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Effects of the Parkinsonian toxin MPP$^+$ on electrophysiological properties of nigral dopaminergic neurons

Andrew G. Yee [a,c,1], Sun-Min Lee [a,c,1], Morag R. Hunter [b,c], Michelle Glass [b,c], Peter S. Freestone [a,c], Janusz Lipski [a,c,*]

[a] Department of Physiology, Faculty of Medical and Health Sciences, University of Auckland, Auckland 1142, New Zealand
[b] Department of Pharmacology, Faculty of Medical and Health Sciences, University of Auckland, Auckland 1142, New Zealand
[c] Centre for Brain Research, Faculty of Medical and Health Sciences, University of Auckland, Auckland 1142, New Zealand

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ABSTRACT

Although MPP$^+$ (1-methyl-4-phenylpyridinium) has been widely used to damage dopaminergic neurons of the Substantia Nigra pars compacta (SNc) and produce animal and cellular models of Parkinson's disease, the action of this toxin on ion channels and electrophysiological properties of these neurons remains controversial. Previous work has attributed the early effects of MPP$^+$ on the membrane potential and firing frequency of SNc neurons either to block of hyperpolarisation-activated (I_h) current, or to activation of ATP-sensitive K^+ (K_{ATP}) channels. Using a combination of electrophysiological and pharmacological techniques, we investigated the acute effects of MPP$^+$ (20 μM) on SNc neurons in rat midbrain slices. Our results show that MPP$^+$ inhibits the activity of these neurons in distinct stages involving different mechanisms. The early phase of inhibition was dependent on D2 autoreceptors, but [^{3}H]raclopride membrane binding and cAMP production assays demonstrated that the toxin (0.001–100 μM) did not directly bind to these receptors nor activated the G_i-linked signalling pathway. Depletion of vesicular dopamine with Ro4-1284 attenuated the early inhibitory effect, indicating that D2 autoreceptors were activated by dopamine released from the somato-dendritic region. After longer exposure (>10–20 min), MPP$^+$ produced a late phase of inhibition which mainly involved activation of K_{ATP} channels, and required uptake of the toxin *via* dopamine transporter. Although I_h current mediated by hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels was reduced by MPP$^+$, neither inhibition of firing nor membrane potential hyperpolarisation was significantly attenuated by blocking HCN channels with ZD7288. Our results indicate that the initial cellular events that lead to activation of cell death pathways by MPP$^+$ are complex and include K_{ATP}, and dopamine-dependent components, and show that the inhibitory effect of the toxin is independent of I_h block.

1. Introduction

MPP$^+$ (1-methyl-4-phenylpyridinium), the active metabolite of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), is a neurotoxin capable of producing relatively selective loss of nigral dopaminergic neurons and severe parkinsonism in humans (Langston et al., 1999). As similar effects can be reproduced in non-human primates and rodents, MPTP exposure or direct MPP$^+$ application have become widely used to create animal and cellular models of Parkinson's disease (*eg.* Jackson-Lewis and Przedborski, 2007; Smeyne and Jackson-Lewis, 2005; Tipton and Singer, 1993). MPP$^+$ is taken up by dopaminergic neurons through dopamine transporter (DAT; Javitch et al., 1985; Schultz et al., 1986) and stored in vesicles by vesicular monoamine transporter 2 (VMAT2; Speciale et al., 1998). The toxin also accumulates in mitochondria where it

Abbreviations: ACSF, artificial cerebrospinal fluid; ATP, adenosine triphosphate; DA, dopamine; DAT, dopamine transporter; GIRK, G protein-coupled inwardly rectifying potassium channels; HCN, hyperpolarisation-activated cyclic nucleotide-gated channels; I_h, hyperpolarization-activated current; K_{ATP}, ATP-sensitive potassium channels; K_{Ca}, calcium-activated potassium channels; LC, locus coeruleus; MPP$^+$, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ROS, reactive oxygen species; SNc, Substantia Nigra pars compacta; TTX, tetrodotoxin; VMAT2, vesicular monoamine transporter 2; VTA, ventral tegmental area.

* Corresponding author at: Department of Physiology, Faculty of Medical and Health Sciences, University of Auckland, Auckland 1142, New Zealand.
E-mail addresses: andrew.yee@auckland.ac.nz (A.G. Yee), slee544@aucklanduni.ac.nz (S.-M. Lee), m.hunter@auckland.ac.nz (M.R. Hunter), m.glass@auckland.ac.nz (M. Glass), peter.s.freestone@gmail.com (P.S. Freestone), j.lipski@auckland.ac.nz (J. Lipski).
[1] The first two authors contributed equally to this work.

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inhibits Complex I of the electron transport chain (Ramsay and Singer, 1986), resulting in superoxide production and reduced ATP synthesis (Chan et al., 1991; Zawada et al., 2011). In addition, MPP^+ can open the mitochondrial permeability transition pore, release cytochrome *c*, and activate pro-apoptotic and/or necrotic pathways (Cassarino et al., 1999; Nicotra and Parvez, 2002), as well as displace and oxidize vesicular dopamine (DA; Lotharius and O'Malley, 2000).

The toxic effects of MPTP/MPP^+ are remarkably fast, as indicated by the fact that Parkinsonian symptoms develop in humans within days of drug intake (Ballard et al., 1985), and that severe striatal DA depletion and damage of dopaminergic neurons within the Substantia Nigra pars compacta (SNc) occur within 0.5–2 h after MPP^+ exposure in rats (Turski et al., 1991; Ambrosio et al., 1996; cf. also Lotharius and O'Malley, 2000). However, in spite of the importance of understanding the early cellular events that lead to nigro-striatal damage, the mechanism of these changes remains controversial. It has previously been shown that MPP^+ inhibits pacemaker activity of SNc neurons due to mitochondrial Complex I inhibition and activation of ATP-sensitive potassium (K_{ATP}) channels, and that silencing of these neurons through K_{ATP} channel activation promotes neural damage (Liss et al., 2005). However, a recent study reported that MPP^+-induced cell membrane hyperpolarisation and rapid inhibition of firing of SNc neurons do not involve K_{ATP} channel activation, and that these effects solely depend on the direct ability of MPP^+ to block hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels (Masi et al., 2013).

In light of this controversy, we aimed to reassess the contribution of HCN and potassium channels to the electrophysiological effects of MPP^+ on dopaminergic SNc neurons, to provide better insight into the mechanisms initiating its toxic action.

2. Materials and methods

2.1. Tissue preparation

All experimental procedures were approved by the Animal Ethics Committee of the University of Auckland. Following brief CO_2 anaesthesia, the brain was quickly removed from P17–24 Wistar rats and placed in cooled (5–8 °C), carbogenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) containing (mM): 127 NaCl, 3 KCl, 2 $CaCl_2$, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 10 glucose (290 mOsm L^{-1}, pH 7.4). Transverse or horizontal midbrain slices (250 μm) containing the SNc were cut with a vibratome (VT1200S, Leica). After a minimum incubation period (40 min, 34 °C), a single slice was transferred to a recording chamber (volume 0.6 mL) and superfused with ACSF (3.5 mL/min, 34 °C) for electrophysiological recording.

2.2. Electrophysiology

Conventional extracellular (single-unit) and intracellular recordings were performed as described (Guatteo et al., 2013). In extracellular recordings conducted in transverse midbrain slices, dopaminergic SNc neurons were identified using the following criteria: (a) location along the ventral border of the medial lemniscus; (b) slow and regular firing frequency (1.7 ± 0.2 Hz, interspike interval coefficient of variation $3.4 \pm 0.4\%$); (c) long spike duration (2.1 ± 0.2 ms); and (c) inhibitory response (ΔHz: -1.2 ± 0.1) to bath applied DA (30 μM, 45 s). Neurons classified as non-dopaminergic were in a similar location but displayed a higher firing rate (6.6 ± 0.7 Hz), higher coefficient of variation ($7.0 \pm 0.9\%$), shorter spike duration (1.7 ± 0.3 ms), and were not inhibited by DA.

The criteria used for identification of dopaminergic neurons in intracellular recordings conducted in horizontal slices were as follows: (a) location next to the medial terminal nucleus of the accessory optic tract; (b) depolarising 'sag' in response to hyperpolarising current steps (−250 pA, 450 ms); (c) spike frequency adaptation during depolarising current steps (50–150 pA, 450 ms); and (d) cell membrane hyperpolarisation and inhibition of firing in response to DA application. Cell membrane potential and input resistance (R_{in}) were measured in the presence of tetrodotoxin (TTX, 0.5 μM). R_{in} was calculated from the initial voltage drop evoked by small hyperpolarising current pulses (50–150 pA, usually 150 ms) applied at 0.1 Hz. Sag amplitude (see Fig. 2C) was measured using stronger and longer duration pulses to promote activation of HCN channels.

Whole-cell patch clamp recordings were conducted in horizontal slices as described previously (Freestone et al., 2009). Patch pipettes (2–5 MΩ) were filled with (mM): 135 K-methanesulphonate, 2 $MgCl_2$, 10 HEPES, 0.1 EGTA, 0.045 $CaCl_2$, 2 ATP-Mg^{2+}, 0.3 GTP-Na^+ (280 mOsm L^{-1}, pH 7.3). In addition to the previously listed criteria used in intracellular recordings, dopaminergic neurons were identified by: (a) large (diameter ≥20 μm), multipolar appearance when visualised with infrared differential interference contrast; and (b) in voltage-clamp, activation of I_h current in response to negative voltage commands from −40 (V_{hold}) to −120 mV (20 mV, 1 s steps).

2.3. [^{3}H]raclopride binding and cAMP assays

HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS) and appropriately selected antibiotics, and grown at 37 °C with 5% CO_2 in a humidity-controlled incubator. Cells were stably transfected with the recombinant human D2 (long isoform; GeneBankNM_000795) receptor, N-terminally tagged with FLAG in pcDNA3.1+, and selected with 400 μg/ml G418 (Gibco, Life Technologies). Cells were suspended in sucrose buffer (mM: 200 sucrose, 50 Tris–HCl, 5 $MgCl_2$ and 2.5 EDTA) and membranes were disrupted using a glass homogeniser. Following removal of the nuclear pellet by centrifugation (1000 × *g*), membranes were isolated by further centrifugation (20,000 × *g*), suspended in sucrose buffer and protein concentration was measured using a DC Protein Assay (Bio-Rad), prior to storage at −80 °C.

Membrane binding assays were performed using 20 μg of membrane, 2.5 nM [^{3}H]raclopride (Perkin Elmer) and the test ligand in 200 μL of assay buffer (mM: 50 HEPES, 1 $MgCl_2$, 1 $CaCl_2$, 0.2% BSA; pH 7.4). After 1 h incubation at 30 °C, triplicate samples were transferred to G/C glass fibre filter plates (Perkin Elmer), vacuum filtered and washed with a buffer (mM: 50 HEPES, 500 NaCl, 0.1% BSA; pH 7.4). Scintillation fluid (Irgasafe, Perkin Elmer) was applied and samples were read for 2 min per well in a Wallac 1450 MicroBeta TriLux. Production of cAMP was measured using the CAMYEL cAMP biosensor (Jiang et al., 2007). Transient transfection of the CAMYEL biosensor, cell stimulation and assay detection were performed as described (Cawston et al., 2013). In order to see D2-mediated inhibition of cAMP production, adenylate cyclase activity was stimulated by application of forskolin (5 μM).

Membrane binding and cAMP assay data were analysed using Prism (v6, GraphPad) and Statistica (v10, StatSoft) software. For membrane binding experiments, corrected counts per minute were plotted against the logarithm of displacer concentrations, and fitted with a one site competition curve. Binding in the absence of displacer and filter blank values were measured with no test ligand and no membrane, respectively. Area under the curve of inverse bioluminescence resonance energy transfer ratios (460/535 nm) was conducted to analyse changes in cAMP production as described (Cawston et al., 2013).

2.4. Drugs

MPP^+ iodide, DA, tolbutamide, glibenclamide, sulpiride, cocaine, raclopride, and Ro4-1284 were obtained from Sigma-Aldrich.