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NeuroToxicology 26 (2005) 869–881

NeuroToxicology

Acute Effects of 6-Hydroxydopamine on Dopaminergic Neurons of the Rat Substantia Nigra Pars Compacta In Vitro

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Received 10 December 2004; accepted 10 January 2005
Available online 10 May 2005

Abstract

6-Hydroxydopamine (6-OHDA) is a neurotoxin which has been implicated in the degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNc) in Parkinson's disease (PD), and is frequently used to produce animal models of the disease. The aim of our study, conducted on midbrain slices obtained from young Wistar rats, was to determine the little known acute effects of this toxin (0.2–2.0 mM; 10–20 min exposure; 34 °C) on electrophysiological properties, intracellular Ca^{2+} levels and dendritic morphology of SNc neurons. Four experimental approaches were used: extracellular recording of firing frequency, whole-cell patch-clamping, ratiometric fura-2 imaging, and cell labeling with lucifer yellow (LY) or dextran-rhodamine. Extracellular recording revealed a concentration-dependent decrease in the tonic, pacemaker-like firing. In whole-cell recordings in voltage-clamp (V_{hold} −60 mV), smaller doses (0.2–0.5 mM) induced an outward current (or cell membrane hyperpolarization in current-clamp), which could in some cells be reversed with tolbutamide (blocker of ATP-dependent K^+ channels). A higher dose (1.0–2.0 mM) caused rapid reductions of cell membrane capacitance and membrane resistance. Toxin exposure gradually increased the intracellular Ca^{2+} level, which did not subsequently return to control. The increase in Ca^{2+} signal was not prevented by depletion of intracellular Ca^{2+} stores with thapsigargin (10 μM) or cyclopiazonic acid (30 μM), nor by removing extracellular Ca^{2+}. Cell membrane current and Ca^{2+} responses were not prevented by blocking dopamine transporter (DAT). Cells loaded with LY or dextran-rhodamine showed signs of damage (cell membrane blebbing) in dendrites following toxin exposure (1 mM; 10–20 min). These results demonstrate that the oxidative and metabolic stress induced in SNc neurons by 6-OHDA results in rapid dose-dependent changes of cell membrane properties with morphological evidence of dendritic damage, as well as in disturbance of intracellular Ca^{2+} homeostasis.

Keywords: Substantia nigra; Dopamine neurons; Brain slice; 6-OHDA; Parkinson's disease

INTRODUCTION

The main pathological characteristic of Parkinson's disease (PD) is the progressive degeneration of striatum-projecting dopamine (DA) neurons in the substantia nigra pars compacta (SNc), which leads to a debilitating motor dysfunction. The etiology of PD remains obscure, but both environmental and genetic factors have been suggested to play a role (Schapira, 1997; von Bohlen und Halbach et al., 2004). Involvement of epigenetic factors in DA cell death has been suggested following the observation in the early 1980s that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produced

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doi:10.1016/j.neuro.2005.01.014

selective death of nigral neurons in humans and motor symptoms resembling those observed in PD (Langston and Ballard, 1983; Langston et al., 1983). More recently, exposure to other environmental toxins (e.g. pesticides and other agricultural chemicals, as well as certain metals) has been shown to promote DA neuron degeneration and induce PD-like symptoms (Betarbet et al., 2000; Gorell et al., 1998; Priyadarshi et al., 2000; Priyadarshi et al., 2001).

There has been growing interest in the cellular and molecular mechanisms underlying the degeneration of SNc neurons. In particular, dysfunction of complex I of the mitochondrial respiratory chain (Hattori et al., 1991), oxidative stress (Floor and Wetzel, 1998; Lotharius and O'Malley, 2000; Sherer et al., 2002) and apoptotic cell death have been suggested as possible causes (Blum et al., 2001). Indeed, understanding the basis of DA cell vulnerability is important for the development of neuroprotective therapies to prevent or delay progression of PD.

6-Hydroxydopamine (6-OHDA) was the first agent discovered that has specific toxicity for neurons which utilize catecholamines as a neurotransmitter (Breese and Traylor, 1970; Ungerstedt, 1968). When injected into the Substantia Nigra, medial forebrain bundle or striatum it destroys SNc DA neurons, resulting in parkinsonian motor deficits (Deumens et al., 2002; Hefti et al., 1980; Schwarting and Huston, 1996). An explosion of interest in 6-OHDA was precipitated by reports that this toxin occurs naturally in the brain (e.g. from hydroxylation of dopamine in the presence of iron; Blum et al., 2001; Curtius et al., 1974; Linert et al., 1996; Napolitano et al., 1999), and thus may be responsible for degeneration of human SNc neurons and the development of PD (Jellinger et al., 1995; Pezzella et al., 1997). This hypothesis is supported by the finding of 6-OHDA in the urine of PD patients (Andrew et al., 1993). Thus, this substance can be regarded not only as a tool to produce an animal model of the disease, but also as an endogenous neurotoxin contributing to neurodegeneration in the disease itself (Blum et al., 2001; Grunblatt et al., 2000).

Although in some models the 6-OHDA-induced death of SNc neurons may progress for months after a single toxin application (Sauer and Oertel, 1994), in most studies the effects were fast, with the interval to cell death dependant on the model used, the route of administration and the dose (e.g. Jeon et al., 1995; Bywood and Johnson, 2000; Zuch et al., 2000; Ding et al., 2004). The aim of our study was to elucidate the mechanism of the early toxic effects of 6-OHDA on SNc neurons in midbrain slices.

Preliminary results were presented earlier in abstract form (Lipski et al., 2003).

METHODS

Slice Preparation

Standard procedures approved by respective institutional animal ethics committees were used to obtain acute midbrain slices from P16-P21 Wistar rats (Mercuri et al., 1995). In brief, rats were anaesthetized with halothane or 100% CO_2 and killed by decapitation. The brain was removed from the skull rapidly and 200–300 μm thick horizontal or transverse slices containing the substantia nigra and ventral tegmental area were cut in cold artificial cerebrospinal fluid (ACSF) with a vibratome (VT1000S, Leica, Germany). After recovery at 33 °C for at least 1 h, slices were individually placed in a recording chamber mounted on the stage of an upright microscope (Axioscope, Zeiss). They were superfused (3 ml/min; 33–34 °C) with ACSF containing (mM): NaCl 126, KCl 2.5, $MgCl_2$ 1.2, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $NaHCO_3$ 19, glucose 10, 0.01% ascorbic acid; bubbled with carbogen (95% O_2/5% CO_2; pH 7.4).

Extracellular Recording

Conventional extracellular recordings were performed using an AC amplifier (NL104, NeuroLog; bandwidth, 70 Hz–3 kHz) and glass microelectrodes (3–5 MΩ) filled with a solution which contained (mM): NaCl 145, KCl 3, $CaCl_2$ 1, MgCl 1, HEPES 10 and glucose 15. Neuron 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 software (Axon Instr., USA).

Patch-clamp Recording

Neurons were visualized with infrared video microscopy (Hamamatsu, Japan). Whole-cell recordings (in voltage or current-clamp) were obtained using an Axopatch 1D or Multiclamp 700A amplifier (Axon Instr.). Patch pipettes (3–4 MΩ) were made from borosilicate glass (outer diameter, 1.5 mm) and pulled with a PP-88 (Narishige, Japan) or P-97 (Sutter Instr., USA) puller. They were filled with a solution containing (mM): K-methylsulphate 150, $MgCl_2$ 2, EGTA 0.1, HEPES 1, ATP 2, GTP 0.3; pH adjusted to

7.3 with KOH. In some experiments the fluorescent dye fura-2 (250 μM; see below) was added. In voltage-clamp, currents acquired at −60 mV holding potential (V_{hold}) were filtered at 3 kHz, digitized at 10 kHz and analyzed on a PC using pClamp software (Axon Instr.).

Cell Labeling with Lucifer Yellow (LY) or Dextran-rhodamine

In some whole-cell patch-clamp experiments, the fluorescent dye LY was added to the pipette solution (1 mg/ml). Using an appropriate filter block (excitation 450–490 nm, dichroic mirror 505 nm, barrier filter 520 nm), good filling of cell body and dendrites was observed 5–10 min after breaking the gigaseal. Real-time imaging of control neurons and those exposed to 6-OHDA was conducted using a SenSys 1401-G2E digital CCD camera (Roper Scientific Inc., USA). In some sections, SNc neurons were labelled with dextran-rhodamine (MW 100,000; Molecular Probes, USA) using a standard loading protocol (Delaney, 2002). After exposure to ACSF (controls) or 1 mM 6-OHDA (20 min), sections were fixed in 4% paraformaldehyde and mounted with Vectashield (Vector, USA). Dendrites of SNc neurons were imaged using a confocal microscope (Leica TCS SP2, Germany).

Microfluorimetry

Neurons were filled with a Ca^{2+}-sensitive ratiometric dye, fura-2 (pentapotassium salt, Molecular Probes), 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, UK). Images were acquired at 6 or 12 s intervals using IonVision software (ImproVision, UK). The time course of fluorescence changes, corresponding to changes in $[Ca^{2+}]_i$, was calculated over an area that included the cell body ('region of interest', defined as those pixels that exhibied at least 20–30% of maximal specific fluorescence). Values were corrected for background fluorescence measured from the region surrounding the region of interest. Calcium levels were expressed as the ratio, $R = (F_{340soma} - F_{340bg})/(F_{380soma} - F_{380bg})$, where F_{340} and F_{380} are the fluorescence emitted at the excitation wavelengths 340 and 380 nm, respectively, for the soma and background (bg).

Drug Application

Most drugs were applied as ACSF solutions from a separate reservoir (also gassed with carbogen, 33–34 °C) to the inlet of the recording chamber using a three-way stopcock. 6-OHDA (hydrochloride salt; Sigma, USA) was dissolved in water as a ×100 stock solution containing 0.4% ascorbic acid which was bubbled with N_2. This concentrated solution was infused using a syringe pump (SP-101; WPI, USA) into the inflow line of the recording chamber at 30 μl/min (flow ratio 1:100), resulting in a 6-OHDA concentration in the recording chamber of 0.2–2.0 mM. This means of toxin application minimized its oxidation before reaching the target tissue.

The type I metabotropic glutamate receptor agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG; Tozzi et al., 2003) was pressure injected (50 ms pressure pulses applied every 2 min; 300 μM) through an injection pipette placed approximately 100 μm from the recorded cell.

RESULTS

Identification of Dopaminergic SNc Neurons

Recordings were obtained from DA neurons of the SNc identified electrophysiologically. For extracellular recordings, SNc neurons ($n = 33$) met the following criteria (Diana and Tepper, 2002): (i) slow and regular firing (2.2 ± 0.1 Hz); (ii) biphasic or triphasic spike waveform with an inflection on the rising phase; (iii) spike duration ≥1.5 ms; and (iv) inhibition of firing after exposure to 30 μM dopamine (Fig. 1A). In whole-cell voltage-clamp recordings, dopaminergic SNc neurons showed a time-dependent inward current (I_h) evoked by hyperpolarizing voltage steps, and in current-clamp hyperpolarizing current steps evoked a typical 'sag' potential due to activation of I_h current (Mercuri et al., 1995; Fig. 1B, left panel). Cells classified as 'non-DA neurons' did not show these characteristics (Fig. 1B, right panel; Berretta et al., 2000).

Effects of 6-OHDA on Firing Frequency of SNc Neurons

In extracellular recordings, 6-OHDA (10 min; 0.2–2 mM) rapidly inhibited the spontaneous firing of SNc neurons in a dose-dependent manner (Fig. 2A–C). No recovery was observed during a 20 min drug washout

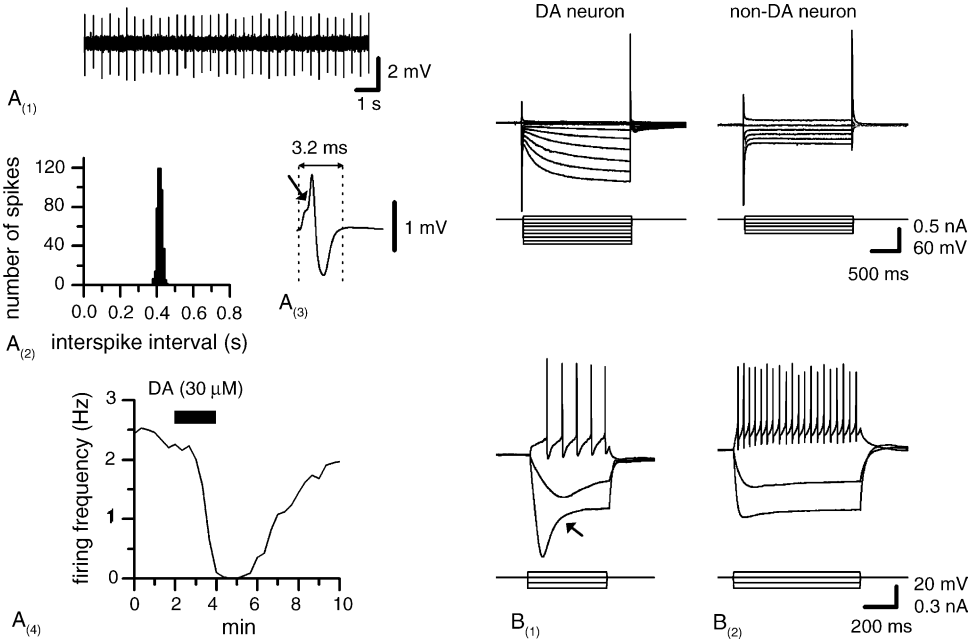

Fig. 1. Identification of SNc neurons in extracellular (A) and whole-cell patch-clamp (B) recordings. (A) Example of extracellular recording from a dopaminergic neuron. A(1): spontaneous firing. A(2): interval histogram demonstrating regularity of spontaneous firing. A(3): shape of individual action potentials (average of 16 consecutive potentials). Note inflection on the rising phase of the action potential (arrow). A(4): inhibition of firing after bath application of dopamine (30 μM). (B) Example of whole-cell patch-clamp recording. B(1): dopaminergic neuron. From top: I_h current induced by hyperpolarizing voltage commands (voltage-clamp), and voltage responses to step current pulses recorded in current-clamp. Note depolarizing sag of the membrane potential (arrow) evoked by hyperpolarizing current pulses. B(2): non-dopaminergic neuron. From above: currents induced by voltage steps (voltage-clamp), and responses to step current pulses (current-clamp).

when the highest concentration of the toxin (2 mM) was used. Lower concentrations resulted in partial recovery. In the presence of the D2 receptor antagonist sulpiride (30 μM; $n = 6$), the inhibition of firing by 0.5 mM 6-OHDA was delayed; subsequently, however, all cells showed complete inhibition with no sign of recovery (Fig. 3A, B). In the presence of the dopamine transporter (DAT) inhibitor nomifensine (10 μM, $n = 8$; Fig. 3A and B) the fast inhibitory effect of 6-OHDA was reduced, but after washout of the toxin the firing rate returned to control (Fig. 3A). However, the time course of the initial inhibition (time to 50% inhibition of firing) was not significantly different from that evoked by the toxin alone (Fig. 3B).

A rapid 6-OHDA-induced inhibition of firing, associated with cell membrane hyperpolarization, was also observed in whole-cell recordings in current-clamp when no current was passed through the recording pipette ($n = 2$, Fig. 2D).

Effects of 6-OHDA on Membrane Properties of SNc Neurons

The effects of 6-OHDA on passive and active cell membrane properties of SNc neurons were investigated in voltage-clamp mode (V_{hold} −60 mV). As illustrated in Figs. 4A, 5, 6A and 7D, the toxin (0.5–1.0 mM) induced an outward current (0.5 mM; 48 ± 16 pA, $n = 6$), associated with an approximately 50% decrease of cell membrane resistance (R_m). The membrane capacitance (C_m) did not change at a low toxin concentration (0.5 mM), but a significant, irreversible decrease was observed at higher concentrations (1–2 mM) of the toxin (Fig. 4B).

The blocker of K_{ATP} channels, tolbutamide (100 μM) was used in an attempt to block the 6-OHDA-induced outward current (corresponding to inhibition of firing, or cell membrane hyperpolarization in extracellular/current-clamp recordings, see above). Tolbutamide was effective in reversing the outward current in a proportion of cells tested (4/7; Fig. 5). However, in the remaining three cells the outward current was not affected by the blocker.

Effect of 6-OHDA on $[Ca^{2+}]_i$ in SNc Neurons

During voltage-clamp recordings (V_{hold} −60 mV), the intracellular calcium level ($[Ca^{2+}]_i$) was measured with the fura-2 technique during slice perfusion with 6-OHDA (0.5–2 mM). As illustrated in Fig. 6A, toxin exposure resulted in a rise in $[Ca^{2+}]_i$ which was time- and concentration-dependent ($n = 33$; Fig. 6C). Only

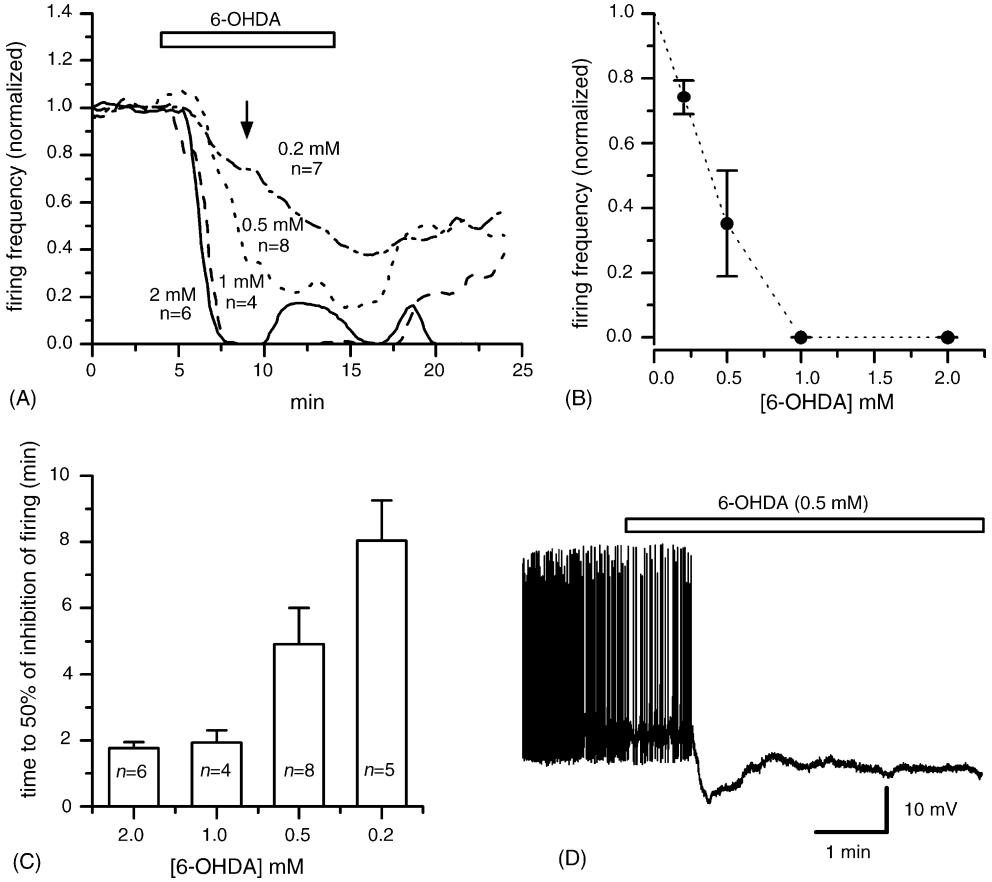

Fig. 2. Effects of 6-OHDA on dopaminergic SNc neuron firing recorded extracellullarly (A–C) and during whole-cell recording (D). A: group data (averaged and normalized) showing concentration-dependent inhibition of firing. (B) Mean firing frequency (±S.E.) for each of the four concentrations at the time point indicated by the arrow in 'A' (5 min after start of 6-OHDA application). (C) Time to reach 50% inhibition of firing at different concentrations of the toxin. (D) Rapid inhibition of firing and cell membrane hyperpolarization observed in current-clamp mode. No current was passed through the patch pipette during the recording.

partial recovery of the calcium signal was observed during a 20 min washout.

Measurements of $[Ca^{2+}]_i$ were also performed in non-DA neurons recorded in the same midbrain area (see Fig. 1B for identification criteria). In such neurons, toxin exposure (0.5 mM) resulted in no significant change in calcium signal ($n = 3$; Fig. 6B). In addition, non-DA neurons exhibited no clear changes of holding current after 6-OHDA exposure ($n = 3$).

Analysis of the Source of $[Ca^{2+}]_i$ Rise

To test the possibility that the 6-OHDA-induced rise in $[Ca^{2+}]_i$ was due to calcium entry from the extracellular space, calcium was removed from the ACSF and replaced with 7 mM Mg^{2+} (in the presence of 1 mM EGTA). In these conditions, 6-OHDA (1 mM) still produced a significant rise in $[Ca^{2+}]_i$ ($n = 3$; Fig. 7A), indicating an intracellular source for the $[Ca^{2+}]_i$ rise.

Cyclopiazonic acid (CPA; 30 μM), a Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum (ER), was used to investigate the possible involvement of intracellular calcium stores. As shown in Fig. 7B, pre-perfusion with CPA virtually abolished the increase of calcium signal induced by close-cell application of DHPG, indicating that the ER calcium stores were emptied by the drug. However, CPA exposure did not prevent the increase in $[Ca^{2+}]_i$ caused by 6-OHDA (1 mM; $n = 5$). Similarly, 6-OHDA (1 mM) still produced an increase of calcium signal in the presence of thapsigargin (10 μm), an irreversible ER Ca^{2+}-ATPase inhibitor (Fig. 7C; $n = 4$).

These observations suggest that intracellular calcium stores other than the ER are responsible for the rise in $[Ca^{2+}]_i$ induced by 6-OHDA.

Effect of DAT Blockade on 6-OHDA-induced $[Ca^{2+}]_i$ Rise

In order to investigate the possible involvement of DAT in the effects of 6-OHDA on $[Ca^{2+}]_i$, experiments were performed in the presence of the DAT inhibitor

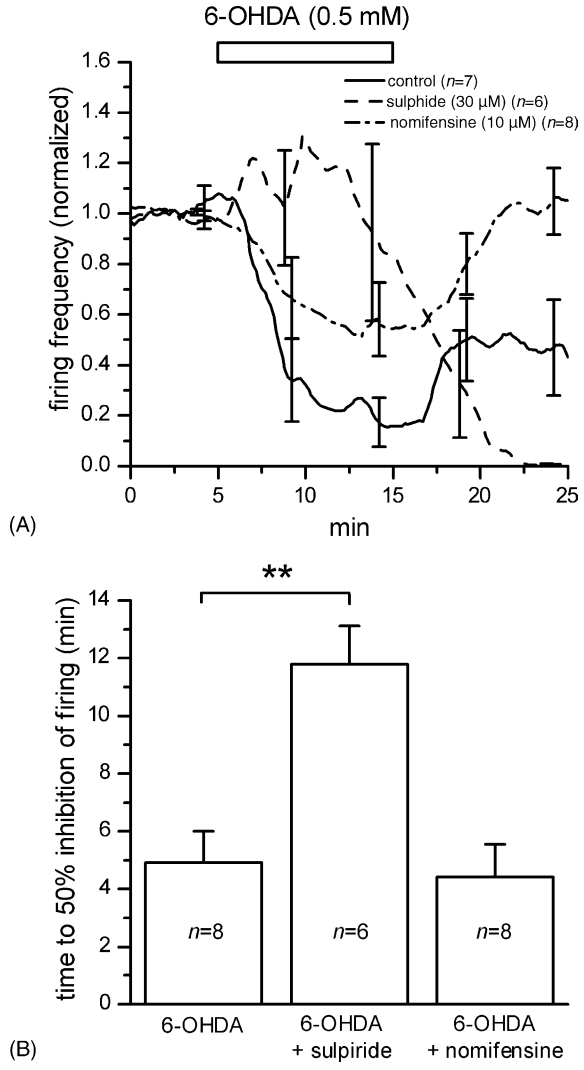

Fig. 3. Effects of sulpiride and nomifensine on 6-OHDA induced inhibition of firing. (A) Group data (averaged and normalized) from extracellular experiments. (B) Time to reach 50% inhibition of firing with 0.5 mM 6-OHDA alone and in the presence of sulpiride or nomifensine. (**) Difference from control; $p < 0.001$.

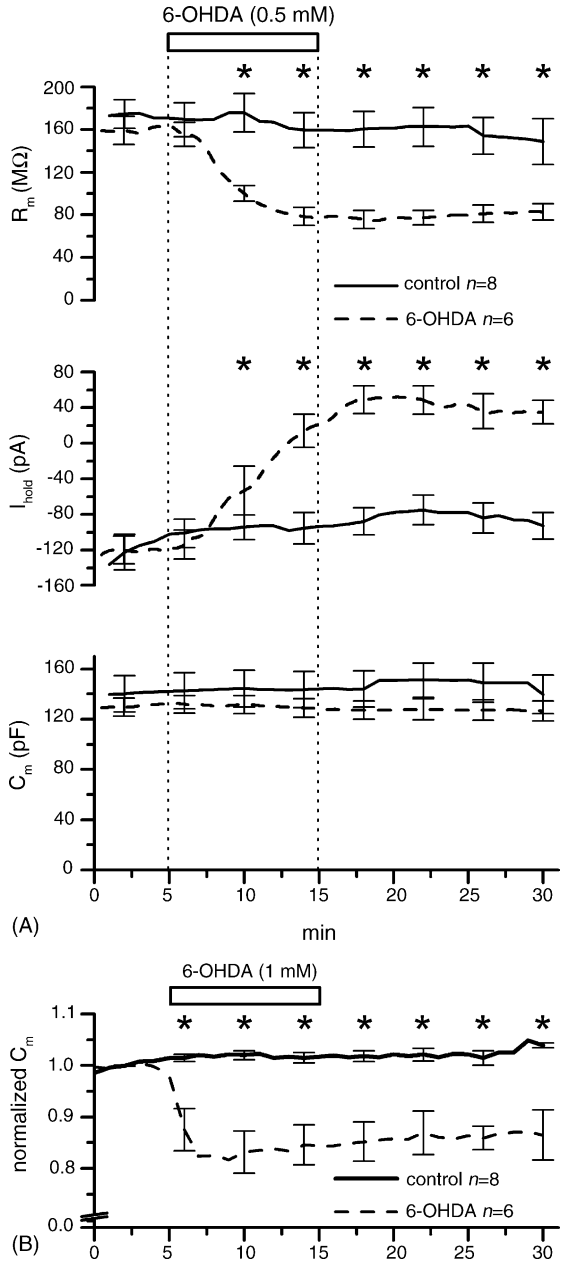

Fig. 4. Effects of 6-OHDA on cell membrane properties of SNc neurons examined in voltage-clamp. (A) Averaged values (±S.E.) of input resistance (R_m), holding current (I_{hold}; V_{hold} −60 mV) and membrane capacitance (C_m) recorded before, during and after application of 0.5 mM of the toxin. (B) A significant, non-recovering decrease in C_m recorded at higher concentration (1 mM). (*) Difference from control; $p < 0.05$.

cocaine. Pre-perfusion with cocaine (50 μM) resulted in an outward current, indicating activation of D2 dopamine receptors due to elevated extracellular DA (Lacey et al., 1990). In the presence of cocaine, the increase in $[Ca^{2+}]_i$ induced by 6-OHDA (0.5 mM) was not prevented ($n = 6$; Fig. 7D).

6-OHDA-induced Dendritic Damage

Fifteen SNc neurons were filled with LY through the patch pipette and real-time images of cell bodies and proximal dendrites were taken before, during and after 10 min exposure to 6-OHDA (1 mM). All cell bodies showed marked swelling. In three neurons, changes in dendritic morphology in the form of membrane 'ebs'-were visible in proximal dendrites during toxin exposure (Fig. 8A).

Labeling of neurons in the SNc region with dextran-rhodamine allowed for assessment of toxin-induced changes in distal portions of the dendritic trees. After 20 min exposure to 1 mM 6-OHDA ($n = 2$), sections were fixed and imaged using confocal microscopy. Both toxin-exposed sections con-

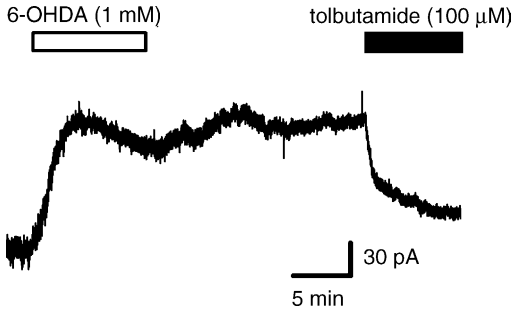

Fig. 5. Effect of K_{ATP} channel blocker. 6-OHDA-induced outward current was partly inhibited by tolbutamide (100 μM). This effect was not observed in all cells tested, indicating that the current is also mediated by other channels (including D_2-receptor activated K^+ channels).

tained neurons with pronounced dendritic blebs and varicosities, as well as signs of fragmentation. All labeled dendrites in control sections ($n = 2$) showed smooth outlines with no morphological signs of damage (Fig. 8B1 and B2).

DISCUSSION

Direct injection of 6-OHDA into the Substantia Nigra, medial forebrain bundle or striatum has been widely used as a way of inducing degeneration of midbrain DA neurons and producing in animals a disorder of motor functions analogous to Parkinson' disease (Deumens et al., 2002; Hefti et al., 1980; Schwarting and Huston, 1996). Heightened interest in 6-OHDA followed publication of evidence that the toxin occurs endogenously in the human brain and might contribute to the disease process itself (see Introduction for references). In spite of the importance of this toxin, the sequence of early cellular events leading to degeneration of DA neurons exposed to it have not yet been well characterized.

Apart from in vivo models, toxic effects of 6-OHDA have been investigated in studies conducted on cultured ventral mesencephalic neurons obtained from

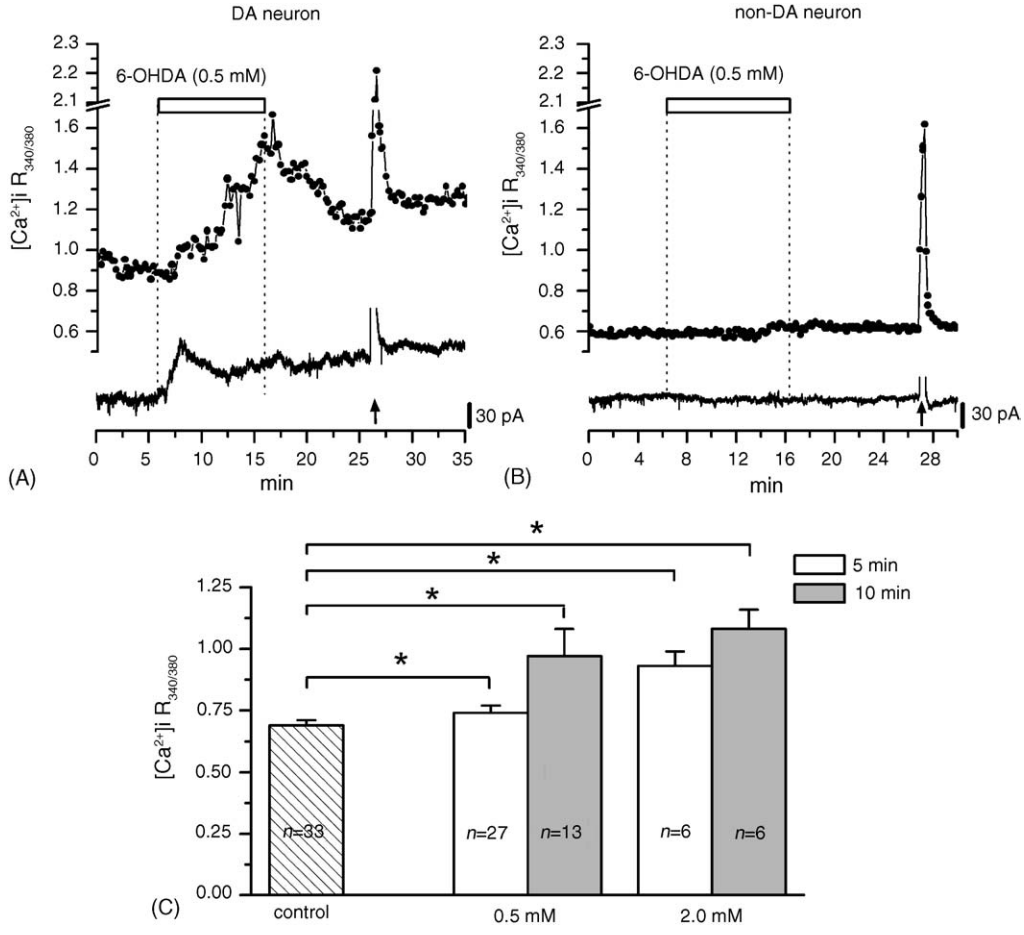

Fig. 6. Effects of 6-OHDA on intracellular free calcium and membrane currents in dopaminergic and non-dopaminergic neurons. (A and B) (upper traces): $[Ca^{2+}]_i$ measured with the fura-2 technique during whole-cell patch-clamp recording from a dopaminergic and a non-dopaminergic neuron. Arrows indicate increases in Ca^{2+} signal evoked by brief (30 s) changes of the V_{hold} from −60 to −30 mV. Lower traces: changes in membrane currents recorded at V_{hold} −60 mV. (C) Group data showing the dose- and time-dependence of intracellular Ca^{2+} rise in dopaminergic neurons. Asterisks indicate statistical significance ($p < 0.05$; paired Student's t-test).

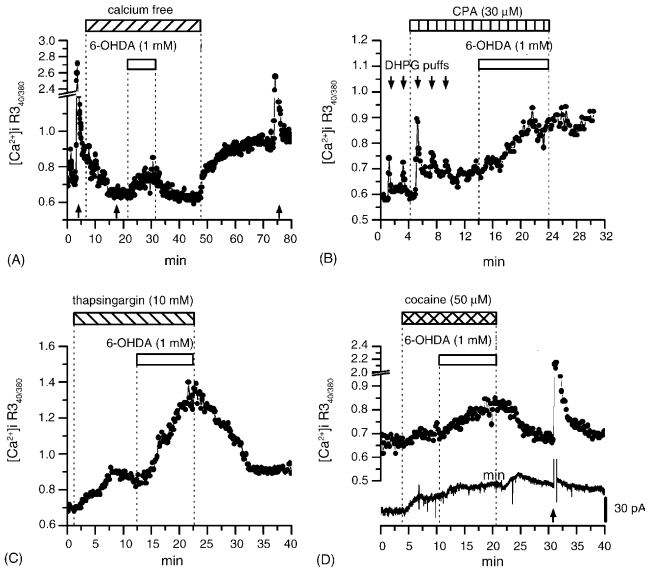

Fig. 7. Effects of 6-OHDA on $[Ca^{2+}]_I$ in calcium-free external solution (A), after emptying intracellular Ca^{2+} stores (B and C), and in the presence of DAT blocker (D). (A) Calcium was removed from the ACSF and replaced with 7 mM Mg^{2+}/1 mM EGTA (upper bar). Arrows indicate times at which V_{hold} was changed from −60 to −30 mV for 30 s. (B) Slice exposed to CPA (upper bar). Arrows indicate time points at which DHPG was applied locally from an injection pipette to test for Ca^{2+} depletion. Note a reduction in response amplitude soon after CPA application. (C) Exposure to thapsigargin (upper bar). Note an increase in $[Ca^{2+}]_i$ after the onset of thapsigargin application. (D) Exposure to cocaine (upper bar). Arrow indicates increase in Ca^{2+} signal evoked by 30 s change of the V_{hold} from −60 to −30 mV (upper trace). Lower trace: changes in membrane current recorded at V_{hold} −60 mV.

fetuses or post-natal pups (Ding et al., 2004; Michel and Hefti, 1990) and on dopamine-synthesizing cell lines (such as PC-12; Schlegel et al., 2004; Yamada et al., 1997). However, the cells used in such studies are immature and often de-differentiate or change their phenotype during culture. An additional drawback of studying 6-OHDA effects in cell cultures relates to the fact that incubation of this toxin in culture media results in auto-oxidation and extracellular generation of oxygen radicals and other reactive species (Blum et al., 2000) which can penetrate the cell membrane. Therefore, observed effects may be 'an artifact of cell culture' (cf. Clement et al., 2002). In contrast, very little work with 6-OHDA has been conducted in brain slices, an in vitro preparation in which SNc neurons demonstrate a more mature phenotype. We are aware of only one report which used this type of preparation (Bywood and Johnson, 2000). However, in contrast to our study which used a combination of electrophysiological, pharmacological, morphological and ion imaging techniques, this previous study focused on toxin-induced morphological and immunocytochemical changes.

The present study provides evidence for remarkably fast effects of 6-OHDA on DA neurons in the rat SNc, indicating cellular stress leading towards neurodegeneration. These early effects include: (i) inhibition of spontaneous firing/cell membrane hyperpolarization associated with a decrease in cell membrane resistance; (ii) a decrease in membrane capacitance (at higher toxin concentrations); (iii) a rise in intracellular free calcium; and (iv) morphological signs of dendritic damage. These effects developed within minutes of toxin exposure and were concentration dependent.

Inhibition of firing was significantly delayed in the presence of the D2 receptor antagonist sulpiride, indicating that the earliest stage of toxin-induced membrane hyperpolarization was mediated by stimulation of D2 autoreceptors (Mercuri et al., 1992). The mechanism of this fast receptor activation is not clear but at least three possibilities can be considered: (a) that 6-OHDA, which is structurally related to dopamine, directly activates D2 receptors; (b) that toxin-induced stress leads to DA release due to reversal of the dopamine transporter (similar to the effect of excessive stimulation with glutamate; Falkenburger et al., 2001), and (c) that there is a leak of DA from toxin-injured dendrites (see below). In addition, the outward current evoked by 6-OHDA (indicative of cell membrane hyperpolarization) could be reduced by the K_{ATP} channel antagonist tolbutamide, suggesting that opening of the K_{ATP} conductance contributes to the subsequent stages of cell membrane hyperpolarization. Functional K_{ATP} channels are known to be expressed in SNc DA neurons and cause hyperpolarization when the cells are exposed to various forms of metabolic stress (such as hypoxia or hypoglycemia; Guatteo et al., 1998; Marinelli et al., 2000). Thus, 6-OHDA may open these channels by impairing ATP synthesis. Indeed, this toxin has been shown to inhibit complexes I and IV of the respiratory chain in isolated mitochondria (Glinka and Youdim, 1995). However, tolbutamide was not effective in reducing the outward current in all cells, which indicates that the sustained inhibition of firing/cell membrane hyperpolarization is a complex process, and that the contribution of K_{ATP} channels may depend on other, poorly understood factors such as the metabolic state of the neuron prior to toxin exposure. Interestingly, only a subpopulation (about 40%) of DA SNc neurons studied in acute mouse brain slices showed signs of activation of K_{ATP} channels in another study in which cellular stress was induced by another mitochondrial complex I blocker, rotenone (Liss and Roeper, 2001).

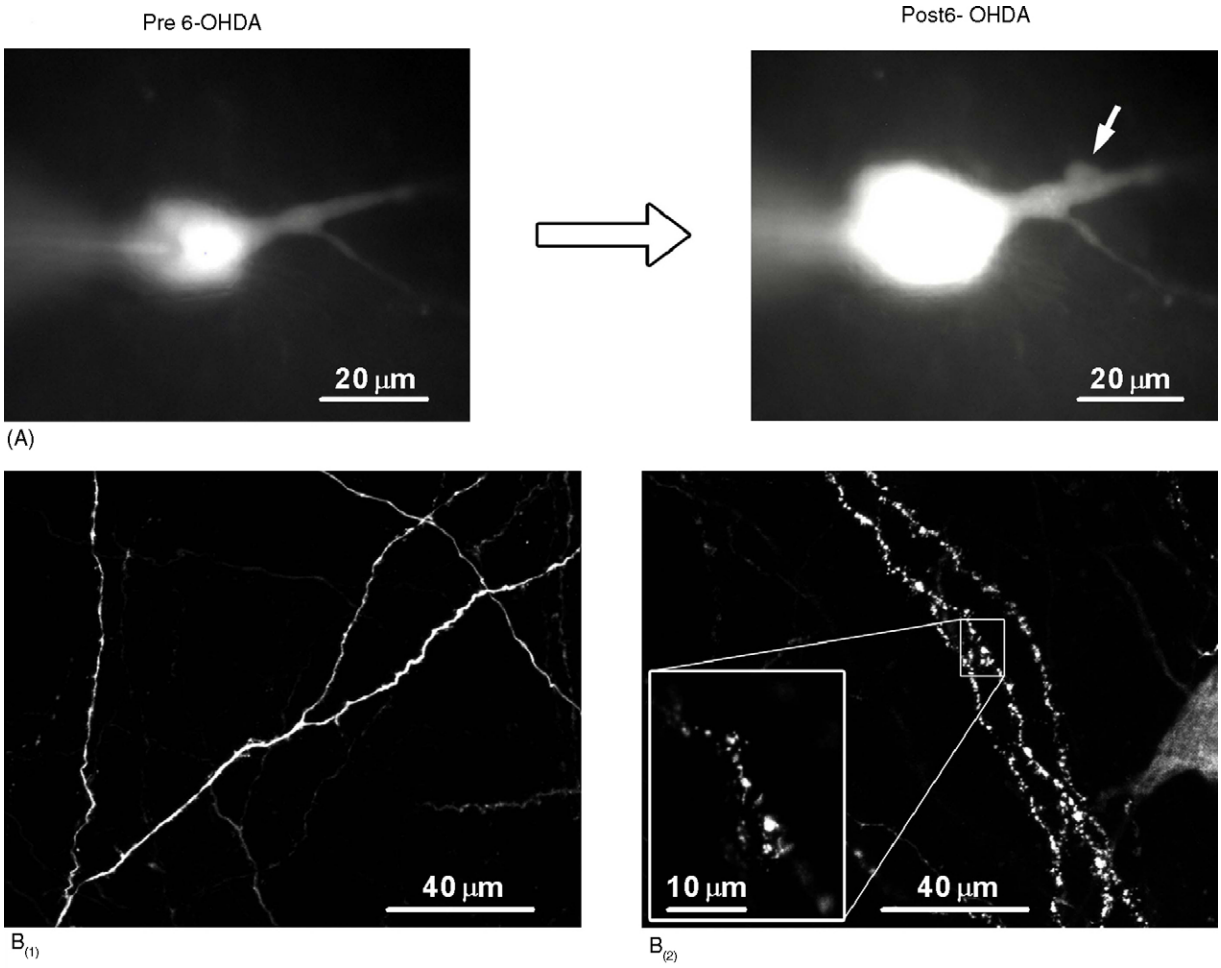

Fig. 8. 6-OHDA-induced damage to dendrites of SNc neurons. A (left): dopaminergic SNc neuron loaded with lucifer yellow via patch pipette before exposure to 6-OHDA for 10 min. A (right): same cell 20 min after onset of toxin exposure (1.0 mM). Note swelling of the cell body and membrane blebbing (arrow) on a proximal dendrite. B1 and 2: confocal projections (30 μm depth) of control section (B1) and section exposed to 6-OHDA (B2; 1 mM, 20 min) showing dendrites loaded with dextran-rhodamine projecting into the Substantia Nigra pars reticulata. Note that toxin-exposed dendrites display a fragmented morphology. Inset in B2 shows a single optical section with enlargement of the boxed region.

The effects of 6-OHDA on whole-cell currents (and cell membrane resistance) were largely permanent within 30 min of recording after toxin application. This irreversibility was dependent on DAT as recovery was seen after this transporter has been pharmacologically blocked. Similar prolonged inhibition has been described in these neurons following exposure to rotenone (Liss and Roeper, 2001). Thus, DA SNc neurons remain hyperpolarized for a relatively long period when subject to stress. Short lasting hyperpolarization and inhibition of firing is normally regarded as a form of cellular defense by which energy is conserved due to reduced ATP consumption by Na/K ATPase. However, a prolonged hyperpolarization may also lead eventually to cell death, in spite of the fact that it prevents calcium influx through voltage-gated Ca^{2+} channels. In DA SNc neurons, which strongly express HCN channels (particularly HCN-3; Notomi and Shigemoto, 2004), hyperpolarization causes an increased Na^{+} influx due to activation of the I_h conductance (Knopfel et al., 1998). Insufficient energy for maintenance of ionic homeostasis may be available due to concomitant impairment of mitochondrial function. Alternatively, Liss and Roeper (2001) suggested that the prolonged hyperpolarization may lead to reduced expression of activity-dependent genes that promote survival of DA SNc neurons.

A novel observation is that 6-OHDA evoked a prominent increase of intracellular free calcium, indicating a rapid impairment of intracellular calcium homeostasis. The main source of increased Ca^{2+} appears to be mitochondrial, as neither removal of this ion from the extracellular medium nor pharmacological blockade of endoplasmic reticulum Ca^{2+}-ATPase with CPA prevented the rise in calcium signal. This conclusion is supported by previous reports which described oxidative stress associated with a rise in reactive oxygen species (particularly hydrogen peroxide, Blum et al.,

2001) and a collapse of the mitochondrial membrane potential in toxin-exposed midbrain DA neurons (Lotharius et al., 1999). It should be noted that the 6-OHDA-induced rise in intracellular calcium did not return to baseline after toxin washout, which indicates a sustained effect on calcium regulation.

At higher doses permanent toxic effects were also reflected in permanent drop of membrane capacitance (C_m), which indicated a decrease in the total surface area of the cell membrane. Decrease in C_m occurred in spite of swelling of the cell bodies, suggesting a prevailing effect on dendrites. This was confirmed by imaging of neurons filled with LY or dextran-rhodamine, which demonstrated signs of dendritic damage in the form of cell membrane blebs and fragmentation. These findings are consistent with data from the literature indicating that dendrites are more susceptible to toxic and other forms of damage than cell bodies. With respect to 6-OHDA, rapid (after 2-h exposure; 0.1 mM) loss of dendrites of SNc neurons was previously reported in acute brain slices obtained from 3-week-old rats by Bywood and Johnson (2000). Our study confirms and extends this observation by showing that the morphological changes in the dendrites occurred almost simultaneously with pronounced changes in the electrophysiological properties and Ca^{2+} homeostasis of DA SNc neurons within minutes of toxin exposure.

It has been proposed that the apparently specific action of 6-OHDA on catecholaminergic neurons is due to the uptake and accumulation of this toxin by DAT in dopaminergic neurons (and by noradrenaline transporter, NET, in noradrenergic neurons) (Blum et al., 2001), as inhibition of the transporter prevents toxic loss of DA neurons (Ding et al., 2004; Gonzalez-Hernandez et al., 2004; Nass et al., 2002; Van Kampen et al., 2000). This idea has, however, been questioned following reports that 6-OHDA does not always enter neurons through DAT (or NET) to exert it toxic effects. These include studies showing that: (a) intracerebral injections of this toxin can result in lesions of both catecholaminergic and non-catecholaminergic neurons (Poirier et al., 1972; (b) in cultures, 6-OHDA toxicity is not selective for dopaminergic neurons (Dodel et al., 1999; Lotharius et al., 1999; Michel and Hefti, 1990; but see Ding et al., 2004); (c) blockers of catecholamine transporters do not always prevent cytotoxocity (see below); (d) even at high concentrations 6-OHDA does not accumulate in some cells expressing the transporters (Decker et al., 1993); (e) catalase, to which cell membranes are not permeable, protects strongly against 6-OHDA toxicity (Abad et al., 1995; Yamada et al., 1997); and (f) 6-OHDA is taken up even by non-neuronal tissue (Jonsson and Sachs, 1971). Thus, it is not clear to what degree toxicity can be ascribed to entry of 6-OHDA into cells via DAT, and how much to its extracellullarly generated oxidation products such as quinones or free radicals. Our results obtained in midbrain slices showed that the effects of 6-OHDA were highly selective for DA SNc neurons, while non-DA neurons in the same area did not respond to the toxin as judged by the firing frequency (data not shown), holding current and intracellular calcium signal. However, the early effects of 6-OHDA on DA SNc neurons could not be entirely ascribed to the entry of 6-OHDA via DAT, since the DAT antagonists nomifensine and cocaine reduced but did not prevent toxin-induced inhibition of firing and Ca^{2+} rise. This conclusion is consistent with other studies which showed that the toxic effects of 6-OHDA were only partially prevented by inhibition of catecholamine uptake into chromaffin cells (Abad et al., 1995) and that the specific DAT inhibitor GBR 12909 did not protect dopaminergic neuroblastoma cell lines against 6-OHDA (Storch et al., 2000).

These results indicate that the relatively selective toxic effects of 6-OHDA on DA SNc neurons are due to extraordinary sensitivity of these neurons to oxidative stress (as compared with non-DA neurons), and that both the intracellular action of the DAT-translocated toxin and other, non-DAT-mediated effects contribute to cell death. This difference in vulnerability to oxidative stress may explain our finding that the toxin did not affect calcium levels in non-DA neurons. On the other hand, non-DAT-mediated effects of 6-OHDA may have been responsible for the $[Ca^{2+}]_i$ rise and whole-cell outward currents in DA neurons after DAT blockade by cocaine.

Finally, a comment has to be made regarding the toxin concentration used in our study (0.5–1.0 mM in most experiments). Although the concentration was higher than that used in many previous studies conducted with cell cultures, it was over an order of magnitude lower than that often used for lesions of DA SNc neurons in vivo (e.g., 24 μg in 8 μl [≈15mM] in the study by Costantini et al., 2001; and 20 μg in 4 μl [≈25 mM] by Sauer and Oertel, 1994).

In conclusion, we studied the cellular effects of 6-OHDA over a short timescale (≤30 min) with electrophysiological, calcium imaging and anatomical techniques in brain slices containing identified DA neurons of the SNc. A number of rapid, dose-dependent effects were observed including inhibition of firing, cell membrane hyperpolarization, intracellular calcium increase and, at high concentration, disruption of the

cell membrane. Most of the responses were irreversible, except after blockade of dopamine transporter, suggesting a predominant action of toxin transported intracellularly via DAT. A novel mechanism involving D2 receptor activation was also demonstrated. Although the toxin-induced sustained increase in $[Ca^{2+}]_i$ may be the key element in triggering the cell death process, further studies with longer recording times are needed to explore the cellular consequences of prolonged membrane hyperpolarization following toxin administration.

ACKNOWLEDGEMENTS

This work was supported by grant RF03.182 from the Ministry of Health (Italy) and by 81439 grant from the Auckland Medical Research Foundation (N.Z.). P. Freestone is a Miller Scholar of the N.Z. Neurological Foundation.

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