al. 2003). Given that all these other channels are unlikely to contribute significantly to the responses of SNc neurons during oxidative stress, we investigated the expression and properties of TRPM2 channels.

TRPM2 mRNA and protein expression in the SNc. Previous studies reported TRPM2 mRNA expression in the human substantia nigra (Nagamine et al. 1998; Uemura et al. 2005).
Consistent with this finding, RT-PCR analysis performed in this study detected TRPM2 mRNA in the rat SNc region. Although a number of human TRPM2 mRNA splice variants have been identified (e.g., Uemura et al. 2005; Wehage et al. 2002; Zhang et al. 2003), no specific splice variants have so far been reported in the rat. In our study, two primer sets were designed based on the rat sequence, which resembles the human full-length variant (TRPM2-L). The results obtained with both sets of primers were consistent, confirming the expression of TRPM2 mRNA in the SNc.

As the tissue collected for mRNA analysis contained a variety of cell types, we used immunohistochemistry to analyze TRPM2 protein expression in individual SNc neurons. Two polyclonal antibodies were used. One was produced by immunization against a human TRPM2 fragment in the channel domain (amino acids 650–700) and the second against a rat TRPM2 fragment located near the COOH terminus in the enzyme domain (amino acids 1430–1508). We have previously tested these antibodies in a study of TRPM2 channel expression in the hippocampus (Bai and Lipski 2010). In the present study, both antibodies produced a similar pattern of staining throughout the midbrain slice. Double-labeling experiments revealed colocalization of TRPM2 and TH, a marker of dopaminergic neurons, providing the first evidence for TRPM2 protein expression in dopaminergic SNc neurons. TRPM2 immunoreactivity was also observed in midbrain slices in regions outside the SNc. This is consistent with reports of TRPM2 channel expression in other cell types (see introduction). A recent study demonstrated that the H2O2-induced increase in spontaneous firing frequency of SNr GABAergic neurons was inhibited by flufenamic acid, consistent with TRPM2 channel expression in these neurons (Lee et al. 2011). The widespread expression of TRPM2 channels suggests that they mediate responses to oxidative stress in many types of cells, and that the high susceptibility of SNc neurons to oxidative damage is not solely due to these channels.

Similar to cultured cortical and hippocampal neurons (Bai and Lipski 2010; Kaneko et al. 2006; Olah et al. 2009), TRPM2 immunoreactivity was not limited to the plasma membrane and a punctuate pattern of staining was observed in the cytoplasm. This could be due to a high level of protein trafficking or to expression in organelles. The latter possibility is supported by a study demonstrating that TRPM2 can also function as a lysosomal Ca2+/H+ release channel (Lange et al. 2009).

Responses of SNc neurons to ADPR. ADPR was used as an activator of TRPM2 channels, to test for their functional expression in SNc neurons. Whereas oxidative stress activates not only these channels but also a large number of other redox-sensitive processes, intracellular ADPR selectively

Fig. 7. Effects of TRPM2 channel blockers on H2O2-induced responses. A1 and B1: averaged changes in whole cell current and [Ca2+]i, in response to H2O2. Slices were preincubated with CLT or N-(p-amylcinnamoyl)anthranilic acid (ACA) for >10 min prior to H2O2 application (control: n = 7; CLT, 5 μM: n = 4; ACA, 20 μM: n = 6). A2 and B2: group data obtained 10 min after the onset of H2O2 application. H2O2-induced [Ca2+]i increase was reduced in the presence of CLT or ACA, whereas the inward current was inhibited only by CLT. C1 and D1: averaged changes in whole cell current and [Ca2+]i, in response to H2O2 in neurons loaded with 3,4-dihydro-5-[4-(1-piperidinyl)butoyl]-1(2H)-isoquinolinone (DPQ) through the patch pipette (control: n = 5; DPQ, 50 μM: n = 9). C2 and D2: group data obtained 15 min after the onset of H2O2 application. No statistically significant effects of DPQ on H2O2-induced responses were observed. *p < 0.05, **p < 0.01.
intracellular application of ADPR under normal conditions did not elicit a current response (Olah et al. 2009). However, when neurons were depolarized to activate voltage-gated Ca\(^{2+}\) channels and raise [Ca\(^{2+}\)], application of ADPR was able to evoke a TRPM2 channel-mediated current.

The ADPR-induced changes in membrane current and [Ca\(^{2+}\)] were strongly inhibited with two nonselective TRPM2 channel blockers, flufenamic acid and clotrimazole. Flufenamic acid is a nonsteroid anti-inflammatory agent that also modulates other TRP channels, as well as voltage-gated Na\(^+\) and K\(^+\) channels, Cl\(^-\) channels, and GABA\(_A\) receptors (Hill et al. 2004b). Clotrimazole is an antifungal agent that also blocks sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase, voltage-gated Ca\(^{2+}\) channels, and Ca\(^{2+}\)-activated K\(^+\) channels (Hill et al. 2004a; Nazirgolu et al. 2007). In the absence of more selective blockers, these drugs have been routinely used to identify TRPM2 channel responses in heterologous expression systems, primary cell cultures, and brain slices (Fonfria et al. 2006; Hill et al. 2006; Olah et al. 2009; Wilkinson et al. 2008). The responses evoked in our study by intracellular application of ADPR, and the inhibition of these responses by TRPM2 blockers, are consistent with functional expression of TRPM2 channels in dopaminergic SNc neurons. However, although unlikely, we cannot exclude the possibility that ADPR acted through a yet-unknown mechanism, independent of TRPM2 channel activation, which was also affected by flufenamic acid and clotrimazole.

**Fig. 8. Dependence of H\(_2\)O\(_2\)-evoked responses on extracellular calcium.** A and C: traces illustrating the changes in whole cell current and [Ca\(^{2+}\)], during exposure of the slice to H\(_2\)O\(_2\). Standard artificial cerebrospinal fluid (aCSF) (containing 2.4 mM Ca\(^{2+}\)) was replaced with “zero” calcium aCSF (0 mM Ca\(^{2+}\) and 1 mM EGTA) for a total of 15 min, starting 10 min prior to H\(_2\)O\(_2\) application. Potassium conductance was blocked with Cs\(^+\) and TEA contained in the pipette solution. B and D: group data showing significantly smaller H\(_2\)O\(_2\)-induced responses in the absence of extracellular calcium (n = 5). *P < 0.05.

binds to the NUDT9 domain of TRPM2 protein. The ADPR concentrations required for channel activation have been reported to be in the range of 50 \(\mu\)M to 1 mM, depending on the experimental model. These concentrations lead to inward current and [Ca\(^{2+}\)] rise in heterologous expression systems and in cultured neurons (Hill et al. 2006; Kaneko et al. 2006; Olah et al. 2009; Sano et al. 2001). In the present study, a similar response was observed in SNc neurons in acute brain slice. Both the ADPR-evoked whole cell current and [Ca\(^{2+}\)] rise were dose dependent and desensitized over time. Rundown of TRPM2 channel response has been previously reported in whole cell recordings (Sano et al. 2001) and in excised membrane patches (Csanady and Torocsik 2009; Hill et al. 2006). While the mechanism underlying the desensitization is unknown, it was suggested to be due to a reduction in the number of active channels (Csanady and Torocsik 2009). Alternatively, the rundown can be due to enzymatic degradation of ADPR or insufficient levels of free [Ca\(^{2+}\)], needed to sustain the response. Previous studies have demonstrated that intracellular calcium is required both for TRPM2 channel activation and to maintain their open state (Csanady and Torocsik 2009; Starkus et al. 2007). Therefore, it is possible that the baseline [Ca\(^{2+}\)] (93 \pm 11 nM, n = 13) and the Ca\(^{2+}\) buffering capacity of the pipette solution permitted channel activation but were unable to sustain the response. This interpretation is in line with a previous study conducted on hippocampal neurons, in which...
Responses of SNc neurons to H$_2$O$_2$. Exogenous application of H$_2$O$_2$ was used to determine whether TRPM2 channels are involved in the response of SNc neurons to oxidative stress. H$_2$O$_2$ was chosen since this oxidant has been suggested to involve in the response of SNc neurons to oxidative stress. The nigrostriatal system, H$_2$O$_2$ regulates dopamine release from a signaling molecule (Avshalumov et al. 2007; Veal et al. 2007). In the nigrostriatal system, H$_2$O$_2$ regulates dopamine release from SNc neurons via a K$_{ATP}$ channel-dependent mechanism (Chen et al. 2001, 2002). Although the effects of H$_2$O$_2$ on K$_{ATP}$ channel activation in SNc neurons are well documented (Avshalumov et al. 2005; Geracitano et al. 2005), it is not known whether this oxidant also activates TRPM2 channels in these neurons.

The effects of H$_2$O$_2$ have been tested in brain slices typically at high micromolar or low millimolar concentrations (Avshalumov et al. 2005; Geracitano et al. 2005; Katsuki et al. 1997; Nisticò et al. 2008). Similar concentrations were also used to activate TRPM2 channels in expression systems (Hill et al. 2006; Kaneko et al. 2006; Naziroglu and Luckhoff 2008; Wilkinson et al. 2008). At 0.2–5 mM, H$_2$O$_2$ only moderately affected mitochondrial function in hippocampal slices (Gerich et al. 2009), indicating minimal toxicity. Although in the present study H$_2$O$_2$ was bath applied at 1–5 mM, the actual concentration in the tissue is likely to be significantly lower because of degradation by glutathione peroxidase and catalase (Dringen et al. 2005). For example, in Jurkat T cells, cytoplasmic H$_2$O$_2$ levels were estimated to be one-seventh of the extracellular concentration (Antunes and Cadenas 2000).

H$_2$O$_2$ induced an outward current that was only moderately inhibited in the presence of K$_{ATP}$ channel blockers. We hypothesized that either a residual K$_{ATP}$ current or another unidentified outward current was masking the expected TRPM2 channel-mediated inward current. Consistent with this hypothesis, blocking all K$^+$ conductances with Cs$^+$ and TEA revealed a H$_2$O$_2$-induced inward current that was sensitive to TRPM2 channel blockers (see below).

H$_2$O$_2$ evoked an increase in [Ca$^{2+}$]$_i$, consistent with opening of Ca$^{2+}$-permeable TRPM2 channels. As expected, this response was insensitive to K$_{ATP}$ channel blockers, but, surprisingly, the [Ca$^{2+}$]$_i$ increase was enhanced when K$^+$ conductances were blocked. This may be a consequence of improved space-clamp conditions associated with increased cell input resistance. Alternatively, it can be hypothesized that, in this experimental model, cesium inhibits Na$^+$-K$^+$-ATPase. Degradation of the transmembrane Na$^+$ gradient would lead to reduced efficiency of the Na$^+$/Ca$^{2+}$ exchanger and a slower rate of Ca$^{2+}$ efflux.

H$_2$O$_2$ failed to evoke a [Ca$^{2+}$]$_i$ rise in the absence of extracellular calcium, demonstrating an extracellular source of this ion. This is consistent with activation of TRPM2 channels in the plasma membrane. In both cultured striatal and cortical neurons, the H$_2$O$_2$-evoked [Ca$^{2+}$]$_i$ rise was also dependent on extracellular calcium (Kaneko et al. 2006; Smith et al. 2003). In contrast, Gerich et al. (2009) reported that in cultured hippocampal neurons the H$_2$O$_2$-evoked [Ca$^{2+}$]$_i$ rise was due to Ca$^{2+}$ release from intracellular stores. The discrepancy between these and our results suggests that the mode by which H$_2$O$_2$ induces [Ca$^{2+}$]$_i$ rise is cell type specific. Alternatively, it could be due to differences in experimental conditions. Gerich et al. studied calcium responses in cultured neurons that, unlike cells in brain slices, are deprived of their normal environment and hence may differ in calcium handling. In addition, in their experiments the cell membrane potential was not clamped, and therefore other voltage-sensitive mechanisms may have influenced [Ca$^{2+}$]$_i$.

Activation of TRPM2 channels highly depends on resting [Ca$^{2+}$]$_i$, a property believed to be mediated by calmodulin (Starkus et al. 2007; Tong et al. 2006). In fact, sufficiently high [Ca$^{2+}$]$_i$ can activate TRPM2 channels by itself (Du et al. 2009). In the present study, the effect of baseline [Ca$^{2+}$]$_i$ on the H$_2$O$_2$-evoked [Ca$^{2+}$]$_i$ rise was tested by increasing [Ca$^{2+}$]$_{pipette}$ and, consequently in patched neurons. In this condition ([Ca$^{2+}$]$_i$, 115 ± 18 nM, n = 7), application of H$_2$O$_2$ evoked a larger [Ca$^{2+}$]$_i$ increase, consistent with this property of TRPM2 channels.

The H$_2$O$_2$-evoked [Ca$^{2+}$]$_i$ rise was significantly reduced by ACA and clotrimazole. ACA is another nonselective TRPM2 channel blocker that is structurally similar to flufenamic acid (Hartenbeck et al. 2007). ACA was used in these experiments since flufenamic acid caused instability during prolonged patch-clamp recordings, possibly due to a mild toxicity effect. The H$_2$O$_2$-induced inward current, unmasked with Cs$^+$ and TEA, was reduced after preincubation with clotrimazole but not ACA. The inconsistent effect on whole cell current suggests that a significant component of the response was mediated by other unidentified channels. However, the effectiveness of both blockers in inhibiting the [Ca$^{2+}$]$_i$ rise supports TRPM2 channel activation in SNc neurons.

It is still debated whether H$_2$O$_2$-mediated activation of TRPM2 channels requires ADPR as a diffusible messenger. In support of a direct gating mechanism, others have reported TRPM2 activation by H$_2$O$_2$ in not only whole cell but also membrane patch recordings (see, e.g., Hill et al. 2006; Naziroglu and Luckhoff 2008). Our results are consistent with this mechanism since H$_2$O$_2$-evoked current and Ca$^{2+}$ responses were observed despite cytoplasmic dialysis associated with whole cell patch-clamp recording. However, there is evidence indicating that oxidative stress-mediated activation of TRPM2 channels requires PARP-1 (Buelow et al. 2008; Fonfria et al. 2004; Perraud et al. 2005), an enzyme involved in the synthesis of ADPR and commonly associated with repair of damaged DNA (Kuhn et al. 2005). In our experiments, the PARP inhibitor DPQ did not inhibit the H$_2$O$_2$-evoked inward current and [Ca$^{2+}$]$_i$ rise. While this suggests that in our system PARP-1 activity is not required for oxidative stress-mediated activation of TRPM2 channels, ADPR may still act as a diffusible messenger that originates from another enzymatic pathway or is released form an intracellular store (Partida-Sanchez et al. 2007).

Comparison between responses evoked by ADPR and H$_2$O$_2$. Our results indicate activation of TRPM2 channels by exogenous ADPR and H$_2$O$_2$ in SNc neurons. However, two major differences were observed between the responses evoked by these two stimuli. First, ADPR evoked a significantly larger inward current. Second, nonselective TRPM2 blockers were more effective at blocking ADPR-evoked responses. These results are consistent with the concept that ADPR is a potent, selective activator of TRPM2 channels. In contrast, H$_2$O$_2$...
potentially not only activates TRPM2 channels but also modulates other redox-sensitive processes.

Conclusions. Our results provide novel information on TRPM2 mRNA and protein expression in SNc neurons and characterize changes in membrane currents and [Ca$^{2+}$]$_i$, evoked by TRPM2 channel activators. Intracellular application of ADPR evoked a rise in [Ca$^{2+}$]$_i$, and an inward current, while H$_2$O$_2$ evoked a complex response comprising a [Ca$^{2+}$]$_i$ rise and an inward current that was masked by activation of K$^+$ conductance. Although our study focused on the oxidative stress-sensing function of TRPM2 channels, there are also considerable data on the role of these channels in mediating cell death (see introduction). In the context of PD, a disorder in which a number of factors contribute to degeneration of SNc neurons (see, e.g., Sulzer 2007), TRPM2 channel may act as a central element that integrates factors such as oxidative stress, calcium dysregulation, and mitochondrial dysfunction (Free- stone et al. 2009). These channels may also play a role in other neurodegenerative and acute brain disorders associated with an increased level of oxidative stress. These considerations, as well as our observations regarding TRPM2 channel function in SNc neurons, warrant further studies to determine the pathophysiological role of these channels in neurological disorders.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K. K. H. C. and J. L. conception and design of research; K. K. H. C. and P. S. F. performed experiments; K. K. H. C. and P. S. F. analyzed data; K. K. H. C., P. S. F., and J. L. interpreted results of experiments; K. K. H. C. and P. S. F. performed experiments; K. K. H. C. and P. S. F. analyzed TRPM2 channel function in SNc neurons, warrant further studies to determine the pathophysiological role of these channels in neurological disorders.

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