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Rotenone induces neuronal death by microglial phagocytosis of neurons

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Rotenone, a common pesticide and inhibitor of mitochondrial complex I, induces microglial activation and loss of dopaminergic neurons in models of Parkinson's disease. However, the mechanisms of rotenone neurotoxicity are still poorly defined. Here, we used primary neuronal/glial cultures prepared from rat cerebella to investigate the contribution of microglia to neuronal cell death induced by low concentrations of rotenone. Rotenone at 2.5 nM induced neuronal loss over several days without increasing the numbers of necrotic or apoptotic neurons, and neuronal loss/death could be prevented by selective removal of microglia. Rotenone increased microglial proliferation and phagocytic activity, without increasing tumour necrosis factor-α release. Rotenone-induced neuronal loss/death could be prevented by inhibition of phagocytic signalling between neurons and microglia with: cyclo(Arg-Gly-Asp-D-Phe-Val) (to block the microglial vitronectin receptor); MRS2578 (to block the microglial $P2Y_6$ receptor); or either annexin V or an antibody against phosphatidylserine (to block exposed phosphatidylserine, a well-characterized neuronal 'eat-me' signal). As inhibition of phagocytosis by five different means prevented neuronal loss without increasing neuronal death, these data indicate that rotenone neurotoxicity is at least partially mediated by microglial phagocytosis of otherwise viable neurons (phagoptosis). Thus, neuronal loss in Parkinson's disease and other neurological diseases might be prevented by blocking phagocytic signalling.

Introduction

Rotenone, a commonly used pesticide derived from the roots of certain plant species, is a specific and potent inhibitor of mitochondrial NADH dehydrogenase (complex I) [1,2]. Chronic systemic rotenone exposure induces selective degeneration of dopaminergic neurons in the nigrostriatal pathway of the midbrain, and, like exposure to another complex I inhibitor, 1-methyl-1,2,3,6-tetrahydropiridine (MPTP), is used to model sporadic Parkinson's disease (PD) *in vivo* [1,2].

The mechanism by which rotenone kills neurons is not entirely clear, but has been attributed to: (a) energy depletion – rotenone inhibits complex I, depleting neurons of ATP, resulting in necrosis, which is potentially exacerbated by excitotoxicity [3,4]; (b) oxidative stress – rotenone inhibits complex I, stimulating mitochondrial reactive oxygen species (ROS) production, and/or indirectly stimulates ROS production from NADPH oxidase [3,5–7]; (c) microtubule disruption – rotenone binds to and disrupts microtubules, increasing ROS production [8]; and/or (d) microglia-mediated cell death – rotenone activates microglia, potentially via increased ROS

Abbreviations
cRGDfV, cyclo(Arg-Gly-Asp-D-Phe-Val); DNP, 2,4-dinitrophenol; IB4, isolectin-B4; LME, L-leucine methyl ester; MFG-E8, milk fat globule epidermal growth factor-like factor 8; MPTP, 1-methyl-1,2,3,6-tetrahydropiridine; PD, Parkinson's disease; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; SEM, standard error of the mean; TMRM, tetramethylrhodamine methyl ester; TNF-α, tumour necrosis factor-α.

production, and the activated microglia kill neurons [2,5,7].

Microglial cells are specialized macrophages of the central nervous system. Once activated by inflammatory or pathological changes in the surrounding microenvironment, they can become highly phagocytic [9]. Microglia can phagocytose dead or dying neurons, and thereby protect the brain by preventing the release of proinflammatory and damaging intracellular components [10]. However, accumulating evidence suggests that activated microglia can also eat viable but stressed neurons, and thereby induce neuronal death [11–14]. Cell death resulting from the cell being phagocytosed has recently been termed 'phagoptosis' [11], with the defining characteristic that inhibition of phagocytosis prevents cell death [11–14]. Inflamed microglia can induce the reversible exposure of the 'eat-me' signal molecule phosphatidylserine (PS) on neurons, and this can be bound by the extracellular bridging protein milk fat globule epidermal growth factor-like factor 8 (MFG-E8), which can induce phagocytosis of neurons via the microglial vitronectin receptor [11–14]. Furthermore, microglial phagocytosis of otherwise viable neurons has also been demonstrated in models of Alzheimer's disease [13] and frontotemporal dementia [15], thus indicating that primary phagocytosis is potentially involved in a variety of brain pathologies [16].

PD is characterized by progressive motor dysfunction and loss of dopaminergic neurons in the substantia nigra of the brain. The causes of neuronal loss are thought to include: α-synuclein inclusions; glial inflammation; and mitochondrial dysfunction [17,18]. α-Synuclein inclusions spread through the brain with PD symptoms; genetic variants of α-synuclein are associated with PD; and α-synuclein can induce glial inflammation and microglial phagocytosis [17,19]. A role for glial inflammation is suggested by the following findings: (a) inflamed glia and proinflammatory cytokines are found in the substantia nigra of patients and animal models; (b) proinflammatory agents, e.g. lipopolysaccharide, cause loss of substantia nigra neurons in culture and *in vivo*; and (c) anti-inflammatory drugs provide protection in patients and animal models [18,20]. A role for mitochondrial dysfunction is implicated by the findings that: (a) complex I activity is reduced in the substantia nigra of PD patient brains; (b) environmental toxins that are complex I inhibitors, MPTP and rotenone, can induce a PD-like pathology in humans and/or rodents; and (c) a number of genes with variants linked to PD (*parkin* and *PINK1*) regulate mitochondrial turnover [17]. A potential link between complex I inhibition and glial inflammation is that: glial inflammation can cause complex I inhibition (via reactive oxygen and nitrogen species), and inhibition of complex I (e.g. by rotenone) can cause glial inflammation (possibly via ROS) [18,20,21].

Using primary rat neuronal/glial cultures, we investigated whether rotenone, at a concentration insufficient to cause direct toxicity to neurons or microglia, induced neuronal loss by microglial phagocytosis of viable cells, i.e. through phagoptosis. We showed that rotenone-induced neuronal loss was dependent on the presence of microglia, and was decreased by inhibition of phagocytic signalling.

Results

Nanomolar rotenone induces progressive neuronal loss only in the presence of microglia

Whereas high concentrations of rotenone (μM) cause rapid and direct neuronal death within minutes to hours of exposure [22–24], low concentrations (nM) induce a loss of dopaminergic neurons in midbrain cultures that requires the presence of activated microglia [7,25]. On the basis of these reports, primary mixed rat cerebellar cultures (consisting of 81 ± 7% neurons, 8 ± 3% astrocytes, and 6 ± 4% microglia) were treated with 2.5 nM rotenone for 3 or 7 days. Addition of 2.5 nM rotenone induced a loss of ~ 20% of the neurons at 3 or 7 days without increasing the fraction of necrotic or apoptotic cells (Fig. 1A,B,D).

To test whether microglia contributed to the neuronal loss induced by low-dose rotenone, microglia were selectively eliminated from mixed neuronal/glial cultures by application of the lysosomotropic reagent L-leucine methyl ester (LME) without affecting the viability of neuronal or astrocytic cell populations [12,26,27]. Selective elimination of microglia prevented almost all of the neuronal loss induced by 2.5 nM rotenone without affecting the amount of necrotic or apoptotic neurons (Fig. 1D), indicating that rotenone was not directly toxic to neurons at this concentration, but rather required microglia to induce neuronal loss (Fig. 1D). Whether astrocytes play a role in this neuronal death is unclear, but they did not enable rotenone-induced neuronal death in the absence of microglia, and they did not prevent neuronal death in the presence of microglia.

Rotenone enhances phagocytic activity of primary microglia

Despite inducing neuronal loss, rotenone did not kill microglia, but rather increased their proliferation in mixed cultures (Fig. 1A,C).

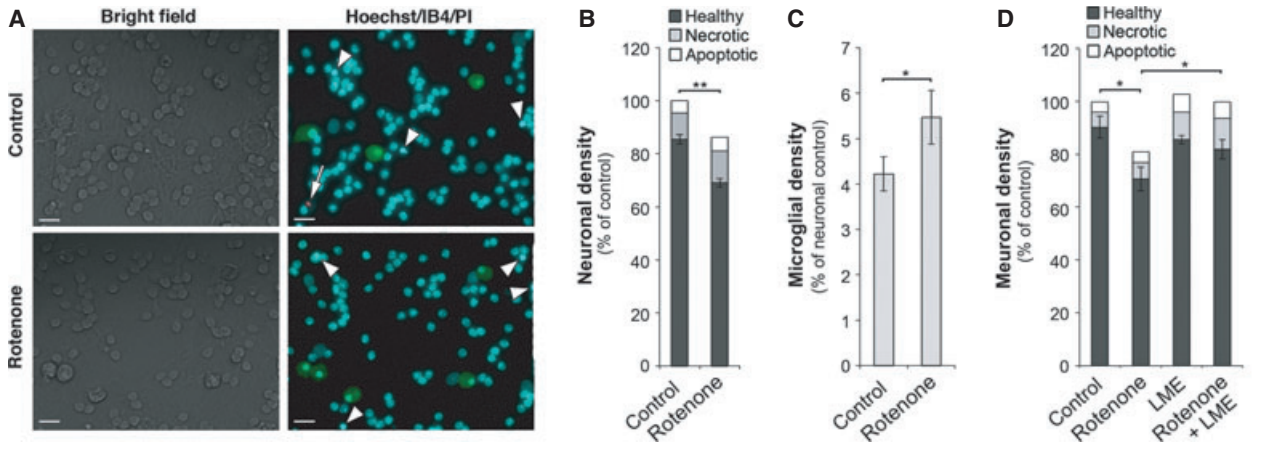

Fig. 1. Rotenone induces neuronal loss in primary mixed neuronal/glial cerebellar cultures only in the presence of microglia. (A) Mixed cerebellar cultures were stimulated with 2.5 nM rotenone for 7 days before microscopic analysis. Hoechst 33342 (Hoechst)-labeled neuronal nuclei are in blue; microglia labeled with Alexa Fluor-labeled IB4 are in green. Cultures contain chromatin-condensed, apoptotic neurons (arrowheads), as well as necrotic, PI-positive cells (arrow). Scale bar: 20 μm. (B, C) Quantification of cell densities 7 days after rotenone stimulation for neuronal and microglial cell populations, respectively. (D) Selective depletion of microglia with LME protects neurons from rotenone-induced death over a period of 3 days. The microglial densities (% of neuronal control) were 2.97 ± 1.48 for control cultures, 3.62 ± 1.71 for rotenone-treated cultures, 0 ± 0.0 for microglia-depleted cultures, and 0.03 ± 0.001 for cultures with both microglia depletion and rotenone treatment. The values represent data from at least three independent experiments. Each experiment was performed in duplicate. Data shown are means ± SEMs (*$P < 0.05$ versus control).

As rotenone inhibits mitochondrial complex I, we tested whether rotenone decreased the mitochondrial and plasma membrane potentials in pure microglia by loading the cells with tetramethylrhodamine methyl ester (TMRM) and then quantifying cellular TMRM fluorescence intensity at 0 and 3.5 min after treatment. After addition of 2.5 nM rotenone, TMRM fluorescence intensity decreased only marginally, indicating little or no immediate decrease in either plasma membrane potential or mitochondrial membrane potential. Addition of 200 μM 2,4-dinitrophenol (DNP), a potent mitochondrial uncoupler, led to a large decrease in membrane potential (Fig. 2A).

Recent evidence suggests that rotenone concentrations as low as 1 nM can effectively increase microglial phagocytic activity in a murine microglia cell line, BV-2 [28,29]. Consequently, we investigated whether 2.5 nM rotenone increased the phagocytic activity of microglia as assessed by their ingestion of 5-μm carboxylate-modified fluorescent beads (mimicking the size and negative surface charge of the PS-exposed neuronal cell body). Microscopic analysis showed that treatment of neuronal/glial cultures with 2.5 nM rotenone for 3 days increased microglial phagocytosis of these beads significantly (Fig. 2B). Because this increase in microglial phagocytic capacity might be secondary to neuronal phagocytosis or an effect of rotenone on neurons, we also repeated this phagocytic assay on glial cultures (containing no neurons) treated with rotenone for 3 days. Again, rotenone increased bead uptake into the microglia within this culture (Fig. 2C), indicating that the rotenone-induced increase in microglial phagocytosis is not secondary to any effect on or of neurons.

Rotenone-induced neuronal loss can be prevented by inhibition of microglial phagocytosis

Exposure of PS is an 'eat-me' signal (inducing phagocytosis of a PS-exposed cell), and is essential for microglial phagocytosis of neurons [12–14,30]. Previously, it has been shown that rotenone induces exposure of PS on neurons [24]. Therefore, we tested whether exposure of this 'eat-me' signal was required for rotenone-induced neuronal loss. The protein annexin V binds PS with high affinity, and effectively blocks phagocytosis mediated by PS [13,14,31]. To assess the role of PS exposure in rotenone-induced neuronal loss, annexin V or an antibody against PS were added at the same time as 2.5 nM rotenone to mixed neuronal/glial cultures. Both annexin V and PS antibody prevented rotenone-induced neuronal loss over a period of 3 days, indicating that PS is an essential recognition signal for rotenone-induced neuronal loss (Fig. 3A).

MFG-E8 is a soluble bridging protein between PS exposed on a target cell and the vitronectin receptor (an $\alpha_v\beta_{3/5}$-integrin) located on a phagocyte, promoting

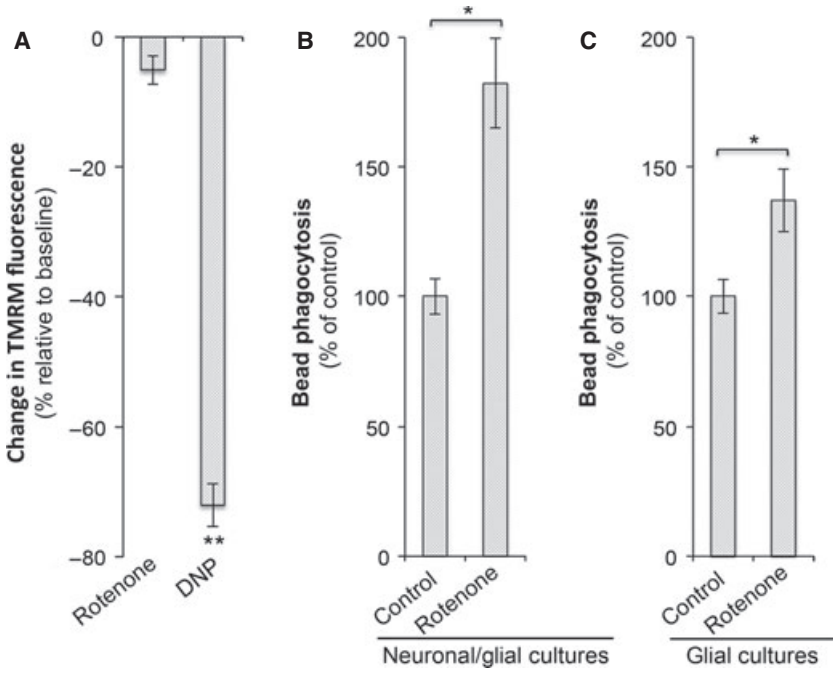

Fig. 2. Stimulation of microglia with nanomolar rotenone enhances phagocytic activity without affecting mitochondrial membrane potential. (A) Quantification of the change in TMRM fluorescence intensity at 3.5 min after treatment with 2.5 nM rotenone or 200 μM DNP. The data shown are means ± SEMs from three independent experiments. Each experiment was performed in duplicate (**$P < 0.01$ versus baseline). (B) Quantitative analysis of microsphere uptake into microglia in mixed neuronal/glial cultures 72 h after stimulation with 2.5 nM rotenone. (C) Quantitative analysis of microsphere uptake into microglia in glial cultures 72 h after stimulation with 2.5 nM rotenone. The data shown are means ± SEMs from at least three independent experiments. Each experiment was performed in quadruplicate (*$P < 0.01$ versus control).

the target cell's engulfment [32]. The cyclic peptide cyclo(Arg-Gly-Asp-D-Phe-Val) (cRGDfV) is a specific antagonist of the vitronectin receptor [33], and effectively inhibits microglial phagocytosis mediated by MFG-E8 in mixed neuronal/glial cultures without having an effect on microglial proliferation or the release of inflammatory mediators [12]. Engulfment of neurons by microglia also requires the microglial purinergic receptor $P2Y_6$, which is activated by UDP released by neurons [31]. The diisothiocyanate derivative MRS2578 is a selective antagonist of the $P2Y_6$ receptor, and blocks microglial phagocytosis of neurons [31]. To test the contribution of phagocytosis to neuronal loss mediated by rotenone, these pathways were blocked by specific antagonists of the vitronection or $P2Y_6$ receptors (cRGDfV or MRS2578, respectively). Inhibition of the vitronectin receptor with cRGDfV resulted in marked neuroprotection at 3 days following rotenone stimulation. Similarly, blocking of $P2Y_6$ receptor activation with MRS2578 significantly reduced neuronal loss (Fig. 3B).

Rotenone did not result in any detectable production of the proinflammatory cytokine tumour necrosis factor-α (TNF-α) (Fig. 3C) in the neuronal/glial cultures, indicating that the microglia are not 'classically' activated by rotenone. In addition, the two blockers of phagocytic receptors – cRGDfV and MRS2578 – also had no effect on TNF-α release, indicating that they inhibit rotenone-induced neuronal loss by blocking inflammation.

Notably, all four different inhibitors of phagocytic signalling between neurons and microglia (annexin V, antibody against PS, cRGDfV, and MRS2578) prevented neuronal loss without increasing the fractions of apoptotic or necrotic cells in the cultures (Fig. 3A, B). If microglia had removed only dead neurons, addition of phagocytosis inhibitors would have increased the numbers of dead (apoptotic or necrotic) neurons without affecting the number of live ones. Thus, these results indicate that neuronal loss and death were caused by microglial phagocytosis of viable neurons, i.e. by phagoptosis, which is characterized as cell death prevented by the inhibition of phagocytosis [11].

Discussion

The data presented here support a contribution of microglial phagocytosis to the loss of otherwise viable

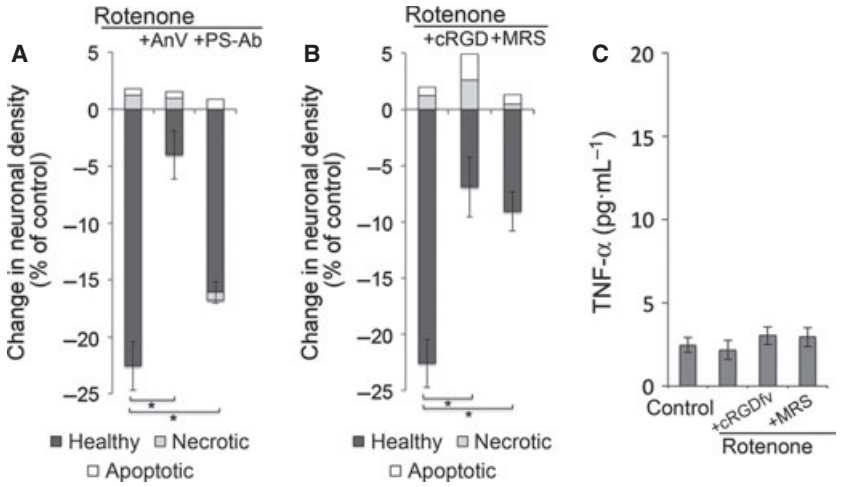

Fig. 3. Rotenone-induced neuronal loss depends on microglia and phagocytic phosphatidylserine (PS) recognition, and can be prevented by inhibition of phagocytosis. (A) Blocking the neuronal 'eat-me' signal PS with annexin V (AnV) or antibody against PS (PS-Ab) protects against rotenone-induced neuronal loss over a period of 3 days without affecting microglial viability and proliferation ($n = 3$). (B) Inhibition of phagocytosis with vitronectin receptor antagonist or $P2Y_6$ receptor antagonist [cRGDfV or MRS2578 (MRS), respectively] prevents loss of neurons over a period of 3 days ($n = 4$). (C) Rotenone does not stimulate microglial activation in mixed neuronal/glial cultures over a period of 3 days as assessed by the production of the proinflammatory TNF-α ($n = 4$). Each experiment was performed in duplicate. The data shown are means ± SEMs (*$P < 0.05$ versus control).

neurons in response to a low concentration of rotenone. Rotenone increased the phagocytic activity of microglia and induced neuronal loss in mixed neuronal/glial cultures without increasing the number of necrotic or apoptotic neurons. Selectively depleting microglia from these cultures prevented neuronal loss, demonstrating that microglia were required for neuronal loss. Furthermore, neuronal loss was preventable by inhibiting different steps involved in the phagocytic process: (a) by blocking exposed PS with annexin V or an antibody against PS; (b) by blocking the vitronectin receptor with cRGDfV; or (c) by blocking the $P2Y_6$ receptor with MRS2578 (Fig. 3). For each of these compounds, inhibition of phagocytosis prevented neuronal loss without significantly increasing the number of dead or dying cells, indicating that neuronal death was executed through microglial phagocytosis rather than being secondary to apoptosis or necrosis.

Neuronal loss occurred with 2.5 nM rotenone. Higher concentrations of rotenone (> 25 nM) resulted in almost all neurons being dead (necrotic or apoptotic) at 3 days, without any significant change in microglial density (data not shown). This is consistent with neuronal loss induced by low rotenone concentrations being mediated by microglial activation, and higher rotenone concentrations inducing direct neurotoxicity. Previous studies found that direct neurotoxicity required rotenone concentrations of 10–50 nM, and was associated with a collapse of the mitochondrial membrane potential and the rapid death of neurons resulting from energy depletion [3,24,34]. Thus, the 2.5 nM rotenone concentration used here to induce microglia-mediated neuronal loss is probably lower than the threshold concentration needed to induce direct neurotoxicity. Gao *et al.* [7,25] showed that 1–15 nM rotenone induced loss of dopaminergic neurons, which was greatly enhanced by the presence of activated microglia.

The use of cerebellar glial/neuronal cultures in this work is not ideal, as PD has little effect on the cerebellum. However, the aim of this work was not to model PD, but rather to investigate the mechanism by which low concentrations of rotenone induce neuronal death. Our finding that the sensitivity of cerebellar cultures to rotenone is similar to that of dopaminergic neurons in mid-brain cultures suggests that the mechanisms of neuronal death may be similar, and this is supported by the finding that microglia are required for neuronal death in both cases [7,25].

Low nanomolar rotenone has been reported to stimulate microglial superoxide production from NADPH oxidase, increase microglial production of proinflammatory mediators, and induce morphological changes resembling an activated state in primary microglial cultures, without inducing any death of microglia [7,28,35]. *In vivo*, Sherer *et al.* showed that systemic exposure of rats to rotenone induced extensive microglial activation, similar to the glial pathology observed in PD [2]. Thus, rotenone can activate microglia

and potentially induce neuronal loss via activated microglia.

A number of environmental toxins, including rotenone and MPTP, can induce PD-like syndromes and loss of dopaminergic neurons in the substantia nigra, apparently via inhibition of mitochondrial complex I [5,17,21]. Also, the substantia nigra of idiopathic PD patients after death appears to have strong inhibition of complex I activity [17]. Thus, it is possible that the mechanism of neuronal death induced by rotenone may have wider validity in PD. If so, it may be that inhibition of phagocytic signalling may be protective in idiopathic PD.

The ability of annexin V or antibody against PS to prevent neuronal loss indicates that rotenone-induced neuronal loss was mediated by PS exposure. PS is a phospholipid that is usually found on the inner leaflet of plasma membranes of healthy cells, but PS exposure can occur on cells as a result of apoptosis or necrosis, or reversibly on viable cells as a result of elevated calcium or oxidants, or of energy depletion [36,37]. Viable but stressed neurons can reversibly externalize PS when exposed to oxidants or activated microglia, and we have shown that this mediates the neuronal loss induced by activated microglia [12–14]. Thus, rotenone might induce microglial generation of ROS, which, in turn, induces PS exposure on neurons, thus promoting their engulfment.

Rotenone neurotoxicity might be further enhanced by direct stimulation of microglial phagocytic activity, as demonstrated here by the cellular uptake of negatively charged fluorescent beads (Fig. 2B,C). This finding is supported by recent evidence showing that rotenone concentrations as low as 1 nM can effectively increase microglial phagocytic activity in a murine microglia cell line, BV-2 [28,29]. Disruption of microtubule dynamics causing increased ROS production has been implicated in the rotenone-induced death of dopaminergic neurons [8,38]. However, phagocytosis requires microtubules, and most microtubule-destabilizing agents block phagocytosis [39,40], suggesting that the rotenone-induced increase in phagocytosis is not mediated by a direct action of rotenone in disrupting microtubules.

Collectively, these results suggest that rotenone directly stimulates microglial phagocytic activity and promotes neuronal death through phagocytosis of viable neurons, i.e. through phagoptosis. Inhibition of different steps of the phagocytic process prevented neuronal loss without increasing neuronal death. This suggests the possibility that blocking phagocytic signalling may prove beneficial for the treatment of PD and other neurological diseases.

Experimental procedures

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986). All cultures were grown in a humidified atmosphere of 5% CO_2 and 95% air, at 37 °C.

Primary cell cultures

Primary mixed neuronal/glial cultures were prepared from Wistar rat cerebella on postnatal days 5–7, as previously described [41]. Cells were seeded at a density of 5×10^5 cells·cm^{-2} onto 24-well plates coated with poly(L-lysine) (Sigma, St Louis, MO, USA), and were allowed to mature *in vitro* for at least 6 days before treatment.

Mixed microglial/astrocytic cultures and pure microglial cultures were prepared at postnatal days 5–7 from Wistar rat cerebral hemispheres, as previously described [41].

Selective microglial elimination

When desired, microglia were selectively eliminated from mixed neuronal/glial cultures after 7–8 days *in vitro* with LME (50 mM; Sigma) for 4 h. Following the incubation, the medium was replaced with conditioned medium from sister cultures.

Culture treatment

Cultures were stimulated with rotenone (Sigma) at 7–8 days *in vitro*. cRGDfV (50 μM; Bachem, Bubendorf, Switzerland), antibody against PS (5 μg·mL^{-1}; Abcam, Cambridge, UK) and annexin V (100 nM; BioVision, Dunstable, UK) were added once, together with the stimulant. MRS2578 (1 μM; Tocris, Bristol, UK) was added daily. PS antibody was repeatedly washed with NaCl/P_i (Invitrogen, Paisley, UK) by use of an Amicon Ultra-4 centrifugal filter device (10-kDa cut-off; Millipore, Watford, UK). The protein concentration was then determined with the Bradford assay (Sigma), according to the manufacturer's instructions. To prevent recognition of the antibodies through their Fc-domains by microglial Fcγ receptors, they were incubated with a five-fold molar excess of Fc-region-specific anti-[goat F(ab)2 fragment] (Jackson Immunolaboratories, West Grove, PA, USA) for 0.5 h at 37 °C with shaking, before they were applied concomitantly with the stimulant.

Assessment of inflammatory mediators

Inflammatory mediators were assessed in culture media at 3 days after stimulation, with rat Quantikine ELISA Kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Quantification of cell densities

Cell densities were assessed in live cultures at 3 or 7 days after stimulation. Cultures were incubated with the nuclear stains Hoechst 33342 (5 $\mu g \cdot mL^{-1}$; Sigma), propidium iodide (PI) (1 $\mu g \cdot mL^{-1}$; Sigma) and Alexa 488-conjugated *Griffonia simplicifolia* isolectin-B4 (IB4) (1 $\mu g \cdot mL^{-1}$; Invitrogen) for 30 min at 37 °C in 5% CO_2. The nuclear morphology of the cell (chromatin condensation and fragmentation) was studied with the readily membrane-permeable dye Hoechst 33342 to assess for the presence of apoptotic cells. Cells with homogeneously stained blue nuclei were considered to be viable, whereas those with condensed and fragmented brightly stained nuclei were counted as apoptotic. PI-positive cells were counted as necrotic, as staining occurs only for cells with disrupted membranes. Microglia were detected by their selective IB4 affinity. The characteristic shape and size of the nuclei of the three cell types present in cerebellar granule cell cultures (neurons, microglia, and astrocytes) together with typical morphology and cell-specific staining (IB4) allowed us to distinguish and quantify the different cell types. Cell densities were assessed with a Leica DMI6000 CS microscope. Four microscopic fields per well in two wells per condition were quantified for each experiment.

Phagocytosis assay

Mixed neuronal/glial cultures and microglial/astrocytic cultures were treated with rotenone for 72 h, and 6 $\mu L \cdot mL^{-1}$ medium of a 1 : 2 solution of carboxylate-modified fluorescent microspheres (5.0–5.9 μm, carboxyl fluorescent nile red particles; Spherotech, Lake Forest, IL, USA) was added and incubated for 2 h at 37 °C in 5% CO_2. Cultures were washed once with ice-cold NaCl/P_i to remove excess beads. After staining with Hoechst 33342 (5 $\mu g \cdot mL^{-1}$) and Alexa 488-conjugated IB4 (1 $\mu g \cdot mL^{-1}$), the number of beads per cell was counted for at least 50 cells per condition.

Assessment of mitochondrial membrane potential

TMRM is a cell-permeable fluorescent indicator that accumulates inside mitochondria in response to their negative membrane potential. Microglial cultures in complete growth medium were loaded with 3 nM TMRM (Sigma) for 30 min at 37 °C in 5% CO_2. This concentration of TMRM was insufficient for aggregation in the mitochondrial matrix. Thus, a decrease in either the plasma membrane or mitochondrial membrane potential was indicated by a reduction in whole cell fluorescence (that is, the experiment was performed in 'non-quenching mode') [42]. Fluorescence was quantified with a Leica DMI6000 CS microscope and IMAGEJ software. Briefly, for each condition, eight random fields containing approximately 5–10 cells each were selected for quantitative analysis. All cells from each field were outlined, and the mean fluorescence intensity of the red channel was determined. Background fluorescence intensity was obtained from areas located next to the cells, and was subtracted from the mean fluorescence intensity of each field. After determination of baseline fluorescence, cells were treated with rotenone (2.5, 10, or 50 nM) or the mitochondrial protonophore DNP (200 μM; Sigma) for 3.5 min, and the change in TMRM fluorescence was assessed thereafter. Loss of TMRM fluorescence indicated a collapse in mitochondrial membrane potential upon treatment. A minimum of 40 cells was quantified for each condition.

Statistical analysis

Statistical analyses were performed with IBM SPSS statistics (IBM, Portsmouth, England). All data shown represent results from at least four independent experiments. Statistical analysis was performed with the Mann–Whitney test. Results were considered to be statistically significant at $P < 0.05$. In all graphs, data are shown as means ± standard errors of the mean (SEMs).

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