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Age-related changes in H_2O_2 production and bioenergetics in rat brain synaptosomes

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ABSTRACT

Detrimental changes to mitochondrial function have been shown to occur with age. In this study we examined the levels of H_2O_2 production, *in situ* mitochondrial membrane potential ($\Delta\psi_m$), oxygen consumption (JO_2) and electron transport chain (ETC) enzyme activities in synaptosomes isolated from rats of two age groups, 6 and 18 months. The rate of H_2O_2 production in synaptosomes was found to be higher in the 18-month old group compared to that of 6-month old. $\Delta\psi_m$ was found to be significantly lower in synaptosomes from the older rats, which also correlated with a reduction in JO_2. Measurement of the individual electron transport chain enzyme activities revealed that reduced complex II/III and complex IV activities were the possible contributors to the reduced bioenergetic function in synaptosomes from the older rats. These data suggest that ageing may lead to increased nerve terminal H_2O_2 production while simultaneous deleterious effects on bioenergetic function occur in *in situ* synaptosomal mitochondria. In addition, Ca^{2+}-independent glutamate release was found to be increased at lower levels of complex I inhibition in the synaptosomes from older rats, suggesting that reduction of mitochondrial function may potentiate excitotoxic conditions in the ageing brain.

1. Introduction

Mitochondrial dysfunction is thought to be part of the ageing process in the brain [1,2] and has been observed in the common neurodegenerative disorders, Parkinson's disease [3] and Alzheimer's disease [4–6]. Mitochondria are known to be the primary source of reactive oxygen species (ROS) production within the cell [7], and a commonly held view is that oxidative stress progressively damages mitochondrial proteins, lipids and DNA, cumulatively leading to dysfunction, including depolarisation of $\Delta\psi_m$ and reduced ATP production [8,9]. Among the sources of mitochondrial ROS production are the enzymes of electron transport chain (ETC) that produce $O_2\cdot-$ and H_2O_2, and previous reports suggest that the levels of H_2O_2 produced by ETC enzymes increases with age [10–12]. The ETC enzymes are also susceptible to damage by ROS [13,14], which may lead to further increases in ROS production [15,16].

The relationship between $\Delta\psi_m$ and the source of H_2O_2 production in the brain has been the subject of much debate in the literature. Studies using isolated mitochondria have indicated that depolarisation of $\Delta\psi_m$ by uncoupling reduces H_2O_2 production [17–19]. However, it seems that this phenomenon is not apparent in the more physiologically relevant synaptosomal model, which examines the effect of $\Delta\psi_m$ on H_2O_2 production in *in situ* nerve terminal mitochondria [20,21]. Complex I and complex III of the ETC are frequently cited as central contributors to mitochondrial H_2O_2 production [22–24] and inhibition of electron transport at these sites using rotenone and antimycin A, respectively, decreases $\Delta\psi_m$ and increases H_2O_2 production in the synaptosomal model [3,21,25]. Inhibition of complex IV activity in *in situ* mitochondria by KCN and NO has also been shown to induce an increase in ROS production in astrocytes [26].

In this study, the relationships between H_2O_2 production, $\Delta\psi_m$, JO_2 and ETC enzyme activities in isolated whole rat brain nerve terminals of two age groups (6 months and 18 months) were examined and compared. It was found that elevated levels of H_2O_2 production in the older group parallel reductions in $\Delta\psi_m$, JO_2 and ETC enzyme activities in this group compared to the younger group.

2. Methods

2.1. Materials

Chemicals were supplied by Sigma Chemical Co., Poole, Dorset, UK. Amplex Red was supplied by Molecular Probes, Eugene, OR, USA. Bio-Rad reagent used for determination of protein concentration was supplied by Bio-Rad Laboratories GmbH, Munich, Germany. Male Wistar rats were supplied by the Bioresources Unit, Biochemistry Department, Trinity College, Dublin.

2.2. Synaptosomal preparation

Two rats of each age group (6 months and 18 months) were killed per preparation by cervical dislocation and synaptosomes and nonsynaptic mitochondria were isolated using a discontinuous ficoll gradient (7.5% w/v and 10% w/v), based on the method of Lai and Clark [27]. Following isolation, synaptosomes were resuspended in ice cold STE (0.32 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4) and the concentration determined using the Bradford protein assay. With the exception of respiration experiments (which were carried out immediately), 1 mg of synaptosomes were added to 1 ml TES buffer (250 mM sucrose/5 mM TES, pH 7.4), centrifuged at 15,000 *g* for 10 min and stored as pellets on ice for use within 2 h of preparation. All experiments were carried out on at least 3 separate synaptosomal preparations to ensure reproducibility of results.

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2.3. Measurement of hydrogen peroxide production

H_2O_2 production was followed using Amplex Red [28]. 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 MgCl2, pH 7.4). Assay sensitivity was optimised and concentrations of 50 μM amplex red and 2.5 U/ml horseradish peroxidase were found to give optimum sensitivity. These concentrations were used for all of the experiments presented in this study. Fluorescence intensity was followed at λ=585 nm (λ=550 nm emission). The fluorescence units were calibrated using a standard curve of known amounts of H_2O_2.

2.4. Measurement of $\Delta\psi_m$

In situ mitochondrial membrane potential was measured with the JC-1 dye (5,5′6,6′ tetrachloro-1,1′3,3′-tetraethylbenzimidazolylcarbocyanine iodide) using a method based on Chinopoulos et al. [29]. Synaptosomes were resuspended in incubation medium (4 mg/ml) and loaded with 6 μM JC-I for 15 min at 37 °C. Following three washes in incubation medium, 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 of inhibitor, where appropriate in ethanol 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 [30]. The ratio of emission at 590 to 535 is a qualitative measure of *in situ* mitochondrial membrane potential [31].

2.5. Oxygen consumption

The rate of *in situ* synaptosomal mitochondrial respiration (JO_2) was followed using Clark-type oxygen electrode with a 7 ml reaction chamber, which was separated from the electrode by an oxygen-permeable Teflon membrane. The rates of oxygen consumption at 37 °C were monitored for 6–8 min in synaptosomes (1 mg/ml) in incubation medium. The samples were then removed from the chamber and stored at −80 °C for measurement of ETC enzyme activities.

2.6. Electron transport chain enzyme activity assays

Samples were freeze-fractured 3 times using liquid nitrogen and were assayed on a Cary UV spectrophotometer at 37 °C. The method used to measure complex I activity was based on a modification of the method of Ragan et al. [32], which involved monitoring the oxidised of NADH at absorbance λ=340 nm, using decylubiquinone as the electron acceptor. 100 μg synaptosomal protein was added to a reaction buffer containing 10 mM $MgCl_2$ and 25 mM potassium phosphate, (pH 7.2) 0.2 mM NADH, 2.5 mg BSA and 1 mM KCN, giving a final volume of 1 ml. Decylubiquinone (50 μM) was added to start the reaction and the rates were monitored for 7–8 min. Rotenone (10 μM) was added to obtain the rotenone-insensitive rate for a further 5–6 min. For complex I activity titration with rotenone, 200 μl synaptosomes (0.5 mg/ml) were incubated in plates at 37 °C with 1 nM–10 μM rotenone for 5 min before being depolarised with 4-aminopyridine, then incubated for a further 10 and stored at −80 °C. Samples were assayed as described, except in a SpectraMAX Plus microplate spectrophotometer with final volume of 200 μl.

Complex II/III activity was determined by following the reduction of cytochrome *c* at absorbance λ=550 nm. Samples (50 μg of protein) were added to plastic cuvettes containing buffer 100 mM potassium phosphate, 0.3 mM potassium-EDTA, (pH 7.4), 1 mM KCN and 100 μM cytochrome *c* with a final volume of 1 ml. The reaction was initiated by the addition of succinate (20 mM). After 7–8 min, myxothiazol (1 μM) was added to calculate the myxothiazol-insensitive rate.

The activity of complex IV was determined using the method of Wharton and Tzagoloff [33]. The oxidation of cytochrome *c* at absorbance λ=550 nm was followed. Reduced cytochrome *c* was prepared by the addition of a few crystals of ascorbic acid to oxidised cytochrome *c* (25 mg/2.5 ml). Excess ascorbic acid was removed by passing the cytochrome *c* sample through a PD_{10} gel filtration column which had been pre-rinsed with 50 ml 1:10 (v/v) dilution of potassium phosphate buffer (100 mM), pH 7.0. The assay cuvettes contained 50 μM reduced cytochrome *c* and 100 μl buffer with a final volume of 1 ml made up with H_2O. The reaction was initiated by the addition of 50 μg synaptosomal samples. The results were expressed as first order decay rate constants (*k*).

2.7. Glutamate release

Glutamate release was measured on a SpectraMAX GeminiXS (Molecular Devices, CA) well plate reader using a continuous fluorimetric assay modified from that described by Nicholls et al. [34]. 100 μl of incubation medium containing 2 mM $NADP^+$, 6.32 U L-glutamic dehydrogenase, was distributed into each of the 96 wells. 2 μl rotenone in ethanol (1 nM–1 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 depolarised after 5 min with 1 mM 4-aminopyridine and rate of increase in NADPH fluorescence at λ=460 nm emission (λ=340 nm excitation) was recorded over a 20 min time period at a 32 s interval following depolarisation. 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 [34] was accounted for when converting rates to pmol/min/mg protein.

2.8. Statistical analysis

Results presented are mean±SEM values. Statistical analyses 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.

3. Results

3.1. H_2O_2 production in synaptosomes

The rate of H_2O_2 production was found to occur at a rate of 0.99±0.03 pmol/min/mg protein in synaptosomes isolated from 6-month old rats (Fig. 1). The rate was found to be approximately 15% higher in 18-month old synaptosomes (1.13±0.02 pmol/min/mg). The difference in the two age groups was still evident after inhibition of complex I activity with 10 μM rotenone, which increased the rate in both groups, with a

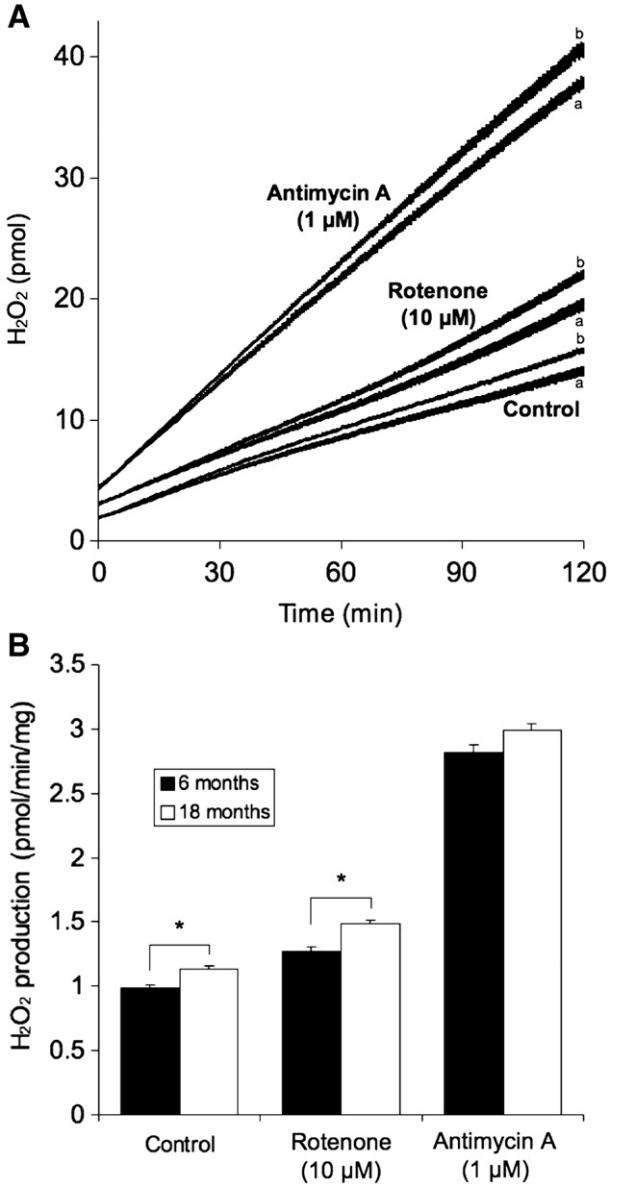

Fig. 1. Age-related increase in H_2O_2 production in rat brain synaptosomes. Synaptosomes (0.5 mg/ml) were incubated with 10 μM rotenone or 1 μM antimycin A where indicated. A: Experimental traces±SEM from synaptosomes isolated from 6-month (traces a) and 18-month (traces b) old rats. B: V_{max} rates of H_2O_2 production±SEM for experiments carried out in triplicate on 3 separate synaptosomal preparations (*=$p<0.05$).

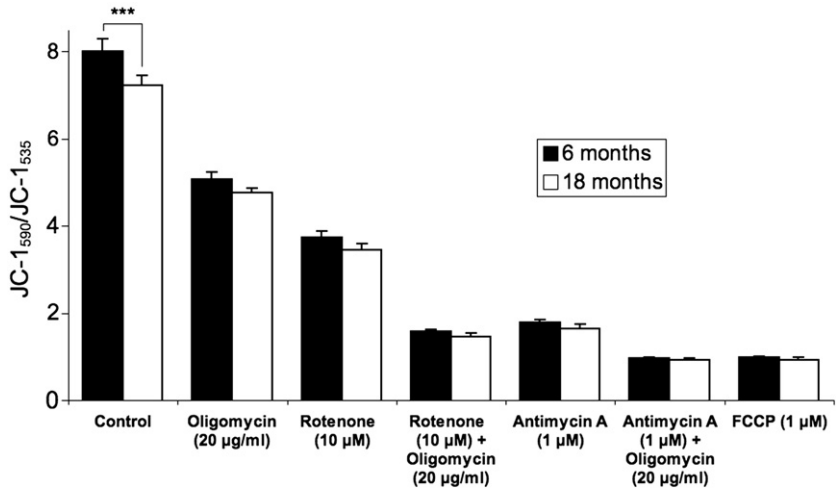

Fig. 2. Age-related decrease in $\Delta\psi_m$ in rat brain synaptosomes. Synaptosomes were loaded with JC-1 and resuspended (0.5 mg/ml) for 10 min at 37 °C with reagents where shown. Fluorescence intensity of the aggregate form of JC-1 (excitation λ=490 nm, emission λ=590) was divided by that of the monomer (excitation λ=490 nm, emission λ=535) under each condition. Bars shown represent the mean±SEM for experiments carried out in triplicate on 3 separate synaptosomal preparations (***=$p<0.001$).

discernable spontaneous increase in rate after 90 min (Fig. 1A). This rate was found to be 1.27±0.04 pmol/min/mg and 1.49±0.03 pmol/min/mg in the two respective groups (Fig. 1B), which corresponds to increases of 29% and 32% compared to controls. Inhibition of complex III activity with antimycin A has previously been shown to cause higher rates of H_2O_2 production in nerve terminals than complex I inhibition with rotenone [21]. In accordance with this work, it was found that 1 μM antimycin A caused a 185% increase in H_2O_2 production from 6-month old and 164% increase in H_2O_2 production from 18-month old synaptosomes, giving rates of 2.81±0.06 and 2.99±0.05 pmol/min/mg respectively (Fig. 1B).

3.2. Membrane potential ($\Delta\psi_m$)

The ratio of aggregate JC-1 (λ=590 nm) to monomer JC-1 (λ=590 nm) is a qualitative indicator of $\Delta\psi_m$ in *in situ* mitochondria, a high ratio indicating aggregation in the mitochondrial matrix due to $\Delta\psi_m$-driven uptake of the dye [30,31]. A reduction in the ratio is therefore thought to reflect depolarisation of $\Delta\psi_m$ [30,31]. $\Delta\psi_m$ was lower in *in situ* synaptosomal mitochondria from 18-month old rats compared to that from 6-month old rats ($p<0.001$, Fig. 2). In synaptosomes from 6-month old rats, the ratio was found to be 8.00±0.29, while in those from 18-month old rats the ratio was found to be 7.22±0.22. Inhibition of complex I activity has been proposed to cause reversal of the complex V (ATP synthase) which results in partial ATP-dependent maintenance of $\Delta\psi_m$ by the protons pumped out of the matrix by complex V [35]. This can be prevented by addition of the complex V inhibitor, oligomycin, to the medium. 10 μM rotenone significantly reduced the ratio in 6- and 18-month old *in situ* synaptosomal mitochondria to 3.73±0.15 and 3.44±0.15 respectively. 20 μg/ml oligomycin with 10 μM rotenone further reduced the $\Delta\psi_m$, resulting in a ratio of 1.57±0.06 in 6-month old and 1.46±0.08 in 18-month old mitochondria. The ratios which resulted from addition of 1 μM FCCP were 0.99±0.02 and 0.93±0.05 in the 6- and 18-month old groups, indicating that $\Delta\psi_m$ was partially maintained under conditions of complete inhibition of complex I activity and complex V activity. This is unsurprising, as proton pumping along the ETC may continue through complex II. In accordance with this interpretation, combined inhibition of complex III and complex V using 1 μM antimycin A and 20 μg/ml oligomycin resulted in total collapse of $\Delta\psi_m$ by 10 min (ratios of 0.96±0.02 in 6-month and 0.93±0.04 in 18-month old samples). A level of $\Delta\psi_m$ was maintained with 1 μM antimycin A in 6-month (1.78±0.24) and 18-month (1.63±0.10) samples, indicating that complex V may partially maintain $\Delta\psi_m$ under conditions of complex III inhibition also. Oligomycin itself has previously been shown to hyperpolarise $\Delta\psi_m$ in guinea pig synaptosomes [29,35]. However using JC-1, it was

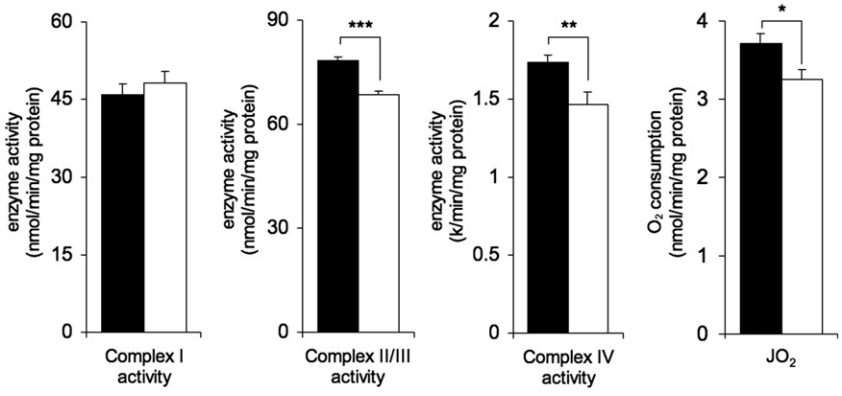

Fig. 3. Age-related decreases in ETC activities and JO_2 in rat brain synaptosomes. Synaptosomes (1 mg) were resuspended in a 1 ml oxygen electrode chamber and JO_2 followed for 5–6 min before being stored at −20 °C for measurement of electron transport chain enzyme activity assays. Bars shown represent the mean±SEM for experiments carried out in triplicate on 3 separate synaptosomal preparations (***=$p<0.001$, **=$p<0.01$, *=$p<0.05$).

found that incubation with oligomycin significantly reduced the membrane potential in both 6-month old and 18-month old rats, giving ratios of 5.08±0.15 in the younger group and 4.77±0.09 in the older.

3.3. Electron transport chain enzyme activities and JO_2

Complex I activity in synaptosomal mitochondria was found to be 45.95±2.10 nmol/min/mg in synaptosomes from 6-month old animals and was not significantly different in those from the 18-month old group (48.11±2.35 nmol/min/mg, Fig. 3A). However, complex II/III activity was found to be 13% lower in the older group (68.46±1 nmol/min/mg, Fig. 3B) compared to 78.29±1.11 nmol/min/mg in the younger ($p<0.001$). The rate of complex IV activity was also found to be lower in the 18-month old group at 1.73±0.04 k/min/mg, which corresponds to a rate 16% slower than the 6-month old group (1.46±0.08 k/min/mg, $p<0.01$). The rate of oxygen consumption (JO_2) by resting synaptosomes was found to be 3.71±0.13 nmol O_2/min/mg in the 6-month old group, and was 12% lower in the older group at 3.25±0.13 nmol O_2/min/mg ($p<0.05$, Fig. 3D).

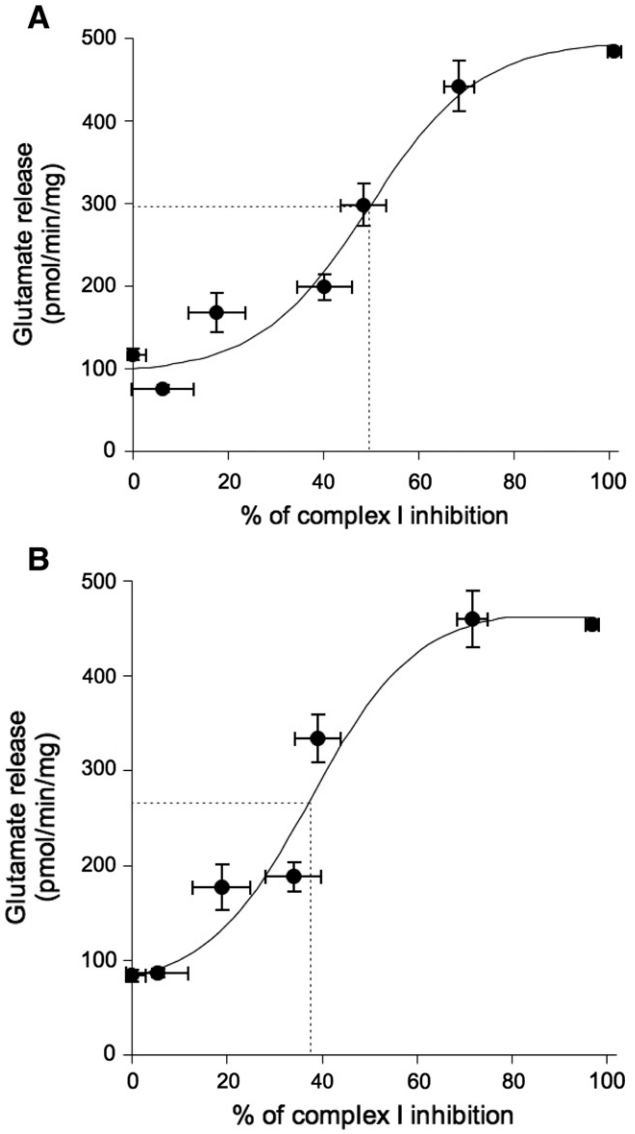

Fig. 4. Age-related differences in complex I control of Ca^{2+}-independent glutamate release from depolarised synaptosomes. Synaptosomes (0.5 mg/ml) were preincubated with rotenone (1 nM – 10 μM) at 37 °C for 5 min before being depolarised with 1 mM 4-aminopyridine. Rates of glutamate release at each concentration of rotenone were plotted against percent inhibition of complex I activity brought about by that concentration of rotenone. Sigmoid curves were plotted through the results and the 50% of maximum glutamate release rate value calculated using a statistical fit. The value was found to correspond to 49.6±7% in synaptosomes from 6-month old rats (A) and 37.6±1.4% in synaptosomes from 18-month old rats (B). Points shown represent the mean±SEM for experiments carried out in triplicate on 4 separate synaptosomal preparations.

3.4. Glutamate release

We have recently reported that Ca^{2+}-independent glutamate release from depolarised synaptosomes was increased by partial complex I inhibition with rotenone [36]. In the present study, we used 1 nM–1 μM rotenone to inhibit complex I activity in a dose-dependent manner and correlated the inhibition of complex I activity with the release rates of glutamate in the two age groups. The pattern of release rates over the titration in synaptosomes from 6-month old rats was similar to that of our previous experiments [36] and the value at which 50% of maximum release rate was reached was found to occur at 49.6±7.0% inhibition of complex I activity (Fig. 4A). It was apparent that the threshold of complex I inhibition was lowered to 37.6±1.4% in the 18-month old rats before 50% of maximum glutamate release rate was reached. (Fig. 4B).

4. Discussion

The ageing process involves changes to mitochondria including increased levels of ROS production and reduction in bioenergetic capacity. This study finds elevated rates of H_2O_2 production in synaptosomes from 18-month old rats compared to those from 6-month old. Simultaneously, $\Delta\psi_m$ and JO_2 were both found to be lower in the older group. Assays of ETC components revealed compromised complex II/III and complex IV activity synaptosomes from older animals. These data indicate that an age-related decline in ETC enzyme activities in nerve terminal mitochondria may have implications for the ability of the nerve terminal to maintain adequate energy requirements. In accordance with the 'vicious cycle' theory of oxidative damage [15,16], age-related increased rates of H_2O_2 production suggest higher levels of oxidative stress, which is likely to result in further damaging effects on mitochondrial function.

Elevated H_2O_2 production in resting synaptosomes was correlated with lower $\Delta\psi_m$ and oxygen consumption rates. The lower level of respiration may be due to lower complex II/III and complex IV activities demonstrated by our enzyme assay results. Age-related reductions in complex II and complex III activities have previously been demonstrated in mouse brain mitochondria [37], and decreased complex IV activity with age has been widely reported in rat brain [13,38]. Low-level pharmacological inhibition of complex II/III and complex IV activities in synaptic mitochondria has been found to reduce oxygen consumption and ATP synthesis by up to 15% in synaptosomal mitochondria [39]. Major changes in oxidative phosphorylation parameters occur when the complex activities are inhibited by approximately 60% and above. Under conditions of reduced electron flow through the electron transport chain, a reduction in proton pumping out of the mitochondrial matrix would be expected, resulting in a lower level of $\Delta\psi_m$. Whether the elevated H_2O_2 production seen in the older group is a cause or effect of reduced levels of ETC enzyme function is not clear.

In agreement with previous studies, inhibition of complex I activity with rotenone increased synaptosomal H_2O_2 production [21,25] and decreased $\Delta\psi_m$ [29,35]. The complex I iron–sulphur centres [40,41] and the flavin moiety [42] have been cited as primary sites of mitochondrial ROS production. The difference in rate of H_2O_2 production between the two age groups was not eradicated by complex I inhibition with rotenone, suggesting that ROS production at the rotenone-binding site in complex I is not responsible for the elevated H_2O_2 production in the older group [43]. Furthermore, complex I activity was not found to be reduced in the older group, suggesting no oxidative damage at this site of the ETC in the 18-month old nerve terminals. Inhibition of complex III using antimycin A produced higher levels of H_2O_2 production and completely

depolarised $\Delta\psi_m$ when reversal of complex V was blocked by oligomycin. However, at least some of the H_2O_2 production induced by this inhibitor is due to reverse electron transport through complex I, and can be inhibited by rotenone [25]. Complex III has been implicated in mitochondrial H_2O_2 production [24] and the rate of H_2O_2 production in the older group was not found to be different to that in the younger group after inhibition at this site of the ETC, suggesting the antimycin A binding site at complex III may partially contribute to the elevated level of H_2O_2 production in the uninhibited older group. Other mitochondrial enzymes are known to simultaneously produce and be susceptible to damage by H_2O_2 in the nerve terminal, most notably α-ketoglutarate dehydrogenase [44,45], and this enzyme may also be among the sources of the excess H_2O_2 produced in the older synaptosomes.

We have recently reported on how partial reduction of complex I activity increases Ca^{2+}-independent glutamate release from depolarised synaptosomes [36]. In the study, we described how our model could be relevant to the neurotransmitter release patterns by glutamatergic nerve terminals during Parkinson's disease, during which complex I activity has been shown to be reduced [46–48]. Flux control analysis of mitochondria isolated from rat brain shows that complex I has a higher flux control coefficient than other ETC components in synaptic mitochondria compared to nonsynaptic mitochondria. Similarly, when complex I activity is inhibited by 25%, major changes in oxidative phosphorylation occur, whereas the energy threshold is 60% in nonsynaptic mitochondria [39,49,50]. An age-related impairment in flux through the oxidative phosphorylation system of *in situ* synaptosomal mitochondria has previously been demonstrated [51]. Results in our present study indicate that a lower threshold of inhibition of complex I activity exists in the nerve terminals of older rats compared to younger rats that may result in increased release of glutamate following depolarisation. The tighter control of complex I over glutamate release in the older group suggests that at the 40% reduction in complex I activity observed in substantia nigra during Parkinson's disease may lead to higher rates of glutamate release with age.

In the present study we used rats of two age groups, 6 months and 18 months. Rats usually reach sexual maturity at 3–4 months and 18-month old rats are considered to be 'late middle aged' [52]. 'Old age' is the highest risk factor for many common chronic neurodegenerative disorders in which deterioration of mitochondrial function is implicated in their aetiology [6]. Our results show elevated levels of nerve terminal H_2O_2 production and reduced level of mitochondrial function in the 'late middle aged' group, indicating that this process may begin before the onset of 'old age'. Although the differences between the two age groups appear to be relatively subtle, they may be among the key events likely to trigger a 'vicious cycle' of mitochondrial deterioration due to increased levels oxidative stress [15,16]. This increase in ROS production and associated decline in mitochondrial function may play a role in the pathogenesis of chronic neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease [4,6,53].

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