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Research Paper

Paradoxical lower sensitivity of Locus Coeruleus than Substantia Nigra *pars compacta* neurons to acute actions of rotenone

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ABSTRACT

Parkinson's disease (PD) is not only associated with degeneration of dopaminergic (DAergic) neurons in the Substantia Nigra, but also with profound loss of noradrenergic neurons in the Locus Coeruleus (LC). Remarkably, LC degeneration may exceed, or even precede the loss of nigral DAergic neurons, suggesting that LC neurons may be more susceptible to damage by various insults. Using a combination of electrophysiology, fluorescence imaging and electrochemistry, we directly compared the responses of LC, nigral DAergic and nigral non-dopaminergic (non-DAergic) neurons in rat brain slices to acute application of rotenone, a mitochondrial toxin used to create animal and *in vitro* models of PD. Rotenone (0.01–5.0 μM) dose-dependently inhibited the firing of all three groups of neurons, primarily by activating K_{ATP} channels. The toxin also depolarised mitochondrial potential (Ψ_m) and released reactive oxygen species (H_2O_2). When K_{ATP} channels were blocked, rotenone (1 μM) increased the firing of LC neurons by activating an inward current associated with dose-dependent increase of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). This effect was attenuated by blocking oxidative stress-sensitive TRPM2 channels, and by pre-treatment of slices with anti-oxidants. These results demonstrate that rotenone inhibits the activity of LC neurons mainly by activating K_{ATP} channels, and increases $[Ca^{2+}]_i$ *via* TRPM2 channels. Since the responses of LC neurons were smaller than those of nigral DAergic neurons, our study shows that LC neurons are paradoxically less sensitive to acute effects of this parkinsonian toxin.

1. Introduction

Although Parkinson's disease (PD) is primarily regarded as a motor disorder, patients also suffer from a range of non-motor symptoms which cause significant disability and impaired quality of life (Shulman et al., 2002). These non-motor symptoms often occur years before the onset of motor dysfunction (Langston, 2006) and are associated with degeneration of noradrenaline (NA)-producing neurons in the Locus Coeruleus (LC) (Delaville et al., 2011). The LC is profoundly affected in PD (Bertrand et al., 1997; German et al., 1992; Patt and Gerhard, 1993), and degeneration of LC neurons can exceed the loss of dopaminergic (DAergic) neurons of the Substantia Nigra *pars compacta* (SNc) (Perry et al., 1990; Zarow et al., 2003). Furthermore, Lewy bodies accumulate in the LC before their occurrence in the SNc (Braak et al., 2003; Del Tredici et al., 2002), consistent with early development of non-motor symptoms. Together, this suggests that LC neurons are more vulnerable to damage than nigral DAergic neurons.

Rotenone, a potent inhibitor of mitochondrial complex I, is a widely used pesticide (Degli Esposti, 1998) which has been linked to increased prevalence of PD (Dhillon et al., 2008; Tanner et al., 2011). Interest in rotenone was bolstered by its ability to reproduce behavioural, biochemical and pathological features of PD in rodents (Betarbet et al., 2000; Pan-Montojo et al., 2010), causing relatively selective degeneration of both LC and nigral DAergic neurons (Chaves et al., 2010; Hoglinger et al., 2003; Lin et al., 2008). In comparison, nigral non-dopaminergic (non-DAergic) neurons are less affected by rotenone (Radad et al., 2008) and resist degeneration in PD (Patt et al., 1991). Previous studies have investigated acute effects of rotenone on nigral DAergic neurons (Freestone et al., 2009; Liss et al., 1999; Liss et al., 2005; Roper and Ashcroft, 1995), but its actions in the LC are unknown. Rotenone activates ATP-sensitive K^+ (K_{ATP}) channels in nigral DAergic neurons by decreasing production of ATP (Dukes et al., 2005) and/or increasing generation of reactive oxygen species (ROS) (Freestone et al., 2009; Radad et al., 2006). In addition, the toxin elevates cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in these neurons by activating oxidative stress-sensitive, transient receptor potential melastatin-2 (TRPM2) channels (Freestone et al., 2009; Mrejeru et al., 2011). Since LC neurons express K_{ATP} channels

Abbreviations: ACA, N-(p-amylcinnamoyl) anthranilic acid; DA, dopamine; DAergic, dopaminergic; FSCV, fast-scan cyclic voltammetry; K_{ATP}, ATP-sensitive potassium channel; LC, Locus Coeruleus; NA, noradrenaline; NAergic, noradrenergic; Non-DAergic, non-dopaminergic; PD, Parkinson's disease; SNc, Substantia Nigra *pars compacta*; SNr, Substantia Nigra *pars reticulata*; TRPM2, transient receptor potential melastatin-2 channel; TH, tyrosine hydroxylase; ROS, reactive oxygen species.

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(Karschin et al., 1997) and TRPM2 mRNA (Cui et al., 2011), rotenone may exert similar effects on these neurons. The present study characterised the early effects of rotenone on LC neurons, and directly compared these responses with those evoked in nigral DAergic and non-DAergic neurons. Contrary to our initial hypothesis that LC neurons are more sensitive to this parkinsonian toxin, our data show that cellular effects were smaller in LC neurons compared to nigral DAergic neurons.

2. Methods and materials

2.1. Tissue preparation

All experiments were approved by the Animal Ethics Committee of the University of Auckland, in accordance with the New Zealand Government Animal Welfare Act. Transverse slices (250 μm) containing the LC or Substantia Nigra (SN) were obtained from P15–19 Wistar rats (n = 117) as described previously (Freestone et al., 2009; Guatteo et al., 2013; Williams and Marshall, 1987). Brain slices were submerged in carbogenated (5% CO_2 in O_2) artificial cerebrospinal fluid (ACSF), containing (mM): 127 NaCl, 3 KCl, 2 $CaCl_2$, 1.25 NaH_2PO_4, 2 $MgSO_4$, 10 glucose, 26 $NaHCO_3$ (290 mOsm/L, pH 7.4, 34 °C). In some experiments Ca^{2+} was replaced by Mg^{2+}, with addition of 1 mM EGTA ('zero'-Ca^{2+} ACSF). Slices were placed in a recording chamber (Warner Instr.; ~1 mL) and superfused at 3.5 mL/min during electrophysiological, fluorescence and electrochemical measurements.

2.2. Electrophysiology

Extracellular single-unit recordings were conducted using AC amplifiers (NL104, NeuroLog; 0.05–5 kHz) and glass microelectrodes (5–10 MΩ) filled with (mM): 145 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 5 glucose. Multiple simultaneous recordings were conducted from the same slice using independent microelectrodes (Guatteo et al., 2013). Noradrenergic LC neurons (n = 136) were identified by their slow firing (1.35 ± 0.05 Hz), relatively irregular firing rate (inter-spike interval coefficient of variation, CV: 14.3 ± 0.8%), long-duration action potentials (2.81 ± 0.11 ms), and inhibitory response to NA (30 μM). In midbrain slices, nigral DAergic neurons (n = 88) fired slow (1.89 ± 0.06 Hz) and regular (CV: 5.38 ± 0.55%) action potentials with long duration (2.39 ± 0.10 ms). In comparison, nigral non-DAergic neurons (n = 44) recorded in the Substantia Nigra *pars reticulata* (SNr), fired faster (7.59 ± 0.56 Hz) and less regular (CV: 8.9 ± 1.3%) action potentials with relatively short duration (1.06 ± 0.05 ms). Distinctively, the firing of nigral DAergic neurons was inhibited by DA (30 μM), while the activity of non-DAergic neurons was enhanced.

Whole-cell patch clamp recordings were conducted under infrared differential interference contrast (IR-DIC; Nikon Eclipse E600FN) using a Multiclamp 700B amplifier (Molecular Devices) with patch pipettes (2–5 MΩ) filled with (mM): 135 K^+- or Cs^+-methanesulfonate, 2 $MgCl_2$, 10 HEPES, 0.1 EGTA, 0.045 $CaCl_2$, 2 ATP-Mg^{2+}, 0.3 GTP-Na^+ (280 mOsm/L, pH 7.3). In voltage-clamp experiments, neurons were held at −60 mV after correcting for liquid junction potential (7.5–8.0 mV). Whole-cell currents were normalised to cell capacitance (pA/pF). LC neurons (n = 161) were identified under IR-DIC as densely packed, medium-sized cells (diameter > 20 μm) which showed spike frequency adaptation following depolarising current injection (50 pA, 800 ms) and linear responses to hyperpolarising voltage steps (−70 to −110 mV, 800 ms). Nigral DAergic neurons (n = 57) also showed spike frequency adaptation, but displayed voltage- and time-dependent I_h current in response to hyperpolarising voltage steps. These features were not observed in nigral non-DAergic neurons (n = 29), which were also distinguished by their smaller size (10–15 μm, compared to >20 μM DAergic neurons).

2.3. Measurement of $[Ca^{2+}]_i$ and $\Delta\Psi_m$

Changes of cytosolic free Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) were measured in voltage-clamped neurons using the ratiometric dye, Fura-2 (pentapotassium salt; 250 μM; Invitrogen), loaded through the patch pipette (Cs^+-based pipette solution). Neurons were illuminated with a xenon lamp (175 W; Sutter Instruments) and filter wheel to provide 340 and 380 nm excitation wavelengths (Optospin; Cairn Research). Background subtracted fluorescence ratios (340/380 nm) were converted to $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985).

Changes of mitochondrial membrane potential ($\Delta\Psi_m$) were measured in voltage-clamped neurons with Rhodamine-123 (Rh-123; 26.6 μM) loaded through the patch pipette (K^+-based pipette solution; excitation: 535/25 nm; emission: 590 nm) (Schuchmann et al., 2000). Background subtracted changes of fluorescence were normalised to baseline (ΔF/F) and converted to Ψ_m using a two-point calibration (Freestone et al., 2009; Schuchmann et al., 2000). Maximal Ψ_m hyperpolarisation (−200 mV) was induced by oligomycin (10 μg/mL) and maximal depolarisation (−60 mV) by FCCP (5 μM).

2.4. Electrochemistry

Changes of extracellular H_2O_2 concentration ($\Delta[H_2O_2]_o$) were measured using fast-scan cyclic voltammetry (FSCV) (Spanos et al., 2013). Carbon fibre microelectrodes were made using a carbon fibre (diameter 7 μm; GoodFellow, Cambridge Ltd.) aspirated into a glass capillary. A tight seal was formed using a horizontal puller (Narishige) and the exposed carbon fibre was trimmed to ~100 μm. FSCV was performed using Demon Voltammetry and Analysis software (Yorgason et al., 2011), National Instrument cards (NI-DAQ: 2× PCI-6221) and a Chem-Clamp amplifier (Dagan Corporation). Electrode scanning followed a triangular waveform (−0.4 V to 1.4 V, 400 V/s) at 10 Hz. Electrodes were pre-treated with isopropyl alcohol (10 min) before insertion into the slice at a depth of ~100 μm.

2.5. Immunohistochemistry

Animals were deeply anesthetized with chloral hydrate (400 mg/kg) and fixed by transcardial perfusion of PBS followed by paraformaldehyde (4% in PBS). Brain sections (50 μm) were cut by vibratome (Leica, 1000S). Cell membranes were permeabilised with Triton X-100 (1%) in PBS, and non-specific binding was blocked with bovine serum albumin (BSA; 5% in PBS; Medica). Sections were incubated overnight (4 °C) with primary antibodies against tyrosine hydroxylase (TH; mouse anti-TH; 1/1000; Millipore) and TRPM2 (rabbit anti-TRPM2; 1/500; Novus Biologicals), followed by secondary antibodies (2 h, room temperature): Alexa 488-conjugated goat anti-mouse (1/500; Invitrogen) and Alexa-568 goat anti-rabbit (1/500; Invitrogen). Sections were mounted in ProLong Gold (Invitrogen) before confocal imaging (FV1000; 60×, NA 1.35).

2.6. Drugs and reagents

Unless stated otherwise, drugs and reagents were purchased from Sigma-Aldrich. Concentrated stock solutions (250×) of drugs were applied using computer-controlled syringe pumps (WPI) near the inlet of the recording chamber at 14 μL/min (i.e. 1/250 of main flow rate: 3.5 mL/min). Stock solutions were either dissolved in distilled H_2O or DMSO (<0.1% after dilution in bath).

2.7. Data analysis

Electrophysiological data were analysed with Clampfit (v.10.2; Molecular Devices), fluorescence signals with Imaging Workbench (v.5.2; Indec Biosystems), and electrochemical data with Demon Voltammetry and Analysis software (Yorgason et al., 2011) using principal