

Rotenone and MPP⁺ preferentially redistribute apoptosis-inducing factor in apoptotic dopamine neurons

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Rotenone and 1-methyl-4-phenylpyridinium produce parkinsonian models and we determined whether their mitochondrially mediated actions differentially redistributed the apoptogenic proteins, apoptosis-inducing factor and cytochrome *c*. Cultured rat mesencephalic dopamine neurons were exposed to rotenone (30 nM) and 1-methyl-4-phenylpyridinium (300 μ M, 24 and 48 h) and apoptosis and mitochondrial redistribution of cytochrome *c* or apoptosis-inducing factor were quantified. Tyrosine hydroxylase-positive dopamine neurons underwent apoptosis (shrinkage, less neurites) and 40%

released apoptosis-inducing factor with rotenone (24 h), whereas cytochrome *c* release reached this value at 48 h when 70% of cells had released apoptosis-inducing factor-positive. 1-Methyl-4-phenylpyridinium produced similar redistribution patterns for both proteins. Preferential redistribution of apoptosis-inducing factor before cytochrome *c* in dopamine neurons indicates caspase-independent mitochondrial proapoptotic signalling predominates in these parkinsonian models. *NeuroReport* 18:307–312
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Introduction

Mitochondrial proapoptotic signalling makes important contributions to neuronal injury [1,2] and in parkinsonism there is evidence for mitochondrial oxidative stress both in experimental models [3,4] and in human postmortem brain [5]. Indeed, the toxins rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium (MPP⁺) exert a key component of their actions via inhibition of mitochondrial complex I and are known to injure dopamine (DA) neurons [6,7]. The 'intrinsic' pathway of caspase activation involves the release of proapoptotic proteins after permeabilization of the outer mitochondrial membrane with cytochrome *c* (cyt *c*) integrally involved in the formation of the 'apoptosome' complex [2]. Although various interventions affecting mitochondrial signalling can attenuate damage induced by MPP⁺ [8–10], the literature is divided as to whether rotenone and MPP⁺ exert their cytotoxic effects via caspase-dependent pathways [11,12]. In addition to mitochondria initiating caspase-dependent apoptosis via release of cyt *c*, they also can release apoptosis-inducing factor (AIF) that then translocates from cytosol to the nucleus inducing large-scale DNA fragmentation [13]. AIF seems to act in a caspase-independent manner [13,14]. Nonetheless, the exact pattern of recruitment of AIF in neuronal injury is not resolved [13], so its proapoptotic role in the injury of DA neurons remains unclear. Given that mitochondrial pro-

apoptotic signalling seems to involve differential interactions between caspase-dependent/caspase-independent downstream mechanisms, which appear to depend in part upon the nature of the insult and cellular type [13,14], we sought to determine the patterns of redistribution of these proapoptotic proteins induced by rotenone and MPP⁺ in mesencephalic dopamine neurons maintained in primary culture. On the basis of our application of a novel double immunocytochemical procedure [15], we report that the temporally dependent distribution of AIF precedes that of cyt *c* for both rotenone and MPP⁺.

Materials and methods

Primary mesencephalic cultures

Primary cultures of rat mesencephalic neurons were established from embryonic day 17–18 rats as described previously [16]. All procedures received ethical approval according to the guidelines of the National Health and Medical Research Council (Australia). After digestion and isolation, cells (0.4×10^6 cells/well) were resuspended in Neurobasal medium containing 2% B27, 25.4 mM KCl and 10% dialysed fetal calf serum (all from Gibco, Melbourne, Australia) in 24-well plates (Medos, Mt. Waverly, Australia) containing 13 mm round glass coverslips precoated with poly-D-lysine (1 mg/ml). At 24 h, the medium was replaced

with serum-free growth medium. Cultures were maintained in a humidified incubator (5% CO₂, 8.5% O₂ and 37°C) and half-medium changes were performed at 3–4 days *in vitro* (*div*). These cultures are essentially 'neuronal' (>85% of cells MAP-2-positive) with routinely 5% considered DA being tyrosine hydroxylase (TH)-positive (cf. [17]). At 6 *div*, cultures were exposed to MPP⁺ (300 μM) and rotenone (30 nM; both Sigma, Sydney, Australia) in antioxidant-free neurobasal medium for 24 or 48 h in parallel with vehicle-treated controls. These concentrations were chosen from preliminary experiments and with reference to established concentration responses for cell death, when injuries were slow in time course and apoptotic [16].

Cytochemistry

Vehicle-control and drug-treated cultures were fixed with 4% paraformaldehyde in 0.9% phosphate-buffered saline (PBS), pH 7.4, for 15 min, permeabilized with 0.1% Triton X-100 for 10 min and incubated with 1% bovine serum albumin (BSA) for 1 h at room temperature. Cells were then incubated with a rabbit anti-TH antibody (1:500; Boehringer Mannheim, Sydney, Australia) for 48 h at 4°C followed by a secondary antibody conjugated to Alexa 488 (diluted 1:200; Invitrogen-Molecular Probes, Melbourne, Australia) overnight at 4°C. Neurons were again incubated with 1% BSA for 1 h at room temperature, then incubated with antibodies for cyt c (1:100) or AIF (1:200; Apotech, Epalinge, Switzerland) for 48 h at 4°C and subsequently with a secondary antibody conjugated to Alexa 647 (diluted 1:200; Invitrogen-Molecular Probes, Melbourne, Australia) overnight at 4°C. In all steps except that involving incubation with BSA, the cells were rinsed two to three times with PBS. The nuclei of cells were counterstained with propidium iodide (PI, 5 μg/ml, 5 min; Invitrogen-Molecular Probes) to assess nuclear morphology. Following incubation with PI, cells were rinsed twice with PBS and coverslips were mounted on glass slides using Permafluor antifade mounting medium (Lipshaw, Pittsburgh, Pennsylvania, USA). Full details of immunostaining controls are given elsewhere [15].

Confocal microscopy

Immunostained samples were analysed by confocal laser scanning microscopy using either a Leica (Wetzlar, Germany) TCS-NT inverted confocal microscope equipped with a 40×/1.25 oil immersion lens and a ×63 water immersion lens, or an Olympus (Mount Waverly, Australia) FluoView 500 inverted confocal microscope with a ×60 water immersion lens. Details of imaging conditions for double-immunostained cultures have been given previously [15]. Cells were analysed for the distribution of apoptogenic proteins with double immunostaining for TH and either cyt c or AIF, as well as apoptotic nuclei via PI fluorescence [15].

Data analysis

For each cell type, values are the mean ± SEM. A total of five independent experiments were performed, and reported observations by confocal microscopy are from *n*=3 independent experiments each of which employed three to four culture wells. The total number of cells counted in each experiment was typically in the range of 150–300 cells per treatment. Analysis of variance, followed by a Bonferroni

post-hoc test, was performed to detect significant differences among treatments and *P*<0.05 was taken as statistically significant.

Results

Apoptotic changes in neurons induced by rotenone and 1-methyl-4-phenylpyridinium

Previously, we documented the concentration-dependent actions of rotenone and MPP⁺ in rat mesencephalic cultures using [³H]DA uptake and TH immunocytochemistry as indices of the integrity of DA neurons and found that the caspase-3 inhibitor, Z-DVED-FMK, partially attenuated injury [16]. TH-positive DA neurons possessed mainly rounded or oval somata, and exhibited extensive neuritic arbours (cf. [17]; Fig. 1). After exposure to rotenone and MPP⁺, TH-positive somata became more rounded with shrunken morphologies, and their neuritic arbours were greatly reduced in size and in some cases totally absent, notably at 48 h (Fig. 1). The injury induced by both complex I inhibitors was very slow in time course with the number of neurons exhibiting apoptotic morphology (cf. [16]), as monitored by PI labelling, being relatively low at 24 h and appreciably higher at 48 h postinsult (Figs 1 and 2). Our primary focus was on TH-positive neurons, and labelling of the nucleus with PI enabled a clear distinction between nonapoptotic and apoptotic DA neuronal profiles. In particular, apoptotic nuclei were generally smaller and even fragmented (in late apoptosis stage) compared with non-apoptotic nuclei and there was considerable increase in the intensity of PI fluorescence under these conditions because of condensation and aggregation of DNA. Following treatment with rotenone, apoptotic nuclei were detected in 18±4.2 and 30±4.0% of TH-positive neurons at 24 and 48 h, respectively. Comparable values (13±2.3%, 27±4.2%) were also observed in cells treated with MPP⁺ (Fig. 2a). TH-negative neurones within the same culture as TH-positive neurons were also analysed for apoptotic nuclei, and higher proportion of PI-labelled apoptotic nuclei were found in these cells, especially at 48 h (50±8.2%) with rotenone (Figs 1 and 2b).

Characterization of the release of cyt c and apoptosis-inducing factor from mitochondria

Independent analysis of cyt c or AIF immunofluorescence in samples double immunostained with TH identified two types of neurons: those demonstrating no release of either protein from the mitochondria where the immunofluorescence was predominantly cytoplasmic and the 'nuclear void' was clearly discernable, and those in which either protein was redistributed from mitochondria (cf. [15]). Neurons in the latter category generally displayed diffused cyt c or AIF immunofluorescence throughout the cell, including the nuclear region (i.e. the 'nuclear void' is barely visible in these cells). Moreover, these cells typically showed an apoptotic pattern of PI labelling (Fig. 1). Concurrent visualization of immunofluorescence for TH and cyt c, or TH and AIF, in the merged confocal images further reinforced the positive identification of individual TH-positive, DA neurons exhibiting redistribution of either apoptogenic protein from the mitochondria. Neurons were scored for nonrelease or release of either cyt c or AIF based on the above criteria irrespective of nuclear morphology, and nonapoptotic or apoptotic nuclei were scored indepen-

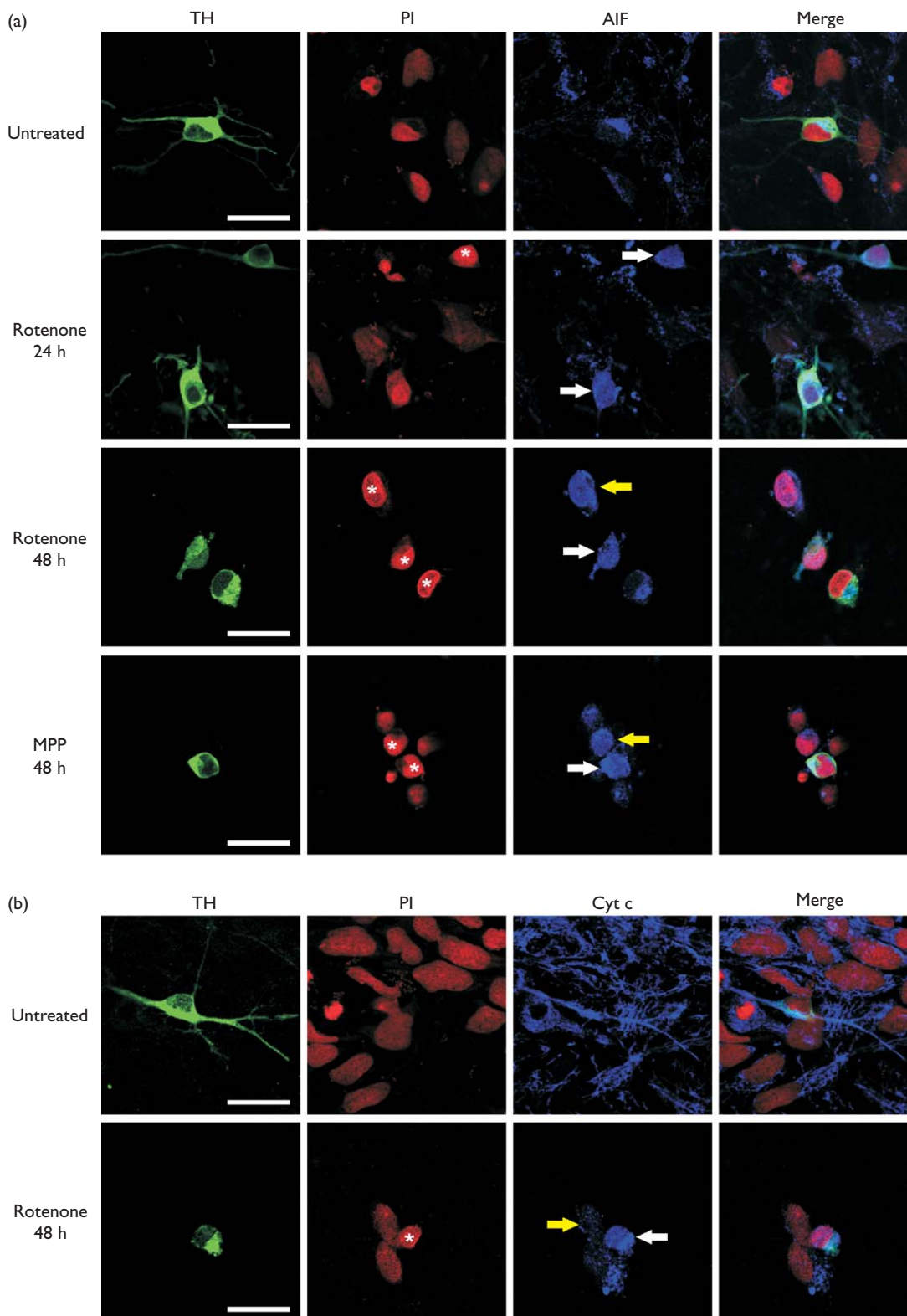


Fig. 1 In-situ detection of intracellular localization of apoptosis-inducing factor (AIF) and cytochrome c (cyt c) following treatment of neurons with rotenone or 1-methyl-4-phenylpyridinium (MPP⁺) for 24 and 48 h. (a) Control neurons, rotenone-and MPP⁺ treated neurons showing tyrosine hydroxylase (TH) immunofluorescence (green) and AIF immunofluorescence (blue). (b) Control neurons and rotenone-treated neurons showing TH immunofluorescence (green) and cyt c immunofluorescence (blue). For both (a, b), the nuclei were counterstained with propidium iodide (PI) (red) and examples of those that are apoptotic are marked with asterisks. Release of either AIF or cyt c from mitochondria to the cytosol is depicted by white arrows in TH-positive neurons and yellow arrows in TH-negative neurons. Note that in neurons in which each protein has redistributed to the cytosol, the nuclear PI fluorescence appears more or less pink in colour rather than red (except when there is a strong PI fluorescence relative to the blue immunofluorescence) owing to colocalization of the blue immunofluorescence and red PI fluorescence. Scale bar=20 μm.

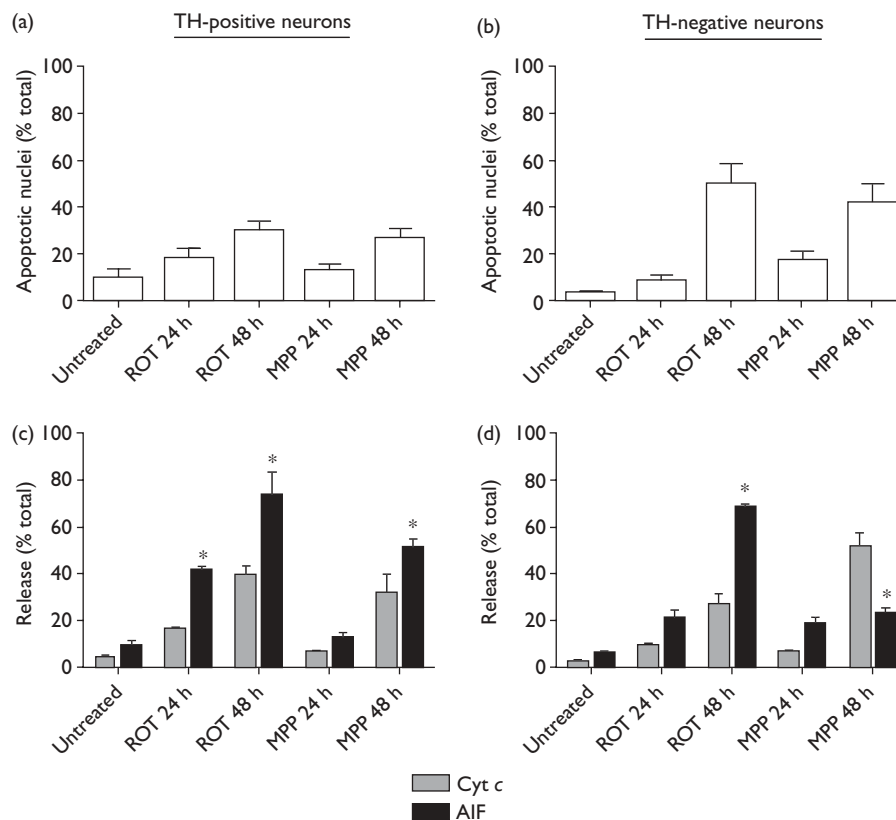


Fig. 2 Effects of rotenone and 1-methyl-4-phenylpyridinium (MPP⁺) on the release of apoptosis-inducing factor (AIF) and cytochrome c (cyt c) from mitochondria and downstream apoptotic nuclear changes. (a, b) Proportion of tyrosine hydroxylase (TH)-positive neurons and TH-negative neurons showing apoptotic nuclei as determined by propidium iodide labelling. Both treatments produced significant increases in the number of apoptotic nuclei ($P < 0.001$) but differences were not observed between neuronal populations. (c, d) Proportion of TH-positive neurons and TH-negative neurons scored for release of either AIF (black bars) or cyt c (grey bars) following treatment with rotenone or MPP⁺ for 24 and 48 h. In all cases, data for each treatment group represent the mean proportion of cells scored for a particular event out of the total number of cells counted, which was typically in the range 150–300 cells per experiment. Values are mean \pm SEM of three independent experiments. Both treatments produced significant increases in AIF and cyt c release, and the effect of rotenone was different to MPP⁺ on both populations of cells (all $P < 0.001$). Asterisks indicate significant differences ($P < 0.05$) between the proportion of neurons showing release of AIF relative to those showing release of cyt c.

dently of protein release. Quantitative analysis of cyt c and AIF release in TH-positive neurons revealed that AIF was released earlier than cyt c in the presence of rotenone and MPP⁺. Specifically, approximately $41 \pm 1.8\%$ of these neurons showed release of AIF, whereas $16 \pm 0.42\%$ showed release of cyt c at 24 h with rotenone, and by 48 h the respective proportions more or less doubled (73 ± 10 and $39 \pm 3.9\%$, respectively). In contrast, significant release of AIF relative to cyt c (i.e. 51 ± 4.0 vs. $31 \pm 8.3\%$) was only observed at 48 h of MPP⁺ treatment (Fig. 2c). Thus, rotenone appeared to induce a faster release of AIF compared with MPP⁺. In comparison, scoring of mitochondrial redistribution of cyt c and AIF amongst TH-negative neurons within the same population produced slightly different outcomes (Fig. 2d). Although AIF was again released earlier than cyt c in the presence of rotenone, the timing seems to be delayed in these neurons compared with that observed in TH-positive neurons, occurring at 48 h. Interestingly, cyt c was released before AIF following exposure to MPP⁺. These observations accord with evidence that both complex I inhibitors *in vitro* affect mitochondria of all cell types [18,19]. Additionally, our results suggest that different mechanisms of mitochondrial protein redistribution (at least in the case

of cyt c and AIF) may be activated in different neuronal cell types by rotenone and MPP⁺.

Discussion

A key finding here is that the apoptotic injury of mesencephalic DA neurons by rotenone and MPP⁺ involves the release of the mitochondrial proapoptotic protein AIF. Our confocal observations of the two proapoptotic proteins AIF and cyt c, in concert with immunocytochemistry for TH, allowed precise analyses of the relative timing of release of these two proteins from mitochondria in individual DA neurons, as well as in non-DA neurons. In contrast to cyt c, the classical marker of activation of the intrinsic mitochondrial pathway of caspase activation [2], AIF initiates a caspase-independent mode of apoptotic cell death following its release from the mitochondria and direct translocation to the nucleus where it affects large-scale cleavage of DNA [13,14]. In some modes of injury the release of AIF can precede that of cyt c, as found herein, and AIF can act on the mitochondrion to release cyt c, thus amplifying the proapoptotic signal [13]. Indeed, both oxidative insults used in this study, especially

rotenone, appeared to produce preferentially and more effectively the early mitochondrial redistribution of AIF relative to cyt c. As far as we are aware ours is the first report that rotenone releases both proapoptotic proteins in DA neurons in primary culture. In addition, our comparative study on the temporal patterns of release of cyt c and AIF between DA neurons and non-DA neurons also demonstrated apparent differences in the mechanisms governing the release of these proteins from mitochondria in different neuronal populations subjected to the same insult (cf. [14,22,23]).

Opinions vary as to whether injury induced by rotenone and MPP⁺ involves caspase-dependent and/or caspase-independent cellular injury [11,12,16,20,21], and the different nature of these reports could be related to the recent contention that cellular intrinsic characteristics, including redox status (and probably energetics), influence the recruitment of proapoptotic mitochondrial signalling [14,22,23]. Thus injury recruitment in primary DA neurons is likely to be more sensitive to oxidative damage than DA and non-DA cell lines [19]. While our study was in progress, Chu *et al.* [12] reported in an elegant study that MPP⁺ treatment of primary murine DA neurons in culture produced caspase-independent cell death as we found earlier [16] in which redistribution of AIF and cyt c occurred. We also found here that MPP⁺ released both AIF and cyt c, but we were also able to add important new insights by quantitating the temporal pattern of redistribution of these two proteins wherein AIF release was earlier and predominated over that of cyt c. Moreover, in our hands rotenone produced similar results to MPP⁺ in terms of the pattern of release of these two proteins relative to one another (cf. [12]), but notably rotenone was more effective than MPP⁺ in triggering release of AIF and cyt c in DA neurons (see Fig. 2c). Our study has not determined whether the release of either cyt c or AIF from mitochondria in the presence of rotenone or MPP⁺ is itself independent or dependent on prior caspase activation. This issue is contentious, particularly in the case of AIF [14]; it has even been suggested that release of AIF from mitochondria requires the activity of other proteases, including calpain I [24]. To resolve this issue under the present insults would require use of caspase and calpain inhibitors but this is beyond the scope of this work.

Overall, the pattern of mitochondrial redistribution of cyt c reported here for both oxidative stressors is consistent with the late recruitment of the downstream caspase-dependent 'intrinsic' apoptotic pathway. The more extensive and early role for AIF would, however, imply a bias towards a caspase-independent downstream mode of apoptosis upon exposure of DA neurons to rotenone and MPP⁺. Nonetheless, release of both cyt c and AIF from mitochondria is consistent with a model of interactive caspase-dependent/caspase-independent mechanisms [13,14], which here is tipped more in favour of caspase-independent downstream apoptotic mechanisms. In view of new evidence that stress of the endoplasmic reticulum may also contribute to neuronal injury in parkinsonism (e.g. [25]), the differential influence of multiple cell death pathways on pathological outcome would be expected, with the balance between injury cascades likely to change according to temporal stress and cellular milieu.

Conclusion

The preferential redistribution of AIF before cyt c in DA neurons indicates that caspase-independent, mitochondrial proapoptotic signalling is likely to predominate in apoptotic injury induced by the oxidative stressors, rotenone and MPP⁺. In a broader context, our findings highlight the need to fully elucidate the mechanisms contributing to caspase-independent injury in parkinsonism.

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