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Partial inhibition of complex I activity increases Ca^{2+}-independent glutamate release rates from depolarized synaptosomes

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Abstract

Mitochondria have been implicated in the pathogenesis of several neurodegenerative disorders and, in particular, complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) activity has been shown to be partially reduced in postmortem studies of the substantia nigra of Parkinson's disease patients. The present study examines the effect of partial inhibition of complex I activity on glutamate release from rat brain synaptosomes. Following a 40% inhibition of complex I activity with rotenone, it was found that Ca^{2+}-independent release of glutamate increased from synaptosomes depolarized with 4-aminopyridine. Highest rates of glutamate release were found to occur between 60–90% complex I inhibition. A similar pattern of increase was shown to occur in synaptosomes depolarized with KCl. The increase in glutamate release was found to correlate to a significant decrease in ATP. Inhibition of complex I activity by 40% was also shown to cause a significant collapse in mitochondrial membrane potential ($\Delta\psi_m$). These results suggest that partial inhibition of complex I activity in *in situ* mitochondria is sufficient to significantly increase release of glutamate from the pre-synaptic nerve terminal. The relevance of these results in the context of excitotoxicity and the pathogenesis of neurodegenerative disorders is discussed.

Keywords: complex I, glutamate release, mitochondria, neurodegeneration, Parkinson's disease, synaptosomes.

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Following episodes of energy depletion caused by anoxia and hypoglycemia, excitotoxic amounts of glutamate are released from nerve terminals leading to acute neurodegeneration (Doble 1999; Hossmann 2006). A mechanism of slow excitotoxicity has also been suggested to occur in chronic neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Beal 1992a; Mattson 2003) and mitochondrial dysfunction is thought to be involved in this process (Beal 1992b; Doble 1999; Lin and Beal 2006). Mitochondrial electron transport chain deficiencies have been implicated in the neurodegenerative process and, in particular, complex I activity is reduced by 35–40% in substantia nigra homegenates in postmortem studies of Parkinson's disease patients (Schapira *et al.* 1989, 1990a,b). Recent evidence has suggested that a complex I deficiency may be widespread in the parkinsonian brain, as the occurrence of a mutation in mitochondrial DNA in idiopathic Parkinson's disease (Parker and Parks 2005), and an associated reduction in complex I activity in mitochondria from the frontal cortex (Keeney *et al.* 2006; Parker *et al.* 2008) have been reported. This evidence supports the hypothesis that complex I deficiency plays a central role in the initial etiology of the disease (Greenamyre *et al.* 2001; Dawson and Dawson 2003; Tretter *et al.* 2004).

Parkinson's disease is characterised by preferential death of dopaminergic neurons in the substantia nigra pars compacta (Hornykiewicz and Kish 1987). This region receives glutamatergic projections from the medial prefrontal cortex, subthalamic nucleus and pedunculopontine tegmental nucleus, and glutamate released from these neuronal projections to the substantia nigra is increased during Parkinsonism induced by the neurotoxin, MPTP (Bezard *et al.* 1997). The rate of spontaneous firing in glutamatergic nerve terminals in the substantia nigra pars compacta has been shown to be increased in MPTP–induced Parkinsonism, as has the number of neurons firing in bursts (Bergman *et al.* 1994). This is thought to contribute to dopaminergic cell death by excitotoxic mechanisms (Obeso *et al.* 2004), indicating the importance of examining the effects of depolarization on

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glutamate release from nerve terminals with compromised complex I activity.

Previous studies on glutamate release with synaptosomes have examined the effects of metabolic inhibitors on glutamate release (Sanchez-Prieto *et al.* 1987; Sanchez-Prieto and Gonzalez 1988; Rubio *et al.* 1991; Santos *et al.* 1996). The results of one such study demonstrated a time-dependent reduction in Ca^{2+}-dependent glutamate release and a 40% increase in Ca^{2+}-independent glutamate efflux from polarized synaptosomes due to reduced ATP levels after total inhibition of complex I activity with rotenone (Kauppinen *et al.* 1988). However, the effects of partial inhibition of oxidative phosphorylation on glutamate release have not been investigated, and increases in Ca^{2+}-independent glutamate release rates after depolarization have been largely overlooked. Complex I has been shown to have particularly high control over oxidative phosphorylation in isolated nerve terminal mitochondria (Davey *et al.* 1998) compared to non-synaptic mitochondria (Davey and Clark 1996; Davey *et al.* 1997). Therefore, partial inhibition of complex I activity in *in situ* nerve terminal mitochondria to extents that model the decreases in complex I activity observed in Parkinson's disease will have consequences on ATP production and may result in increased glutamate release.

Materials and methods

Materials

Chemicals were supplied by Sigma Chemical Co. (Poole, Dorset, UK) or BDH (Dagenham, Essex, UK). Luciferin/luciferase mixture (ENLITEN® ATP Assay System) was supplied by Promega, Madison, Wisconsin, USA. Female Wistar rats (200–250 g) were supplied by the Bioresources Unit, Biochemistry Department, Trinity College, Dublin.

Synaptosomal preparation

Rats were killed by cervical dislocation and synaptosomes were prepared using a discontinuous ficoll gradient (7.5% w/v and 10% w/v), acording to the method of Lai and Clark (Lai and Clark 1978). Synaptosomes (1 mg) were resuspended in TES buffer (250 mM sucrose/5 mM TES, pH 7.4) and following centrifugation at 15 000 *g* for 5 min were stored as 1 mg pellets on ice for use within 2 h of preparation. All experiments were carried out on at least three separate synaptosomal preparations to ensure reproducibility of results (Nicholls *et al.* 1987).

Glutamate release

Glutamate release was measured on a SpectraMAX GeminiXS (Molecular Devices, CA, USA) well plate reader using a continuous flourimetric assay modified from that described by Nicholls *et al.* (1987). Synaptosomal pellets were resuspended in 1 mL of incubation medium (3 mM KCl, 140 mM NaCl, 25 mM Tris-HCl, 10 mM glucose, 2 mM $MgCl_2$, pH 7.4). 100 μL of incubation medium containing 2 mM $NADP^+$, 6.32 U L-glutamic dehydrogenase, (and 1.4 mM $CaCl_2$ where appropriate) was distributed into each of the 96 wells. 2 μL rotenone in ethanol (1 nM – 10 μM final concentrations) was added followed by 100 μL of resuspended synaptosomes (final concentration 0.5 mg/mL) and each experimental condition was carried out in triplicate on each plate. Synaptosomes were depolarized after 5 min and rate of increase in NADPH fluorescence at λ = 460 nm emission (340 nm excitation) was recorded over a 20 min time period at a 32 s interval following depolarization. Linear rates were fitted to the traces by the SoftMax Pro program, which accompanies the instrument, and these rates were calibrated using a standard curve. Enzyme lag (Nicholls *et al.* 1987) was accounted for when converting rates to nmol/min/mg protein.

Complex I activity assay

Complex I activity was assayed by modification of the method of Ragan *et al.* (Ragan *et al.* 1987). Synaptosomes were added to plates as with the glutamate release experiments, but with the absence of $NADP^+$ and L-glutamic dehydrogenase in the medium. The plates were then incubated for 15 min before being stored at −80°C. All samples were freeze-fractured three times prior to being assayed. 20 μg synaptosomal protein was added to a reaction medium containing 25 mM potassium phosphate pH 7.2, 0.2 mM NADH, 10 mM $MgCl_2$, 1 mM KCN and 2.5 mg bovine serum albumin, final volume 1 mL. 50 μM decylubiquinone was used as the electron acceptor and rates were followed for 5–7 min before addition of 10 μM rotenone to obtain the rotenone-insensitive rate.

ATP determinations

ATP levels were determined using a luciferase coupled assay a modification of the method of Kauppinen and Nicholls (Kauppinen and Nicholls 1986a). Synaptosomes (1 mg/mL) were pre-incubated at 37°C for 5 min in incubation medium containing either 10 mM glucose or 2 mM deoxyglucose. After 5 min 100 μL synaptosomes were added to 100 μL of incubation medium with 2 μL rotenone (25 nM and 10 μM final concentrations) added, where appropriate. One group was prepared for ATP determination immediately at this point, by adding 10 μL 6.5 M perchloric acid and, after centrifugation, neutralizing 150 μL of supernatant with 375 μL 1 M K_2HPO_4. Two groups were depolarized after 5 min (one with 1 mM 4-aminopyridine and another with 40 mM KCl). [ATP] is expressed as a percentage of the control group that contained no rotenone. These groups were perchloric acid extracted in the same way 10 min after depolarization and all samples were then stored at −80°C. 10 μL of extract was added to 100 μL reconstituted luciferin/luciferase ENLITEN® reagent and read immediately. All samples were read within 2 weeks of preparation.

Measurement of $\Delta\psi_m$

In situ mitochondrial membrane potential was measured by using JC-1 (5,5′6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolylcarbocyanine iodide) based on the method of Chinopoulos *et al.* (Chinopoulos *et al.* 1999). Synaptosomes were resuspended in incubation medium (4 mg/mL) and loaded with JC-l, 6 μM) for 15 min at 37°C. Following three washes, synaptosomes were diluted to 1 mg/mL and 100 μL added to a pre-prepared 96-well plate containing 100 μL incubation medium plus 2 μL rotenone in ethanol (1 nM – 10 μM final concentrations) in each well. Each experimental condition was carried out in triplicate on each plate.

Fluorescence intensity at λ = 590 nm and at λ = 535 nm (λ = 490 nm emission) were measured using a SpectraMAX GeminiXS in kinetic mode. These fluorescence peaks correspond to the fluorescent peaks of the aggregate and monomer forms of the dye respectively (Reers *et al.* 1991). The ratio of emission at 590 to 535 is a qualitative measure of *in situ* mitochondrial membrane potential (Reers *et al.* 1995).

Statistical analysis

Results presented are mean ± SEM values. Statistical analysis of the results were determined by doing a one-way ANOVA followed by a Newman-Keuls *post-hoc* test. Values of $p < 0.05$ were taken to be significant.

Results

Partial inhibition of complex I activity increases the rate of Ca^{2+}-independent glutamate release from depolarized synaptosomes

Titration of complex I activity with rotenone caused an increase of glutamate release from synaptosomes depolarized with both 4-aminopyridine (Figs 1 and 2a) and KCl (Fig. 2). 1 mM 4-aminopyridine induces repetitive action potential-like depolarizations of synaptosomes (Tibbs *et al.* 1989, 1996), which cause a Ca^{2+}-dependent release of glutamate from the vesicular pool. In synaptosomes depolarized with 4-aminopyridine, a low level of Ca^{2+}-independent glutamate release was detected in the absence of inhibitor (27.1 ± 7.2 pmol/min/mg, Figs 1 and 2a). This rate was not different from the Ca^{2+}-independent glutamate efflux rate from polarized synaptosomes (25.5 ± 7.2 pmol/min/mg, Fig. 3, 0% inhibition). When the blank trace was subtracted it was clear that 1 mM 4-aminopyridine did not elicit any Ca^{2+}-independent release of glutamate in the absence of inhibitor (Fig. 1, bottom trace). When depolarized with 4-aminopyridine in the presence of 25 nM rotenone, the rate of release was increased to 101.6 ± 15.5 pmol/min/mg ($p < 0.05$, Figs 1 and 2a). At this concentration of rotenone, complex I activity was inhibited by 37.8 ± 6.8% (Fig. 2a). Highest Ca^{2+}-independent glutamate release rates of were observed between 60% and 90% inhibition of complex I activity, and 217.7 ± 30.8 pmol/min/mg was released in the presence of 1 μM rotenone. This corresponded to a release of 21% of the initial total glutamate pool over the 20 min period after depolarization, whareas 2.6% was released in the absence of inhibitor. In the presence of 1.4 mM $CaCl_2$, synaptosomes released 107.8 ± 16.1 pmol glutamate/min/mg when depolarized with 4-aminopyridine (10.4% of the total pool over 20 min), suggesting a Ca^{2+}-dependent glutamate release rate of approximately 80 pmol/min/mg in the absence of complex I inhibitor. A decrease in release from the Ca^{2+}-dependent pool was evident as levels of complex I inhibition were increased.

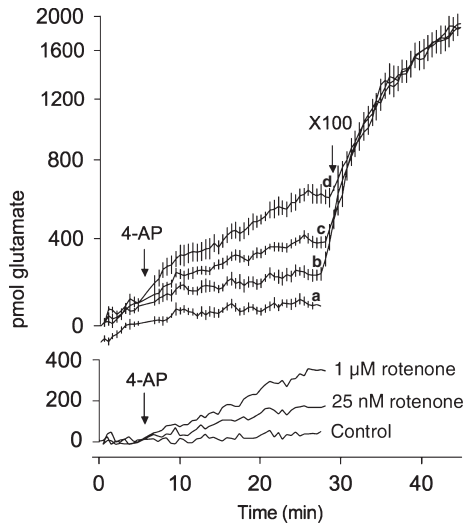

Fig. 1 Representative traces showing the effect of rotenone on Ca^{2+}-independent release of glutamate from synaptosomes depolarized with 4-aminopyridine. Synaptosomes were pre-incubated at 1 mg/mL for 5 min before addition to assay mixture and readings were taken over a 32-s period. Trace **a** shows the Ca^{2+}-independent efflux of glutamate from polarized synaptosomes ('blank rate'); trace **b** shows release after depolarization with 4-aminopyridine; traces **c** and **d** included 25 nM and 1 μM Rotenone respectively. 1 mM 4-aminopyridine (4-AP) was added to traces **b, c** and **d** 5 min into the assay as shown. Lower traces indicate the differences between traces **b, c** and **d** and the 'blank' trace respectively. Subsequent figures represent rates taken over the 20 min period following depolarization. Triton X-100 (0.5% vol/vol) was added 20 min after depolarization to assay the total glutamate pool which was found to be 20.69 ± 0.14 nmol/mg. Traces represent the mean ± SEM of experiments done in triplicate on a single preparation. [Correction added on 27 June 2008; after first online publication: in Fig. 1, 25 μM rotenone has been changed to 25 nM rotenone.]

As well as causing a Ca^{2+}-dependent release from the vesicular pool, depolarization with KCl causes a Ca^{2+}-independent release of cytoplasmic glutamate from synaptosomes (Nicholls and Sihra 1986; Nicholls *et al.* 1987). Figure 2(b) shows that a Ca^{2+}-independent release rate of 102.3 ± 9.4 pmol glutamate/mg/min (9.9% of the total pool after 20 min) was found in synaptosomes depolarized with 40 mM KCl. The Ca^{2+}-independent release rate was also higher after depolarization with 40 mM KCl than 1 mM 4-aminopyridine at all concentrations of rotenone. In the presence of 25 nM rotenone, the rate was increased to 192.5 ± 14.7 pmol/min/mg ($p < 0.001$). This concentration of rotenone brought about 42.7 ± 5.6% inhibition of complex I activity. When depolarized with KCl, 100 nM rotenone, which caused 68.4 ± 6.3% inhibition of complex I, gave the highest rate of glutamate release (310.2 ± 18.4 pmol/min/mg, $p < 0.001$, 30% of total pool after 20 min). Addition of 1.4 mM $CaCl_2$ to the medium caused release of 198.5 ± 11.3 pmol glutamate/mg/min from control, suggesting a Ca^{2+}-dependent release rate of 96.2 pmol/min/mg with

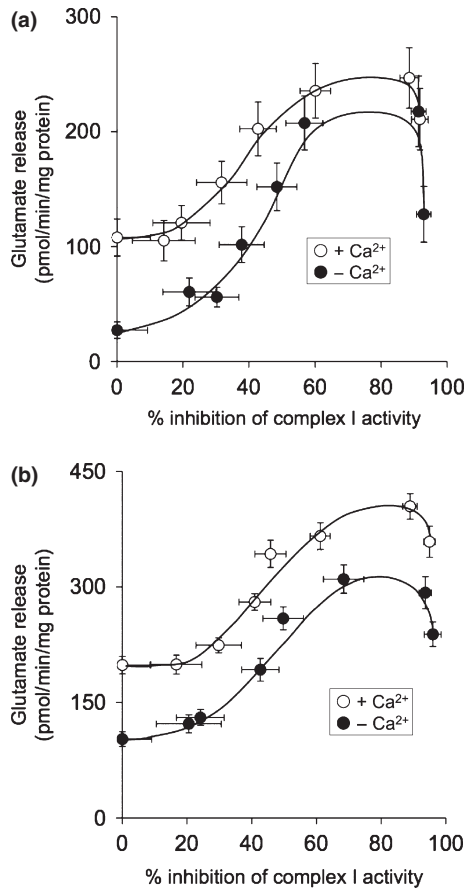

Fig. 2 Partial inhibition of complex I activity increases release of non-vesicular glutamate stores from depolarized synaptosomes. Synaptosomes (0.5 mg/mL) were pre-incubated at 37°C for 5 min in the presence (○) or absence (●) of 1.4 mM $CaCl_2$ before being depolarized with (a) 1 mM 4-aminopyridine or (b) 40 mM KCl. Rates of glutamate release at each concentration of rotenone were plotted against percent inhibition of complex I activity brought about by that concentration of rotenone. Freehand curves were drawn through the results. Points shown represent the mean ± SEM for experiments carried out in triplicate on at least three separate synaptosomal preparations.

KCl which remained constant during the rotenone titration. As shown in Fig. 1(a) and (b), inhibition of complex I activity by more than 90% significantly reduced the maximum level of glutamate released during 60–90% inhibition of complex I activity. Addition of 0.5 mM Na-EGTA had no effect on the Ca^{2+}-independent release rates (results not shown).

Inhibition of complex I potentiates the increase in glutamate efflux from glycolytically inhibited synaptosomes

Glutamate efflux from polarized synaptosomes was found to occur at a rate of 25.5 ± 6.5 pmol/min/mg (Fig. 3). Titration of complex I activity with rotenone did not produce any significant increase in glutamate release from polarized synaptosomes.

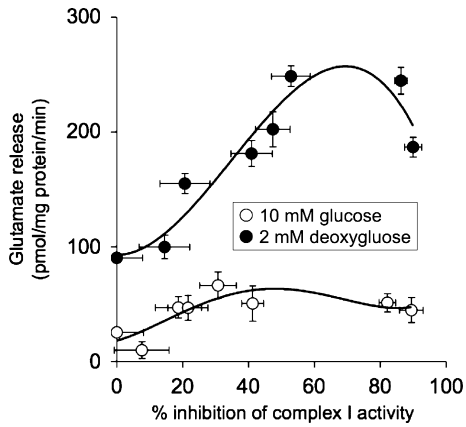

Fig. 3 Partial inhibition of complex I activity combined with inhibition of glycolysis increases glutamate efflux from synaptosomes. Synaptosomes (0.5 mg/mL) were incubated with 10 mM glucose (○) or 2 mM deoxyglucose (●) and rotenone (1 nM – 10 μM) and in the presence of 1.4 mM $CaCl_2$. Rates of glutamate release at each concentration of rotenone were plotted against percent inhibition of complex I activity brought about by that concentration of rotenone. Freehand curves were drawn through the results. Points shown represent the mean ± SEM for experiments carried out in triplicate on three separate synaptosomal preparations.

Substitution of 10 mM glucose in the medium with 2 mM deoxyglucose, an inhibitor of glycolysis, caused an increase in glutamate efflux to a rate of 90.4 ± 16.7 pmol/min/mg ($p < 0.001$). Addition of rotenone caused a further increase in the rate of glutamate efflux to a maximum of 248.6 ± 9.1 pmol/min/mg between 53–86% inhibition of complex I activity. The total glutamate pool was found to be reduced by deoxyglucose from 20.7 ± 0.14 to 16.8 ± 0.15 ($p < 0.01$) after 5 min and 13.9 ± 0.14 15 min later ($p < 0.01$, results not shown). The rate of glutamate efflux was not changed in the absence of 1.4 mM $CaCl_2$ at any concentration of rotenone (results not shown), indicating that the increase in glutamate efflux is Ca^{2+}-independent.

Partial inhibition of complex I activity combined with inhibition of glycolysis blocks exocytotic release of glutamate from synaptosomes

The presence of the glycolytic inhibitor deoxyglucose increased Ca^{2+}-independent release of glutamate from 27.1 ± 7.2 to 60 ± 14.8 pmol/min/mg and decreased release in the presence of 1.4 mM $CaCl_2$ from 107.8 ± 16.1 to 84 ± 8.2 pmol/min/mg, when depolarized with 4-aminopyridine (Figs 2a and 4a). This suggests that Ca^{2+}-dependent release was blocked under these conditions. Similarly, when depolarized with KCl, exocytotic release of glutamate from synaptosomes is blocked (Fig. 4b). Using both 4-aminopyridine and KCl as depolarizing agents, the overall glutamate release rate in the presence of $CaCl_2$ was reduced when

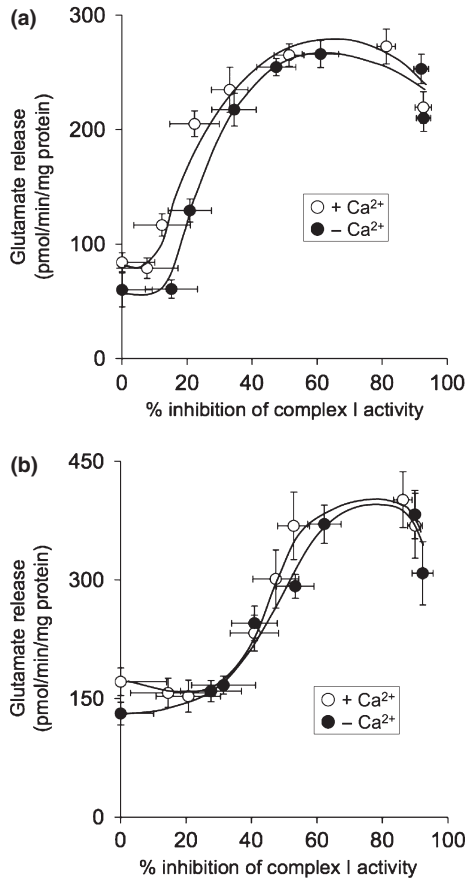

Fig. 4 Inhibition of complex I activity and glycolysis blocks the Ca^{2+}-dependent component of glutamate release from depolarized synaptosomes. Synaptosomes (0.5 mg/mL) were pre-incubated for 5 min with 2 mM deoxyglucose in the presence (○) or absence (●) of 1.4 mM $CaCl_2$ before being depolarized with (a) 1 mM 4-aminopyridine or (b) 40 mM KCl. Rates of glutamate release at each concentration of rotenone were plotted against percent inhibition of complex I activity brought about by that concentration of rotenone. Freehand curves were drawn through the results. Points shown represent the mean ± SEM for experiments carried out in triplicate on three separate synaptosomal preparations.

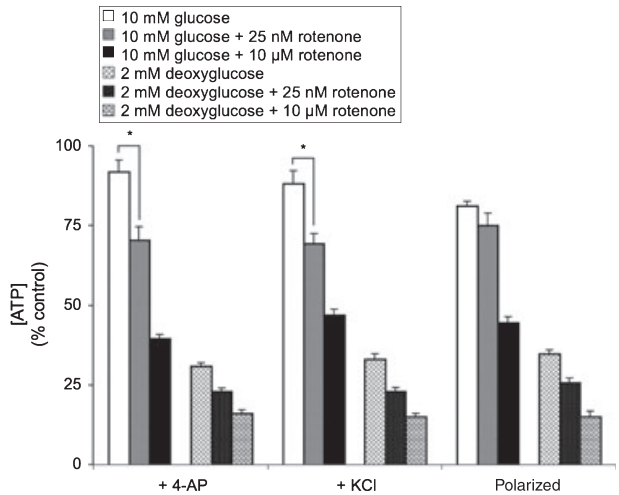

Fig. 5 Partial inhibition of complex I activity controls ATP levels in synaptosomes. Synaptosomes (0.5 mg/mL) were divided into groups and incubated at 37°C with 10 mM glucose or 2 mM 2-deoxyglucose and rotenone as indicated. ATP from synaptosomes was PCA extracted immediately upon resuspension and again 15 min later [where appropriate, groups were depolarized with 1 mM 4-aminopyridine (+ 4-AP) or KCl (+ KCl) 5 min after initial samples were taken]. Results are expressed as a percentage of the initial [ATP] (577.7 ± 62.1 pmol/mg protein) and represent the mean ± SEM for experiments carried out on three separate synaptosomal preparations. *$p < 0.05$.

glycolysis is inhibited (comparing Figs 2 and 4, 0% inhibition) and increased in the absence of $CaCl_2$.

A reduction of complex I activity by 20% approximately doubled the rates of glutamate release from synaptosomes depolarized with 4-aminopyridine and maximal glutamate release was observed at between 50–80% inhibition of complex I activity (Fig. 4a). The peak rate of inhibition, 266 ± 12 pmol/min/mg, is not significantly different from that, induced by deoxyglucose combined with 50–80% complex I inhibition, suggesting that glutamate release is minimally influenced by 4-aminopyridine within this range of inhibition. Release of glutamate from synaptosomes depolarized with KCl also peaked within this range of complex I inhibition (Fig. 4b).

ATP depletion caused by inhibition of complex I and glycolysis

Synaptosomes were shown to maintain 90% of initial ATP concentration 10 min after depolarization with 4-aminopyridine or KCl (Fig. 5). However, incubation with 25 nM rotenone reduced the level to 60% with both depolarisating agents ($p < 0.05$). 10 μM rotenone caused a decrease in the ATP content to 40% ($p < 0.001$) in synaptosomes depolarized with 4-aminopyridine, and 47% ($p < 0.001$) in those depolarized with KCl. In polarized synaptosomes, ATP was reduced to 81% in the absence of inhibitor, and treatment with 25 nM and 10 μM rotenone decreased the levels to 75% and 45% respectively. Inhibition of glycolysis with deoxyglucose reduced ATP levels to 30–35% both in 'polarized' synaptosomes and those depolarized with 4-aminopyridine or KCl. This reduction was potentiated in the presence of 25 nM and 10 μM rotenone, which reduced the ATP levels to 23–26% and 15–16%, respectively, under all three conditions.

Effect on $\Delta\psi_m$ caused by inhibition of complex I and glycolysis

The ratio of red (590 nm) fluorescence of JC-1 (aggregate form) to green (535 nm, monomeric form) was found to be 6.88 ± 0.14 in control synaptosomes after 15 min incubation (Fig. 6). The ratio is a qualitative indicator of $\Delta\psi_m$, a high ratio correlating with a polarized mitochondrial inner membrane (Reers *et al.* 1991, 1995). Inhibition of complex I activity by 21.5 ± 10% (using 1 nM rotenone) caused a

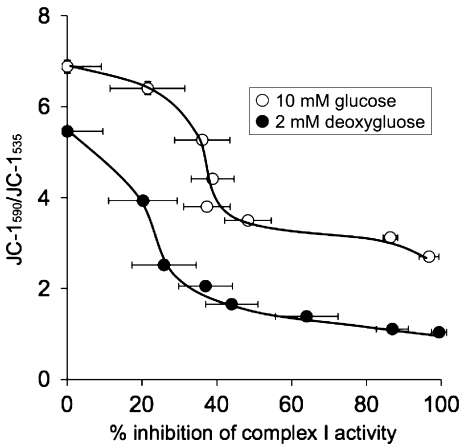

Fig. 6 Partial inhibition of complex I activity causes a fall in $\Delta\psi_m$ in synaptosomes. Synaptosomes were loaded with JC-1 and resuspended (0.5 mg/mL) with 10 mM glucose (○) or 2 mM deoxyglucose (●) and rotenone (1 nM – 10 μM) and in the absence of Ca^{2+}. Fluorescence intensity of the aggregate form of JC-1 (excitation λ = 490 nm, emission λ = 590) was divided by that of the monomer (excitation λ = 490 nm, emission λ = 535) at each concentration of rotenone. The resulting ratios were plotted against percent inhibition of complex I activity brought about by that concentration of rotenone. Points shown represent the mean ± SEM for experiments carried out in triplicate on three separate synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. Addition of FCCP, an ionophore which causes a complete collapse of $\Delta\psi_m$, results in a ratio of 1.08 ± 0.04 in control synaptosomes.

reduction in the ratio to 6.4 ± 0.04. The largest drop in value of the ratio occurs as inhibition of complex I activity approaches 40%, reaching 3.8 ± 0.04 in the presence of 50 nM, corresponding to 37.3 ± 6.3% inhibition. When 1 μM carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) was added to completely collapse $\Delta\psi_m$, the ratio was 1.08 ± 0.04. Using 10 μM rotenone to cause 96.7 ± 2.6% inhibition of complex I activity, the ratio was 2.7 ± 0.04, suggesting a capability of maintaining a low level of $\Delta\psi_m$ for at least 15 min under conditions of almost complete inhibition of complex I in synaptosomes. These results suggest the occurrence of a threshold of complex I inhibition with regards to $\Delta\psi_m$.

The JC-1 ratio was reduced from 6.88 ± 0.14 to 5.45 ± 0.11 in the presence of the glycolytic inhibitor 2-deoxyglucose (2 mM). Also, the threshold was reduced in the presence of deoxyglucose, as 20.2 ± 9.2% (1 nM rotenone) and 25.9 ± 8.6% (10 nM rotenone) inhibition reduced the ratio to 3.93 ± 0.1 and 2.52 ± 0.11 respectively. At 44 ± 7% inhibition of complex I activity (50 nM rotenone), the ratio was reduced to 1.65 ± 0.04. The ratio of 1.04 ± 0.04 at 99.4 ± 2% inhibition of complex I activity (10 μM rotenone) was not significantly different from the value after addition of FCCP, suggesting a complete collapse in $\Delta\psi_m$ under these conditions.

Discussion

In this study we used the synaptosomal model to examine the effect of reduced complex I activity on glutamate release rates. It was found that 40% inhibition of complex I with rotenone significantly increased the Ca^{2+}-independent glutamate release from synaptosomes depolarized with 4-aminopyridine and KCl (Fig. 2). Ca^{2+}-independent release after depolarization with KCl is due to the reversal of the plasma membrane Na^+-cotransport pathway (Nicholls 1989) and results from other studies suggest a large increase in glutamate release from synaptosomes depolarized with KCl when complex I is fully inhibited (Kauppinen *et al.* 1988; Erecinska *et al.* 1996). However, the authors of these studies did not draw attention to this increase, most likely because of the non-physiological 'clamped' depolarization brought about by KCl. Our results show that 4-aminopyridine, an agent which mimics the effect of action potentials on nerve terminals (Tibbs *et al.* 1989) causes a significant increase in Ca^{2+}-independent release of glutamate similar in pattern to the KCl-induced increase when complex I activity is partially inhibited (Figs 1 and 2). Thus, if complex I activity is decreased by 40% in the substantia nigra region in Parkinson's disease, this may be responsible for increasing glutamate release and inducing excitotoxic environments, especially when energy demand is high.

A Ca^{2+}-independent efflux of glutamate in the absence of any direct depolarizing agent has been demonstrated in synaptosomes (Nicholls *et al.* 1987) and metabolic inhibition is known to increase Ca^{2+}-independent efflux from synaptosomes via reversal of Na^+-cotransport pathway (Erecinska 1987; Nicholls *et al.* 1987; Sanchez-Prieto *et al.* 1987; Kauppinen *et al.* 1988; Rubio *et al.* 1991; Santos *et al.* 1996). The suggestion is that reduced ATP levels impede Na^+/K^+ ATPase function resulting in a collapse of plasma membrane potential. Glutamate uptake is thought to occur with cotransport of three Na^+ in and one K^+ out thus relies on the maintenance of the ionic gradients across the membrane (Nicholls 1993). The results from resting synaptosomes (i.e. not depolarized with either 4-aminopyridine or KCl, Fig. 3) suggest that the increase in glutamate release via reversal of uptake may result from a drop below a threshold ATP level (Fig. 5). The 25% reduction in ATP after incubation with 25 nM rotenone does not correlate with a significant increase in glutamate release. Even a 55% reduction in ATP level, (using 10 μM rotenone, which causes 97% inhibition of complex I activity) did not result in glutamate release being significantly increased. This is accordance with the finding that ATP of glycolytic origin is sufficient to maintain the potential across the plasma membrane at close to resting level when complex I activity is inhibited (Scott and Nicholls 1980).

Synaptosomes have been shown to maintain a high rate of ATP production through glycolysis (Kauppinen and Nicholls 1986a) and inhibition of complex I activity with 6 μM

rotenone caused a 10-fold acceleration in the glycolytic rate in synaptosomes which was sustained for over 30 min (Kauppinen and Nicholls 1986b). When glycolysis is inhibited with deoxyglucose, ATP is reduced by 65% in the absence of rotenone (Fig. 5) and glutamate efflux is increased more than 3-fold (Fig. 3, 0% inhibition). Combining inhibition of glycolysis with complex I inhibition results in an even greater increase in glutamate release. 25 nM rotenone (41% complex I inhibition) with deoxyglucose doubles the rate brought about by deoxyglucose alone. ATP is reduced by 75% after 15 min under these conditions (Fig. 5). The highest rates of glutamate release in the absence of 4-aminopyridine or KCl occur when deoxyglucose and 100 nM and 1 μM rotenone are added, and 10 μM rotenone causes 85% reduction in ATP. These results suggest a possible threshold ATP level of 35–45% to prevent reversal of glutamate transporters and below which glutamate release substantially increases.

In each of the glutamate release experiments peak levels of glutamate release occurred at concentrations of < 10 μM rotenone. Nonetheless, 10 μM rotenone was the concentration that consistently brought about the highest levels of complex I inhibition. This may be due to a possible interference with K^+ current across the plasma membrane. Electrophysiological studies have demonstrated the presence of ATP-sensitive K^+ channels in many types of neurons (Mourre *et al.* 1989; Jiang *et al.* 1994; Mercuri *et al.* 1994) and two subtypes have been identified in cortical neurons (Jiang and Haddad 1997). The K_{ATP} channels are closed at normal physiological concentrations of ATP (Ashcroft and Ashcroft 1990), however, the reduction in ATP that follows metabolic impairment results in a hyperpolarization response of membrane potential in different types of neurons (Jiang *et al.* 1994; Nieber *et al.* 1995; Pisani *et al.* 1999; Nicholls 2006). A study using cholinergic neurons showed a hyperpolarization which lasted over 20 min after application of 10 μM rotenone, but show shorter hyperpolarization phases at 3 μM and 1 μM rotenone (Bonsi *et al.* 2004). The duration of hyperpolarization was concentration-dependent. The response to anoxia has been suggested to be a short-term mechanism of self-preservation, which reduces the ATP requirement for the maintenance of Na^+/K^+ pump activity. The use of excess levels of metabolic inhibitors in all previous studies of glutamate release from synaptosomes has meant that this effect has not previously been observed in the nerve terminal model.

The occurrence of threshold effects of ATP production and oxygen consumption in isolated rat brain mitochondria has previously been demonstrated (Davey and Clark 1996; Davey *et al.* 1997, 1998). Complex I of synaptosomal mitochondria has been shown to have a particularly low threshold: when the enzyme is inhibited by > 25% there is an abrupt decrease in ATP production and respiration rate (Davey *et al.* 1998). Figure 6 shows that inhibition of complex I activity by rotenone titration caused a depolarization of $\Delta\psi_m$ and a threshold effect on the mitochondrial membrane potential of *in situ* synaptosomal mitochondria. This threshold may have consequences for the mitochondrial function in substantia nigra nerve terminals of Parkinson's disease sufferers. A reduction of 40% in complex I activity has been demonstrated in postmortem studies of the substantia nigra of patients who suffered from the disease (Schapira *et al.* 1989, 1990a). At this level of inhibition in isolated rat brain nerve terminals, the JC-1 ratio indicator for $\Delta\psi_m$ has almost dropped to the level of total complex I inhibition (Fig. 6).

Inhibition of complex I with rotenone has previously been found to reverse ATP synthase (complex V) activity in guinea-pig synaptosomes (Scott and Nicholls 1980; Chinopoulos *et al.* 1999). This effect has been interpreted as an attempt by the mitochondria to sustain an energised state when the ability of complex I to pump protons from the matrix is compromised (Scott and Nicholls 1980). Under normal circumstances, ATP synthesis is tightly coupled to proton re-entry into the matrix through complex V (Nicholls and Budd 2000). When proton pumping through complex I is reduced, complex V compensates by operating in reverse, thus acting as an alternative proton pump, but resulting in hydrolysis of ATP (Nicholls 2006). The level of $\Delta\psi_m$ maintained in this situation is sub-optimal (Scott and Nicholls 1980; Chinopoulos *et al.* 1999). Removal of the supply of glycolytic ATP by substituting 2-deoxyglucose for glucose causes a complete depolarization of $\Delta\psi_m$ at highest levels of complex I inhibition (Fig. 6). Indeed, the threshold level of inhibition is reduced to < 20% of complex I activity when $\Delta\psi_m$ is affected, suggesting the ATP synthase may begin compensating at low levels of complex I inhibition.

The present study demonstrates that the low threshold of synaptosomal complex I on ATP production and mitochondrial membrane potential has consequences on glutamate release from isolated nerve terminals. The implications of this result for acute neurodegeneration in which the evidence of excitotoxic cell death occurs is hardest are most obvious. During ischemia, oxygen and glucose supply to the affected area is interrupted. Figure 3 models the effect of inhibition of glycolysis on glutamate release resting nerve terminals, and shows that glutamate release is increased under these conditions. Complex I activity is reduced during ischemia by 25% in brain mitochondria (Almeida *et al.* 1995) and 40–50% in synaptsosomes (Allen *et al.* 1995). A 40–50% reduction in synaptosomal complex I activity combined with inhibition of glycolysis correlates to a doubling of the rate brought about by inhibition of glycolysis alone (Fig. 3). Due to the lack of availability of ATP to drive Na^+/K^+ pump, neurons are more likely to depolarize after stimulation during ischemia (Hossmann 2006). Using 4-aminopyridine as a depolarizing agent, a large Ca^{2+}-independent release is observed at 40–50% inhibition of complex I activity which does not increase at higher levels of inhibition (Fig. 4a).

Increased glutamate release from the nerve terminal caused by reduced complex I activity may also have ramifications for the pathogenesis of chronic neurodegenerative disorders. Mitochondrial dysfunction has been implicated in the pathogenesis of Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Lin and Beal 2006) and there is evidence to support a resulting occurrence of slow excitotoxicity in these disorders (Beal 1992a; Doble 1999; Mattson 2003). The principal pathological characteristic of Parkinson's disease is progressive loss of dopaminergic neurons in the substantia nigra pars compacta. The initial etiology is not fully understood, however reduced complex I activity plays a central role in the pathogenesis of sporadic Parkinson's disease (Greenamyre *et al.* 2001; Dawson and Dawson 2003).

Evidence of glutamate excitotoxicity occurring as a result of reduced complex I activity in Parkinson's disease comes largely from the MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) model. MPTP is metabolised in glia by monoamine oxidase-B to MPP^+, a potent complex I inhibitor (Tipton and Singer 1993). NMDA receptor antagonists have been shown to block MPP^+ neurotoxicity in rat substantia nigra (Turski *et al.* 1991) and striatum (Storey *et al.* 1992) and similar protective effects have been demonstrated in primates treated intravenously with MPTP (Zuddas *et al.* 1992; Lange *et al.* 1993). Excitotoxic glutamate thought to be neuronal in origin as decortication prior to treatment with the toxin also results in a protective effect (Srivastava *et al.* 1993).

Glutamatergic projections from the subthalamic nucleus to the substantia nigra are thought to become overactive due to the loss of inhibitory input of dopamine (Bergman *et al.* 1994; Bezard *et al.* 1997). The increased firing activity and bursting patterns of action potentials may in itself lead to further dopaminergic cell death by excitotoxicity (Obeso *et al.* 2004). If the results represented in Figs 1 and 2(a) are regarded as a model for overactive glutamatergic nerve terminals in the substantia nigra region of Parkinson's disease patients, the 40% inhibition of complex I brought about by 25 nM rotenone combined with repetitive firing extrapolates to a substantial release of cytoplasmic glutamate which could contribute to excitotoxicity.

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