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**NERVE TERMINAL
BIOENERGETICS AND CONTROL
OF NEUROTRANSMITTER RELEASE**

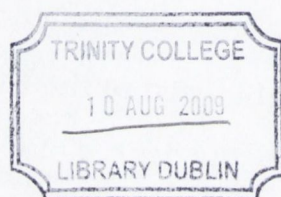
BY

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**A dissertation submitted to the University of Dublin
in candidature for the degree of Doctor of Philosophy**

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DECLARATION

I certify that none of the work presented in this thesis has been submitted for any degree at this or at any other University. I declare that this thesis is entirely my own work. I agree that the library may lend or copy this thesis upon request.

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Seán Kilbride

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SUMMARY

Mitochondrial dysfunction has been shown to develop as part of the aging process and has been implicated in chronic neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease. However, the initial aetiology of these disorders remains unclear, and the role of mitochondrial dysfunction in their pathogenesis has yet to be fully elucidated. However, mitochondrial involvement in excitotoxicity and oxidative stress has been postulated in affected brain regions in these disorders.

In this study, the control of the mitochondrial electron transport chain enzymes of *in situ* nerve terminal mitochondria over glutamate release, mitochondrial membrane potential ($\Delta\psi_m$), plasma membrane potential ($\Delta\psi_p$) and ROS production was examined by titrating out the activities of the specific enzymes with appropriate inhibitors. Inhibition of complex I activity by 40% with rotenone was found to result in increased Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with either 4-aminopyridine or KCl. Highest rates of glutamate release were found to occur between 60 – 90% inhibition of complex I activity. In the absence of any depolarising agent, glutamate efflux was not effected by any level of inhibition of complex I activity. However, when combined with inhibition of glycolysis, the release pattern was found to be similar to that in depolarised synaptosomes. Inhibition of glycolysis blocked Ca^{2+} -dependent glutamate release rates from depolarised synaptosomes. The increase in Ca^{2+} -independent glutamate release rates were found to correlate with a significant decrease in ATP level, and complex I control over these rates was shown to increase with age.

Complexes III and IV were found to have less control over Ca^{2+} -independent glutamate release rates from depolarised synaptosomes: inhibition of complex III by up to 50 – 60% with myxothiazol and up to 85% with antimycin A had no effect on glutamate release rates, nor did inhibition of complex IV activity by up to 85% with KCN. However, inhibition of complex III or complex IV activities above these threshold levels increased Ca^{2+} -independent glutamate release rates from both polarised and depolarised synaptosomes. The increased Ca^{2+} -independent glutamate release rates were correlated to a fall in $\Delta\psi_p$ below a threshold of ~ -50 mV.

Complex I was also found to have tighter control over $\Delta\psi_m$ in *in situ* synaptosomal mitochondria than complex III or complex IV. Inhibition of complex I activity by 40% was found to be sufficient to partially depolarise $\Delta\psi_m$, whereas inhibition of complex III activity by 70% or 90% (with myxothiazol and antimycin A respectively) was required, and inhibition complex IV activity did not affect $\Delta\psi_m$. H_2O_2 production was shown to be reduced by depolarisation of $\Delta\psi_m$ using the uncoupler FCCP. Also, H_2O_2 production was found to be increased in synaptosomes isolated from 18-month old rats compared to those isolated from 6-month old rats, while resting $\Delta\psi_m$ was also found to be decreased in the nerve terminals isolated from the older rats. The relevance of such results in the context of aging and the pathogenesis of chronic neurodegenerative disorders is discussed.

ABBREVIATIONS

ΔG	Gibb's free energy
Δp	Protonmotive force
$\Delta\psi_m$	Electrical potential across the mitochondrial membrane
$\Delta\psi_p$	Electrical potential across the plasma membrane
ALS	Amyotrophic lateral sclerosis
AP	Aminopyridine
ADP	Adenosine diphosphate
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
BSA	Bovine serum albumen
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
Cl^-	Chloride
dH ₂ O	De-ionised water
EAAT1	Excitatory amino acid transporter 1
EAAT2	Excitatory amino acid transporter 2
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
FADH ₂	Flavin adenine dinucleotide (reduced form)
FCCP	Carbonylcyanide <i>p</i> -triflouromethoxyphenylhydrazone
FMN	Flavin mononucleotide
g	Relative gravitational force
GDH	L-glutamic dehydrogenase
GSH	Glutathione
GSSG	Oxidised glutathione
H ₂ O ₂	Hydrogen peroxide
K^+	Potassium
KCl	Potassium chloride
KCN	Potassium cyanide
λ	Wavelength
l	Litre

M	Molar
MgCl ₂	Magnesium Chloride
nm	Nanometres
min	Minute
MPP ⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mV	Millivolts
Na ⁺	Sodium
NaCl ₂	Sodium chloride
NADH ⁺	Nicotinamide adenine nucleotide
NADH	Nicotinamide adenine nucleotide (reduced form)
NADP ⁺	Nicotinamide adenine nucleotide phosphate
NADPH	Nicotinamide adenine nucleotide phosphate
NMDA	<i>N</i> -methyl-D-aspartate
O ₂ ^{·-}	Superoxide radical
OH [·]	Hydroxyl radical
ONOO ⁻	Peroxynitrite
PMPI	Plasma membrane potential indicator
Q cycle	Ubiquinol-ubiquinone cycle
s	Seconds
SEM	Standard error of the mean
SOD	Superoxide dismutase
Tris	Tris(hydroxymethyl) aminomethane
Triton X-100	Iso-octylphenoxypolyethoxyethanol
U	Units of enzyme activity
vATPase	Vesicular ATPase
Vol.	Volume
v/v	Volume per unit volume
w/v	Weight per unit volume

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Chapter 1

General Introduction

1.1 Bioenergetics of the nerve terminal

The metabolic rate in the mammalian central nervous system is extraordinarily high, consuming 20% of the oxygen inspired at rest, despite only accounting for 2% of bodyweight (Silver and Erecinska 1998). Much of the neuronal energy demand is attributable to the nerve terminal, where the ATP is used to maintain ionic gradients across the cell membranes. Nerve terminals and neurons generally are heavily reliant on mitochondrial aerobic metabolism (Gunter *et al.* 1994) and failure to maintain adequate oxygen supply quickly leads to cell death (Navarro and Boveris 2004). At least 70% of nerve terminal ATP is produced by mitochondria (Kauppinen and Nicholls 1986a).

Mitochondria are organelles that consist of two compartments, separated by an inner membrane. The inner membrane is highly invaginated, forming ‘cristae’, and encloses the mitochondrial matrix. The intracristal spaces are continuous with the intermembrane space, the second compartment. The outer membrane is permeable to ions and small molecules via transmembrane channels called ‘porins’. The inner membrane is only permeable to ions via ion-specific transporters, and is impermeable to protons. ATP can be generated by mitochondria because of proton pumping by enzymes across the mitochondrial inner membrane, which creates a proton gradient that is tightly coupled to ATP production.

1.1.1 The electron transport chain and the chemiosmotic theory

The electron transport chain (ETC) consists of prosthetic groups of integral proteins that transfer electrons in series (Navarro and Boveris 2007). The first of the component of the ETC, complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) catalyses the oxidation of NADH to NAD^+ at a redox potential of -300 mV, pumping four protons out of the mitochondrial matrix to the intermembrane space in the process (Fig. 1.1). It is composed of 45 different polypeptide subunits (Carroll *et al.* 2006) including 6 iron-sulphur centres (Hinchliffe and Sazanov 2005) and an FMN-containing flavoprotein (Hatefi 1985). The ‘L-shaped’ form of complex I includes a long hydrophobic arm, which is embedded in the mitochondrial inner membrane and a short hydrophilic arm, which extends into the mitochondrial matrix, and which

contains the FMN and NADH active site (Hatefi 1985). These arms assemble independently due to their separate genetic origin (Hofhaus *et al.* 1991). Rotenone was found to be a specific and stoichiometric inhibitor of complex I activity in isolated mitochondria by Chance and Williams (1956), and all rotenone-insensitive NADH oxidation in NADH dehydrogenase preparations is thought to occur via denatured forms of complex I (Navarro and Boveris 2007).

Complex II (Succinate:quinone oxidoreductase (EC 1.3.5.1) catalyses the oxidation of FADH_2 to FAD^+ , and also functions as a component of the citric acid cycle. Both complex I and complex II can reduce ubiquinol/ubiquinone, which, when donating electrons to complex III (QH₂:cytochrome *c* oxidoreductase, EC 1.10.2.2), causes another four protons to pass from the matrix to the intermembrane space (fig. 1.1). Cytochrome *c* is then reduced by complex III at a redox potential of approximately + 250 mV and donates the electrons to complex IV (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1), which converts O_2 to $2\text{H}_2\text{O}$ with four electrons from the reduced cytochrome *c*. In doing this, it consumes 4 protons from the mitochondrial matrix. The complexes of the electron transport chain are thought to assemble into supercomplexes, with stoichiometric ratios between the individual complexes.

Although research indicates that supercomplexes in cardiac and brain mitochondria contain a I_1III_2 component (Schafer *et al.* 2006), it is not yet clear what the ratio of complex IV to the other complexes is, and the ratio may vary within brain mitochondria (Reifschneider *et al.* 2006). The combined proton-pumping of the supercomplexes scattered throughout the inner mitochondrial membrane creates an electrochemical gradient (of protons) across the membrane (Nicholls and Budd 2000). It is this protonmotive force (Δp) that maintains the electrochemical proton gradient across the mitochondrial inner membrane and allows production of ATP from ADP and a phosphate group by complex V (ATP synthase, EC 3.6.3.14). The passage of three protons through complex V into the mitochondrial matrix results in the generation of one ATP molecule (Fig. 1.1).

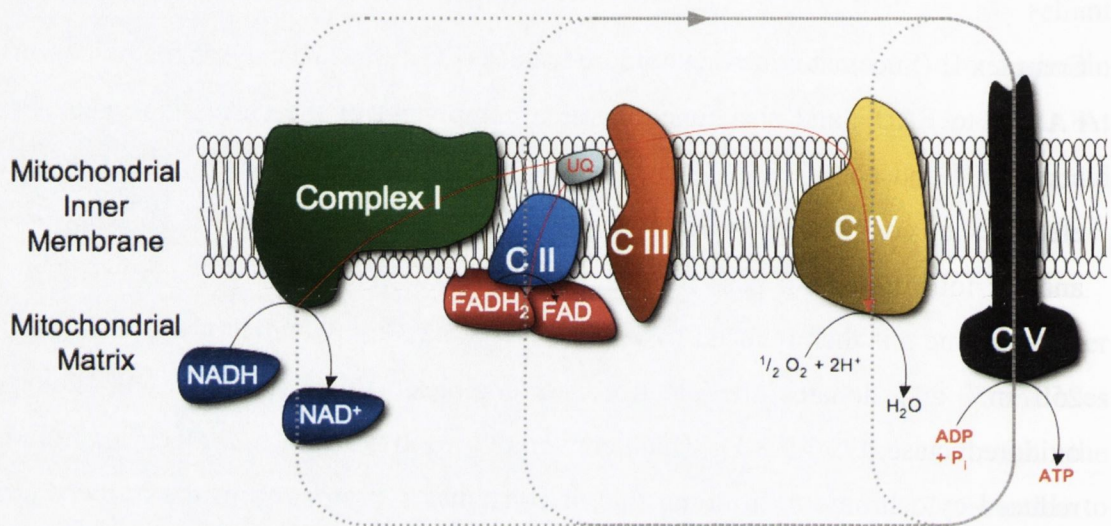


Fig. 1.1 Schematic representation of the electron transport chain and proton circuit of oxidative phosphorylation.

Electrons from NADH and FADH₂ enter the respiratory chain at complex I and II respectively. Complexes I, III and IV pump protons in series out of the matrix into the intermembrane space. Protein re-entry through complex V generates ATP from ADP and an inorganic phosphate. Adapted from Fig. 1A of Nicholls and Budd (2000).

1.1.2 Mitochondrial proton circuit

The fall in redox potential of the electrons being passed through the electron transport chain during respiration is used to generate a proton electrochemical potential gradient (Δp) across the mitochondrial inner membrane.

The ATP synthase is the primary route of proton re-entry into the mitochondrial matrix. The protein complex is in fact an ATP-hydrolysing proton pump that is forced to work in reverse by Δp . Mitochondria maintain the ATP synthesis reaction up to 10 times away from equilibrium (Nicholls 1974), and the free energy in a cell comes from this displacement from equilibrium – the ATP molecule itself is not a ‘high-energy compound’. The Gibb’s free energy (ΔG), a measure of this displacement from equilibrium, is approximately + 60 kJ/mol for ATP synthesis in the cytoplasm (Rosing and Slater 1972).

The proton circuit has been compared to an electrical circuit for instructive purposes (Fig. 1.2). If Δp is the potential term, and the proton conductance of the inner membrane corresponds to the conductance of a component, the resultant proton current around the circuit is subject to Ohm’s Law (Nicholls and Budd 2000). Proton re-entry through the ATP synthase completes the circuit. The rate of ATP synthesis is tightly coupled to re-entry of protons through the ATP synthase, and therefore also to proton current around the circuit. Hence the proton current automatically responds to ATP demand. Proton pumping by each of the electron transport chain complexes is also tightly coupled to electron flux through the respective complex so that electron flux is also controlled by ATP demand (Nicholls and Budd 2000). This control of respiration rate by ATP turnover is termed ‘respiratory control’, and in nerve terminals is dictated mainly by the demand on ATP made by plasma membrane ion pumping (Pauwels *et al.* 1985). The Δp is expressed in electrical potential units and can be calculated in isolated mitochondria using the following equation:

$$\Delta p \text{ (mV)} = \Delta \psi_m - \left(2.3 \frac{RT}{F} \right) \Delta pH$$

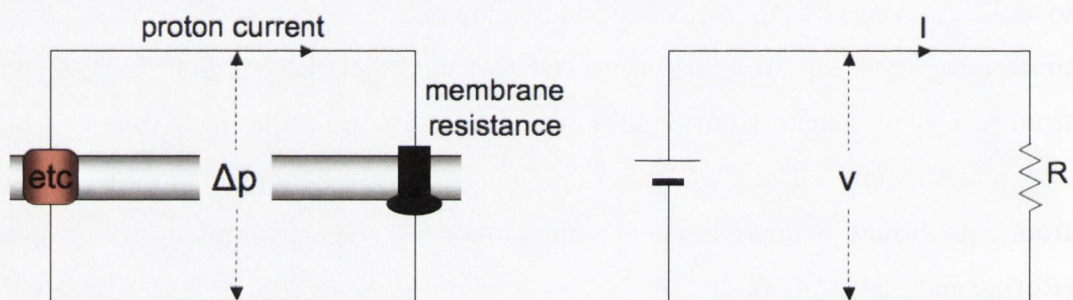


Fig. 1.2 Mitochondrial proton circuit

The mitochondrial proton circuit is comparable to an equivalent electrical circuit and can be quantified by Ohm's law, where proton-motive force (Δp) is equivalent to voltage term (v) and magnitude of proton current (I) is defined by membrane resistance to protons (R). The 'resistance' through complex V is controlled by rate of ATP synthesis. Adapted from Fig. 1B of Nicholls and Budd (2000).

where $\Delta\psi_m$ is the mitochondrial membrane potential, ΔpH is the pH gradient across the inner membrane, R is the gas constant, T the absolute temperature and F the Faraday constant. At 37°C , $2.3RT/F = 60$. In isolated mitochondrial models that use reasonable physiological conditions, $\Delta\psi_m$ accounts for 150 – 180 mV of the total Δp of 200 – 220 mV (Nicholls 1974).

1.1.3 Mitochondrial membrane potential ($\Delta\psi_m$)

As described above, Δp across the mitochondrial inner membrane is composed of the chemical potential of the proton gradient, but mainly the electrical potential, termed $\Delta\psi_m$. $\Delta\psi_m$ acts on all ionic distribution across the mitochondrial inner membrane, and controls transport between the mitochondrion and the cytoplasm. The equilibrium distribution of an ion is related to $\Delta\psi_m$ by the Nernst equation:

$$\Delta\psi_m = 2.3 \left(\frac{RT}{mF} \right) \log_{10} \left(\frac{[X^{m+}]_A}{[X^{m+}]_B} \right)$$

where $\Delta\psi_m$ is the mitochondrial membrane potential, R is the gas constant, T the absolute temperature, m the valence of the ion, F the Faraday constant and $[X^{m+}]_B$ and $[X^{m+}]_A$ the concentration of the ion outside and inside the inner membrane. In *in situ* mitochondria, the most important ions regulated by $\Delta\psi_m$ are Ca^{2+} and Na^+ (Fig. 1.3). From the Nernst equation it can be deduced that divalent cations are accumulated in the mitochondrial matrix to much higher extents than monovalent cations, and that anions are excluded. Under normal physiological conditions, $\Delta\psi_m$ is maintained at approximately -150 mV in *in situ* nerve terminal mitochondria of resting synaptosomes (Scott and Nicholls 1980). The equilibrium distribution of synthetic fluorimetric or radiolabelled cations across the mitochondrial inner membrane forms the basis of experimental measurement of $\Delta\psi_m$.

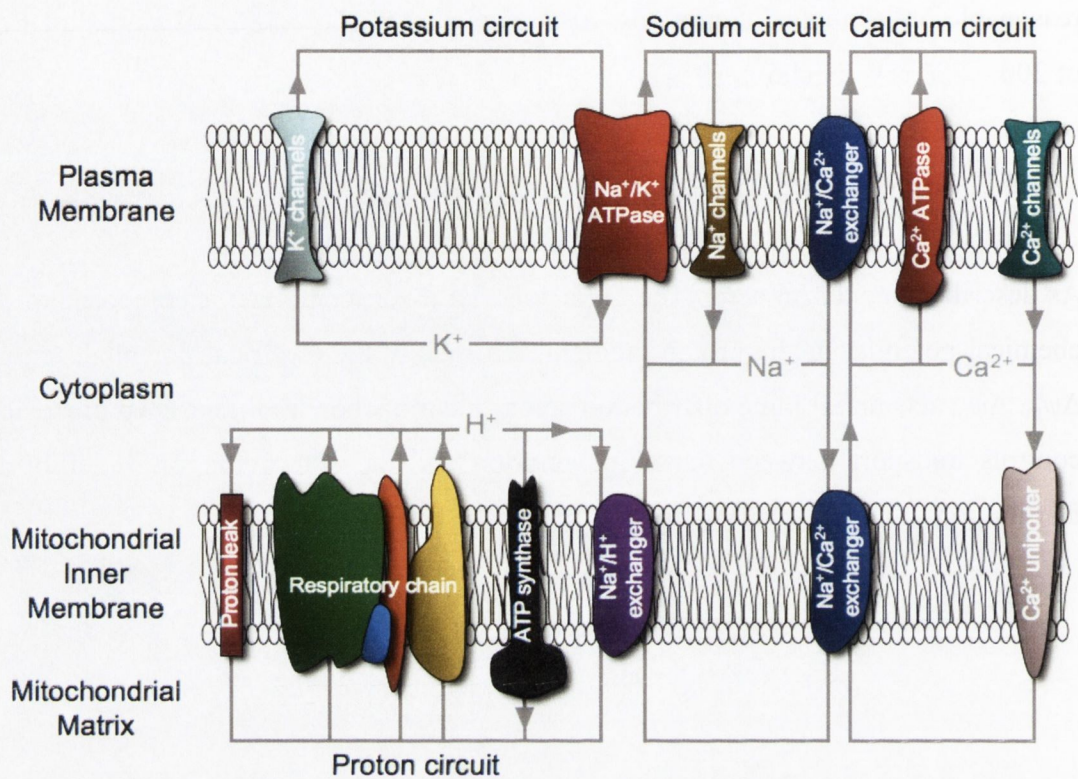


Fig. 1.3 Ion circuitry across mitochondrial and plasma membranes of a typical neuron

The protonmotive force of the respiratory chain drives all of the circuits represented here either directly or indirectly via ATP. Adapted from Fig. 2 of Nicholls and Budd (2000).

1.1.4 Plasma membrane potential ($\Delta\psi_p$)

Ion flux across the plasma membrane of neurons is also governed by electrochemical driving forces. In nerve terminals, where K^+ , Na^+ and Cl^- gradients exist across the membrane, the resting $\Delta\psi_p$ can be quantified by the Goldman equation:

$$\Delta\psi_p = 2.3 \frac{RT}{F} \log_{10} \left(\frac{P_{Na} [Na^+]_{out} + P_K [K^+]_{out} + P_{Cl} [Cl^-]_{out}}{P_{Na} [Na^+]_{in} + P_K [K^+]_{in} + P_{Cl} [Cl^-]_{in}} \right)$$

where P_{Na} , P_K and P_{Cl} are the permeabilities of the respective ions, and $[Na^+]_{out}$ and $[Na^+]_{in}$ the extracellular and intracellular Na^+ concentrations respectively etc.. In neurons, K^+ , Na^+ gradients are maintained by active pumping of these ions by the Na^+/K^+ ATPase (Fig. 1.3). In one cycle, the Na^+/K^+ ATPase exchanges three intracellular Na^+ ions for two K^+ ions, hydrolysing one ATP molecule in the process. The Na^+/K^+ ATPase maintains the $[Na^+]_{out}$ approximately twenty fold greater than the $[Na^+]_{in}$, and $[K^+]_{in}$ is maintained at approximately 10 times $[K^+]_{out}$. A resulting $\Delta\psi_p$ of -80mV (Nicholls 2006) has been measured in neurons in vitro, and -45 mV in nerve terminals (Scott and Nicholls 1980). Ion-specific transporters that use the Na^+/K^+ gradient to pump Ca^{2+} and Cl^- against the electrochemical gradient out of the cytoplasm maintain the Ca^{2+} and Cl^- gradients, and there is also Ca^{2+} -ATPases present on the plasma membrane.

Using the Goldman equation, the following permeability ratios for resting $\Delta\psi_p$ have been elucidated electrophysiologically by Katz and Miledi (1967):

$$P_K : P_{Na} : P_{Cl} = 1.0 : 0.04 : 0.45$$

Under resting conditions, Na^+ permeability is low. However, Na^+ permeability increases via the opening of voltage-dependent Na^+ channels following the depolarisation of $\Delta\psi_p$ to a threshold level, resulting in a sudden influx of Na^+ and the propagation of an action potential. At the peak of an action potential, the permeability ratios change such that:

$$P_K : P_{Na} : P_{Cl} = 1.0 : 20.0 : 0.45$$

$\Delta\psi_p$ rapidly falls and becomes positive during the milliseconds of an action potential, and in response voltage-dependent K^+ -channels open, resulting in a rapid restoration of $\Delta\psi_p$ to a hyperpolarised level. This brief hyperpolarisation prevents immediate propagation of the next action potential, but $\Delta\psi_p$ returns to a resting level within milliseconds. In nerve terminals, voltage-dependent Ca^{2+} -channels respond to action potentials by allowing highly localised Ca^{2+} entry, which triggers exocytosis of neurotransmitter (Nicholls 1974).

1.2 The synaptosomal model of the nerve terminal

In the early 1960s it was noticed that by using differential centrifugation techniques on brain homogenate to extract neuronal mitochondria there appeared to be extra particles in the crude fraction (Gray and Whittaker 1962). When examined under the electron microscope it was evident these particles were intact nerve terminals which had been pinched off from their adjoining axons. They occur in highest concentrations in layered brain regions such as the cerebral cortex and the hippocampus, as cleavage during homogenisation tended to follow the divisions between the layers, resulting in shearing of the terminals off axons. Subsequently, the term ‘synaptosomes’ was given to the particles, and they were defined as being 0.5 – 1 μm in diameter, and containing one or more mitochondria (Nicholls 1993). Often pieces of post-synaptic membrane are adherent to the parts of the synaptosome which contain a high concentration of 50 nm diameter synaptic vesicles (the ‘active zones’). Later, techniques for purifying (and testing the viability of) synaptosomes became standardised, and synaptosomes are now commonly used as models of the nerve terminal.

Synaptosomes possess all of the features of nerve terminals including fully functioning mitochondria, ion channels, vesicles, and neurotransmitter exocytotic and re-uptake equipment (Fig. 1.4). After careful preparation they remain viable for several hours. Glutamate is the most common excitatory neurotransmitter in the brain, and is involved in cognition, memory movement and sensation (Gasic and Hollmann 1992). In any preparation of whole brain synaptosomes, > 90% are glutamatergic (Nicholls 1993).

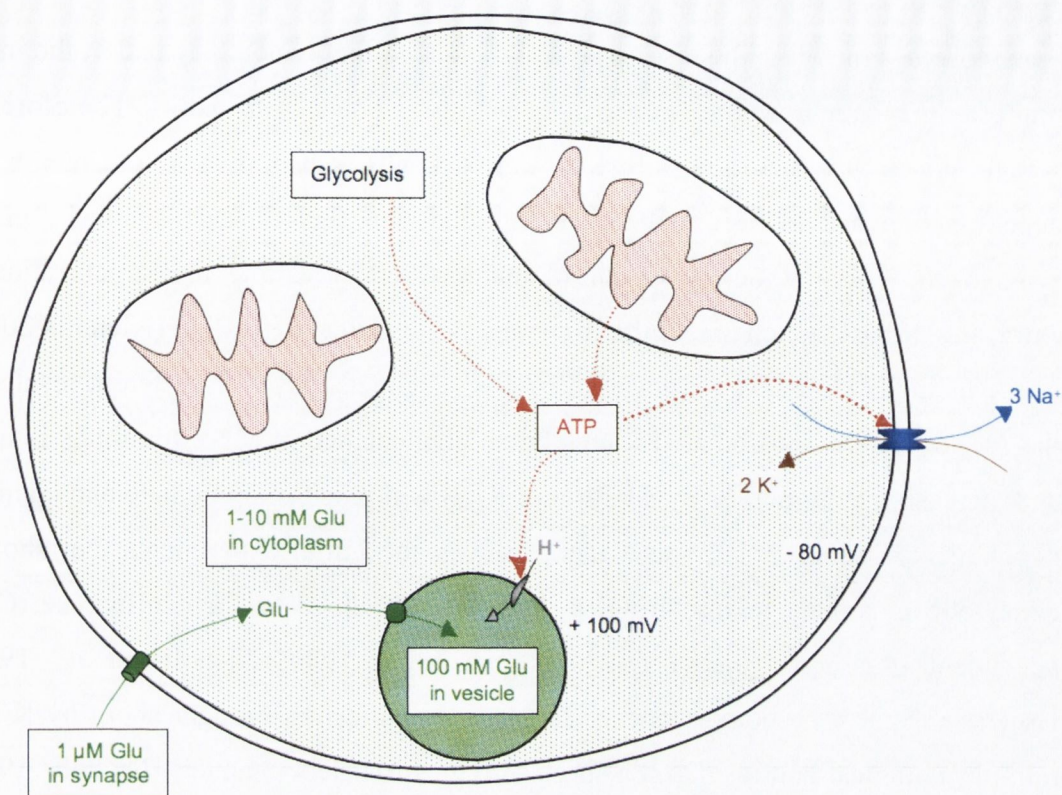


Fig. 1.4 The glutamatergic synaptosome

ATP produced by glycolytic metabolism of glucose or by oxidative phosphorylation in mitochondria is mainly used by the Na⁺/K⁺ ATPase (up to 70% in resting synaptosomes), which maintains a resting $\Delta\psi_p$ of approximately -80 mV. *In vivo*, uptake by glutamate transporters on nerve terminals and surrounding glia maintain a low extracellular glutamate concentration (1 μM), while the cytoplasmic concentration is at least a thousand-fold higher (1 – 10 mM). Glutamate is most heavily concentrated in vesicles (100 mM). ATP drives both of these uptake mechanisms indirectly: uptake across the plasma membrane is driven by the Na⁺ and K⁺ gradients across the membrane, and vesicular uptake is driven by the H⁺ gradient across the vesicular membrane, maintained by the vATPase. Exocytosis of glutamate triggered by Ca²⁺ influx after depolarisation of $\Delta\psi_p$ has also been shown to be ATP-dependent.

1.2.1 Depolarising synaptosomes

Synaptosomes maintain the resting potential close to that which they had in the intact brain, as they are no longer receiving electrical signals from axons. Therefore, to study release of neurotransmitter from synaptosomes, modes of membrane depolarisation had to be obtained. To this end, ion flux through channels on the synaptosomal plasma membrane can be manipulated by various means: elevation of extracellular KCl, K⁺-channel inhibition, and Na⁺-channel activation (Nicholls 2003). These methods all activate voltage-gated Ca²⁺-channels, but with different results on the $\Delta\psi_p$ of the synaptosomes. K⁺ membrane conductance in nerve terminals is high, and increasing external K⁺ (by addition of KCl) will result in immediate and clamped depolarisation. This model was widely used in the initial experiments that showed exocytotic glutamate release from synaptosomes coupled to Ca²⁺ influx, i.e. Ca²⁺-dependent glutamate release (Nicholls and Sihra 1986; Nicholls *et al.* 1987). However, it must be noted that the clamped depolarisation brought about by KCl is non-physiological and results in a single firing of transient channels (Nicholls 1993). Also, the high K⁺ concentration may cause thermodynamic reversal of Na⁺ : amino acid cotransport, causing release of cytoplasmic glutamate (Erecinska 1987). Nonetheless, Ca²⁺-dependent exocytotic release is still distinguishable from Ca²⁺-independent release via transporter reversal (Nicholls *et al.* 1987).

A more physiological pattern of depolarisation is brought about by K⁺-channel inhibition by millimolar amounts of 4-aminopyridine (Fig. 1.4) or nanomolar amounts of the mamba neurotoxin α -dendrotoxin. Both 4-aminopyridine and dendrotoxin inhibit a specific subset of K⁺-channel, I_A channels, which regulate excitability in neurons. I_A channels are thought to hyperpolarise the plasma membrane when open, being activated by presynaptic G-protein linked receptors (in some neurons at least). I_A channel inhibition brings about repetitive firing of Na⁺-channels, resulting in repetitive action potential-like depolarisations of synaptosomes (Tibbs *et al.* 1989; Tibbs *et al.* 1996). 4-aminopyridine has been developed commercially as a treatment of Multiple Sclerosis, an autoimmune disorder in which demyelination of neurons affects the propagation of action potentials along axons. As a drug it has been shown to improve motor and visual abilities and relieve fatigue in sufferers (Judge and Bever 2006).

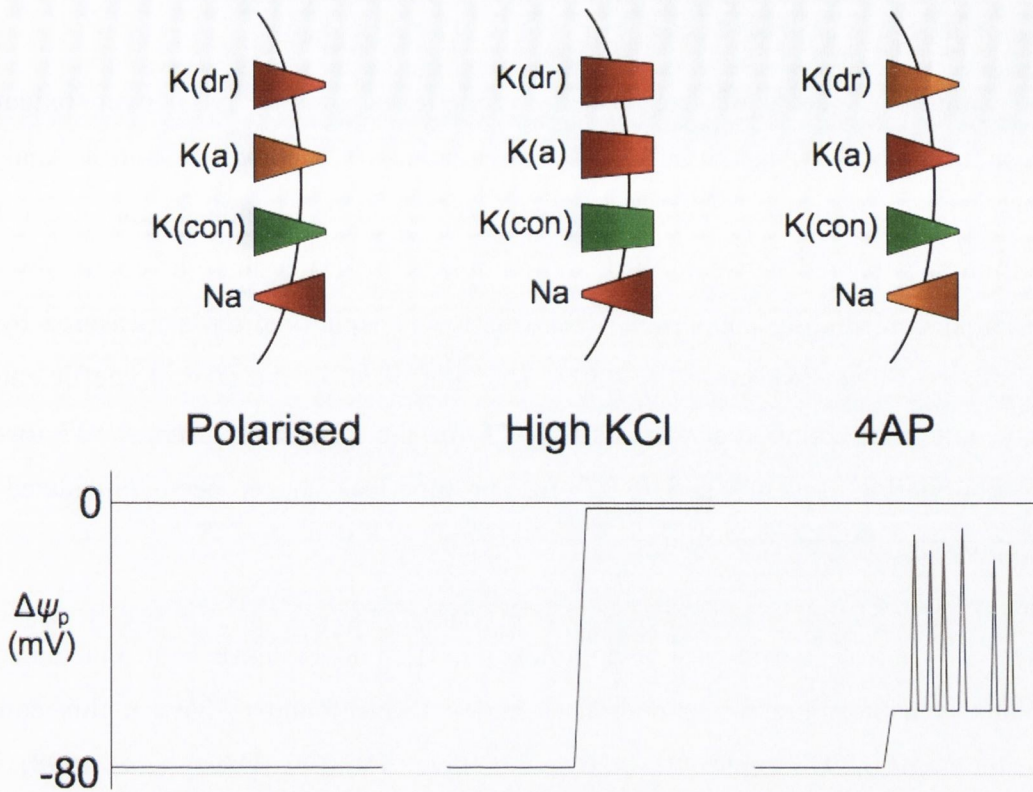


Fig. 1.5 Schematic representation of the most common ways of depolarising synaptosomes

Polygons represent the direction and approximate magnitude of K^+ or Na^+ gradients under each condition between the cytoplasm (left) and extracellular compartments (right). Thus when synaptosomes are polarised the K^+ gradient is outward, but under conditions of high $[KCl]_{out}$ the magnitude of the gradient is decreased. Red infilling means the pathway is essentially inactive under that condition, orange means that the pathway is intermittently active while red means that the pathway is permanently active. Na, Na^+ channel; K(con), constitutive K^+ conductance; K(a), K_A^+ channel (inhibited by 1 mM 4-aminopyridine); K(dr) 'delayed rectifier' K^+ channel. Under polarised conditions the K_A^+ channel helps to stabilise $\Delta\psi_p$ by opening in response to slight depolarisations. With high $[KCl]$ there is a 'clamped' depolarisation mediated by the constitutive K^+ conductance. With 4-aminopyridine the K_A^+ channels are inhibited and the membrane potential is no longer stabilised. Spontaneous action potentials occur involving both the Na^+ channels and 'delayed rectifier' K^+ channels. Adapted from Fig. 3 in Nicholls 1993.

1.3 Flux control of respiration and energy threshold effects

The control of each of each of the complexes of the ETC over oxidative phosphorylation can be elucidated by application of Metabolic Control Analysis (Heinrich and Rapoport 1974). The results of this method indicate that control of respiration is shared between all of the complexes, and the share of control by any particular contributing element to the oxidative phosphorylation is measured by its control coefficient (Mazat *et al.* 2001). The sum of all of the control coefficients is unity, and flux control coefficients of 0.71 for the oxidation system, 0.27 for the phosphorylation system and 0.02 for proton leak have been elucidated in synaptosomes (Joyce *et al.* 2003).

The flux control coefficients of complexes I, III, and IV have been elucidated in isolated synaptosomal mitochondria. Complex I was found to have a flux control coefficient of 0.29, complex III, 0.20, and complex IV, 0.13 (Davey *et al.* 1998). The high complex I control coefficient results in a low threshold of inhibition of complex I activity before rates of oxygen consumption and ATP production are seriously affected. Inhibition of complex I activity by 25% in synaptic mitochondria with rotenone resulted in a major fall in oxygen consumption and ATP production, whereas these changes in oxidative phosphorylation did not occur until 80% inhibition of complex III activity with myxothiazol or 70% inhibition of complex IV activity with KCN (Davey *et al.* 1998). For reasons that have not yet been established (Pathak and Davey 2008), the threshold level of inhibition in isolated nonsynaptic mitochondria was much higher, at 72% (Davey and Clark 1996).

Although numerous defects of mitochondrial enzymes involved in mitochondrial diseases are known to exist and have been shown to be genetic in origin, the pathogenic mechanisms for many of these disorders are usually not well understood. Indeed, the same defect in mitochondrial DNA can often lead to several different clinical syndromes. For example, genetic defects that affect complex I activity have been implicated in Parkinson's disease, Leber's disease, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (Rossignol *et al.* 2003). The heterogeneity of brain mitochondria may suggest that the lower complex I threshold in nerve terminal mitochondria may be acquired, perhaps as part of the aging process.

1.4 Mitochondria in aging

Declines in basal metabolism and physical performance in energy-requiring tasks are typical characteristics of the aging process (Boveris and Navarro, 2008). Age-dependent effects on mitochondrial energy transduction in brain mitochondria have been reported, including decreases in electron transfer rates by ETC enzymes, mild uncoupling of oxidative phosphorylation, and resulting impairment of ATP synthesis (Navarro and Boveris 2007). Enzymatic activity of complexes I and IV have consistently been shown to be reduced with age in isolated brain mitochondria (Benzi *et al.* 1992; Lenaz *et al.* 1997; Kwong and Sohal 2000; Navarro *et al.* 2002). Complex I activity was found to be reduced by 17 – 33% in mice aged 52 – 76 weeks, and complex IV activity reduced by 24 – 36%, compared to mice aged 6 weeks (Navarro *et al.* 2002; Navarro *et al.* 2004; Navarro *et al.* 2005). Decreased neurological function including performance of motor and tasks correlated with the decreased enzymatic function in the respiratory enzymes (Benzi *et al.* 1992; Lenaz *et al.* 1997; Kwong and Sohal 2000; Navarro *et al.* 2002; Navarro *et al.* 2005). Age-related decreased oxygen consumption in isolated brain mitochondria consuming glutamate and malate (operating through complex I) has been reported, while in those consuming succinate (operating through complex II) the respiration rate was unchanged with age (Navarro *et al.* 2005).

Evidence for age-related increased proton permeability across the mitochondrial inner membrane has come indirectly from measurement of $\Delta\psi_m$ in isolated brain mitochondria (LaFrance *et al.* 2005). As well as showing reduced $\Delta\psi_m$, brain mitochondria from older rats have been shown to have larger volumes and increased H₂O permeability when exposed to hypertonic conditions (Navarro *et al.* 2004). An age-related decrease in *in situ* nerve terminal maximal oxygen consumption rate after artificial uncoupling has also been reported (Joyce *et al.* 2003). These age-related declines in mitochondrial function have added to evidence supporting the mitochondrial hypothesis of aging (Beckman and Ames 1998). The free radical theory of aging has been proposed to be at the centre of this hypothesis (Gerschman *et al.* 1954; Harman 1956, 2006).

1.5 ROS, oxidative stress and mitochondria

A free radical is an independent species with one or more unpaired electron and all aerobic metabolism is thought to involve the production of free radicals (Gutteridge and Halliwell 2000). These unstable reactive species have a half-life of only nanoseconds but can damage proteins, lipids and DNA (Floyd *et al.* 2001). Among the most common free radicals produced in cells are the reactive oxygen species (ROS): the superoxide ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^{\cdot-}$). The mitochondrial ETC is thought to be a major source of free radical production (Gutteridge and Halliwell 2000) and 1 – 4% of oxygen consumed by mitochondria has been estimated to be converted to $O_2^{\cdot-}$ and H_2O_2 . The complexes of the mitochondrial respiratory chain are also susceptible to oxidative damage by ROS (Hillered and Ernster 1983; Zhang *et al.* 1990).

Considerable defence mechanisms have evolved to cope with the free radical load produced by mitochondria (Gutteridge and Halliwell 2000). Among these are antioxidants, both enzymatic and nonenzymatic, which regulate the intra and extracellular steady-state free radical content. The primary enzymatic antioxidant of aerobic cells is superoxide dismutase (SOD), which neutralises $O_2^{\cdot-}$ (Fig. 1.6). Three forms of SOD are found in the brain: mitochondrial manganese form (SOD2), a cytoplasmic copper/zinc form (SOD1) and an extracellular form (SOD3) (Schapira and DiMauro 2002). Glutathione (GSH), a thiol-containing tripeptide, is abundant in neurons, and oxidises H_2O_2 in a reaction catalysed by glutathione peroxidase (Fig. 1.6 (Ambani *et al.* 1975). Oxidised GSH can then be reduced by GSH reductase using NADPH (Fig. 1.6, Cross *et al.* 1987). Thus, this antioxidant defence system also prevents the decrease of intracellular thiols (Schapira and DiMauro 2002). H_2O_2 may also be hydrolysed by catalase, but the concentration of this enzyme in the brain is relatively low (Ambani *et al.* 1975). These enzymes help prevent damage to lipids, proteins and DNA by free radicals, cumulatively termed oxidative stress.

Non-enzymatic free radical scavengers, including vitamins A, C and E also play an important role in minimising oxidative stress, and these antioxidants can neutralise $O_2^{\cdot-}$ and $OH^{\cdot}$ (Schapira and DiMauro 2002).

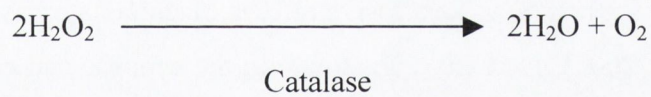
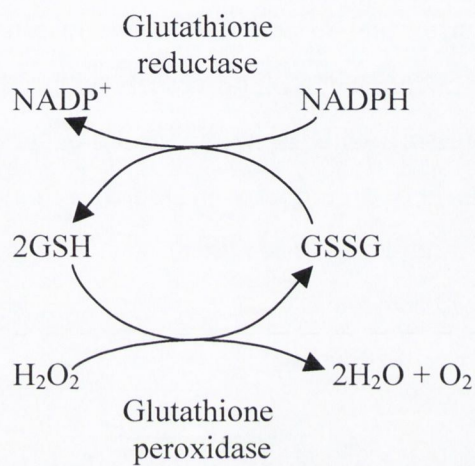
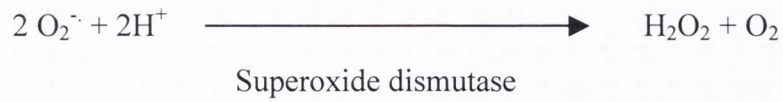


Fig 1.6 Inactivation of reactive oxygen species by antioxidant enzymes

Key:

GSH – Glutathione

GSSG – Oxidised glutathione

H₂O₂ – Hydrogen peroxide

NADP⁺ – Nicotinamide adenine nucleotide phosphate

1.6 Acute neurodegeneration

Acute neurodegeneration refers to widespread neuronal loss over a time span of days, hours or minutes. The most common form of acute neurodegeneration is stroke, which occurs when blood to the brain is interrupted. In contrast to other organs, the brain has no capacity to synthesise or store energy reserves. Therefore, during an ischemic or hemorrhagic event in the brain, the resulting hypoxia and hypoglycaemia is thought to bring about rapid neuronal death by osmotic swelling and necrosis (Doble 1999). Stroke is the second most common cause of death in the world, with 9% of all deaths attributed to it (Murray and Lopez 1997). The origin of stroke is usually vascular: hemorrhagic stroke occurs when blood vessels within the skull rupture, often after the build up of an aneurism, while ischemic stroke occurs when intracranial vessels are blocked by thrombosis or an embolism (Donnan *et al.* 2008). Approximately 80% of all strokes are ischemic (Thrift *et al.* 2001).

1.6.1 Ischemic excitotoxicity

In areas surrounding the core infarction, where there is reduced blood supply, a fall in neuronal ATP levels is thought to occur, causing reduced activity of the Na^+/K^+ ATPase. The ionic gradients across the plasma membranes of neurons and glia are compromised as a result, and excessive release of glutamate has been found to occur (Hurtado *et al.* 2003). Excessive glutamate in the synapse can cause further death of neurons, as constant activation post-synaptic ionotropic glutamate receptors (NMDA, kainate and AMPA receptors) allow a rise in intracellular Ca^{2+} -concentrations to occur. In this region, the penumbra, high intracellular Ca^{2+} -concentrations trigger may trigger apoptosis, causing an increase in the size of the infarction (MacManus and Buchan 2000). Much of the attempts at obtaining neuroprotective agents against stroke have been focusing on taming the effects of the excessive release of glutamate in the penumbra (Doble 1999). Those which act on the NMDA receptor have been proven to be effective in animal models but not in humans, as adverse effects are considered to be too numerous and there is a narrow time window (3 – 6 hrs) after the stroke in which such agents prevent damage (Caplan 2002). Indeed, the oldest method of treatment, hypothermia, which, when performed reduces metabolic requirement in the treated area, remains the most effective against infarction (Polderman 2008).

1.6.2 Glutamate release during ischemic excitotoxicity

Four mechanisms of glutamate release during excitotoxicity have been examined: Ca^{2+} -dependent vesicular release; glutamate release from astrocytes surrounding the synapse through swelling-activated anion channels; release from astrocytes in an indomethacin-sensitive process; and reversal of glutamate transporters (Rossi *et al.* 2000). Though each proposed mechanism has credible evidence that supports its involvement in stroke, the highest level of glutamate release during an ischemic event has been demonstrated to be via reversed glutamate transporters, whose normal function is to reuptake glutamate from the synapse (Jabaudon *et al.* 2000; Rossi *et al.* 2000). Under normal conditions of neurotransmission at a glutamatergic synapse *in vivo*, glutamate is thought to be taken up quickly by high affinity transporters, including EAAT1 and EAAT2 (GLAST and GLT-1 in rats, Rothstein *et al.* 1996; Kanai and Hediger 2003). GLT-1 (EAAT2) shows the highest affinity for glutamate uptake, and there is evidence that it is present in nerve terminals (Suchak *et al.* 2003). Models of anoxia and hypoglycaemia have frequently been shown to increase glutamate release from isolated nerve terminals (Sanchez-Prieto *et al.* 1987; Sanchez-Prieto and Gonzalez 1988; Erecinska *et al.* 1996; Santos *et al.* 1996). Nonetheless, the high cytoplasmic glutamate pool in astrocytes has been most frequently cited as the origin of excitotoxic glutamate (Rothstein *et al.* 1996; Rossi *et al.* 2000).

A mechanism of reversal of glutamate transporters during ischemia has been postulated. Both neurons and astrocytes require maintenance of the plasma membrane electrochemical gradient to be maintained for normal functioning of the glutamate transporters (Peng *et al.* 2001). EAAT1 and EAAT2 transporters each co-transport three Na^+ ions and one H^+ and counter-transport a K^+ ion for each glutamate molecule they transport (Nicholls and Attwell 1990), and reductions in the concentration gradients of these ions across the plasma membrane result in the reversal of the transporters, leakage of cytoplasmic glutamate into synapses and excitotoxicity caused by excessive levels of extracellular glutamate (Rossi *et al.* 2000). Such events can occur due to impaired ATP production by mitochondria and the Na^+/K^+ ATPase activity is reduced (Schinder *et al.* 1996). Impaired mitochondrial ATP production has also been demonstrated in chronic neurodegenerative disorders.

1.6.3 Synaptosomal models of ischemic glutamate release

Synaptosomes have been commonly used to model the effects of ischemic energy depletion on nerve terminal physiology, including glutamate release. Chemical ‘anoxia’ has been induced using mitochondrial ETC inhibitors, including KCN (Sanchez-Prieto and Gonzalez 1988) and rotenone (Sanchez-Prieto *et al.* 1987). Hypoglycaemia has also been modelled by replacing glucose in the medium with iodoacetate (Rubio *et al.* 1991) or deoxyglucose (Santos *et al.* 1996), both inhibitors of glycolysis. The findings of these reports are generally consistent in reporting a Ca^{2+} -independent increase in glutamate efflux from synaptosomes after addition of the inhibiting agent (Erecinska *et al.* 1996). In addition, Ca^{2+} -dependent exocytotic glutamate release has been shown to be blocked in a time-dependent manner by metabolic inhibitors (Sanchez-Prieto and Gonzalez 1988; Rubio *et al.* 1991). The time-dependency has been shown to be related to depletion of the synaptosomal ATP pool to below a threshold level (Nicholls and Attwell 1990), below which there is not enough ATP available in the nerve terminal to support exocytosis, which is an energy dependent process (Sanchez-Prieto *et al.* 1987).

1.7 Slow excitotoxicity and mitochondria

A mechanism of slow excitotoxicity has been proposed to occur in chronic neurodegenerative disorders as a result of mitochondrial dysfunction (Beal 1992). The evidence supporting this hypothesis initially came from *in vitro* models, which indicated that impaired energy metabolism lowers the glutamate threshold concentration required to cause excitotoxicity (Doble 1999). The first study of this kind showed that inhibition of complex IV activity or omission of glucose in the culture medium caused normal levels of extracellular glutamate to become toxic to cerebellar granule cells (Novelli *et al.* 1988). The finding has since been widely replicated both *in vivo* (Zeevalk and Nicklas 1991; Simpson and Isacson 1993) and *in vitro* (Castilho *et al.* 1998; Nicholls and Ward 2000; Ward *et al.* 2000) and notably in cerebellar granule neurons by partial inhibition of complex I activity with low concentrations of rotenone (Yadava and Nicholls 2007).

A number of hypotheses have been developed to explain the mitochondrial involvement in NMDA-receptor mediated excitotoxicity. Reduced ATP supply to the Na^+/K^+ ATPase is thought to cause a lowering of $\Delta\psi_p$ and removal of the magnesium block of the NMDA receptors, resulting in chronic activation of the receptors (Mayer *et al.* 1984; Nowak *et al.* 1984). The resulting increase in intracellular Ca^{2+} levels is thought to add to the mitochondrial Ca^{2+} load and may trigger apoptosis via opening of the mitochondrial permeability transition pore (Nicholls and Ward 2000). Opening of the transition pore is characterised by an initial depolarisation of $\Delta\psi_m$, followed by a failure to regulate intracellular Ca^{2+} levels (delayed Ca^{2+} deregulation) and cell death (Nicholls and Ward 2000). Increased intracellular Ca^{2+} levels after NMDA receptor activation is also known to increase ROS production by the mitochondrial ETC enzymes (Dugan *et al.* 1995; Reynolds and Hastings 1995), which may also induce apoptosis (Rego and Oliveira 2003).

1.8 Parkinson's disease

Parkinson's disease is a chronic neurodegenerative disorder with a prevalence of 0.3% in the population of industrialised countries (Nussbaum and Ellis 2003). The principal pathological feature is preferential loss of dopaminergic neurons in the nigrostriatal pathway, and as the disease progresses other neural populations degenerate, including the serotonergic neurons of the raphe nuclei, dopaminergic neurons in the ventral tegmental area, cholinergic neurons of the basal forebrain, and the noradrenergic neurons in the locus ceruleus, which results in a net increase in inhibitory output from basal ganglia to the thalamus (Jellinger 1991). Other pathologic features such as the development of Lewy bodies and ubiquitinated α -synuclein inclusions are common but not always necessarily present for diagnosis (Weiner 2008). Symptoms include rigidity, tremor, bradykinesia and impaired postural reflexes. Although over 90% of cases are apparently sporadic (de Lau and Breteler 2006), recent research has identified numerous genes that may be involved in the initial aetiology of idiopathic Parkinson's disease (Smigrodzki *et al.* 2004; Parker and Parks 2005; Smigrodzki *et al.* 2005). Multiple biochemical abnormalities have been cited as contributors to the pathogenesis of Parkinson's disease, including mitochondrial dysfunction, excitotoxicity and free radical mediated damage.

1.8.1 Mitochondrial dysfunction in Parkinson's disease

The idea that mitochondrial dysfunction is involved in the aetiology of Parkinson's disease arose after MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) was identified as the substance primarily responsible for inducing parkinsonian symptoms in drug addicts (Langston *et al.* 1983). MPTP was identified as an impurity found in black market synthetic opiates. The mechanism of neurotoxicity was later elucidated: MPTP is converted into MPP⁺ (1-methyl-4-phenylpyridinium) by monoamine oxidase-B, primarily in glia, where this enzyme is primarily localized in adult brain (Youdim *et al.* 2006). MPP⁺ is then taken up via the dopamine transporter into nerve terminals, where it potently inhibits complex I activity (Tipton and Singer 1993). Such inhibition is thought to result in reduced capacity for ATP production and increased ROS production, both of which have been found to lower the threshold of apoptosis, which is controlled by mitochondria (Drechsel and Patel 2008).

Subsequently, it was found that complex I activity was reduced in post mortem samples isolated from the substantia nigra of patients who suffered from Parkinson's disease (Schapira *et al.* 1989). The deficiency was measured to be an approximately 40% reduction in activity compared to controls (Schapira *et al.* 1990a). Decreases in amounts of specific subunits in complex I proteins were found in striatum of Parkinson's disease patients (Mizuno *et al.* 1989) and deficiencies in complex I activity was also found in blood platelets (Parker *et al.* 1989; Schapira *et al.* 1990b). Recent evidence has identified subunits of complex I that are more susceptible to oxidative stress in Parkinson's disease frontal cortex mitochondria than in controls, and suggests the functional impairment is due to misassembly (Keeney *et al.* 2006). The reduced activity in frontal cortex complex I activity has also been suggested to be of genetic origin (Parker *et al.* 2008). This suggests deficient complex I activity plays a role in the initial aetiology of Parkinson's disease.

1.8.2 Excitotoxicity in Parkinson's disease

A number of studies have addressed the role of excitotoxicity in parkinsonism. The substantia nigra 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). Lesions induced by local application of MPP⁺ into rat striatum were found to be reduced in size by both prior surgical removal of the glutamatergic corticostriatal tract, or addition of the NMDA receptor inhibitor, MK-801 (Storey *et al.* 1992). Studies that pharmacologically inhibited glutamate release from the corticostriatal tract using riluzole also showed protective effects on dopaminergic neurons in rats and mice (Boireau *et al.* 1994b; Boireau *et al.* 1994a). The results may be a consequence of a lower threshold of resistance to glutamate in the dopaminergic cells or higher local levels of glutamate release, stimulated by inhibition of complex I activity. Experiments performed using microdialysis indicated that MPP⁺ stimulated glutamate release in the striatum (Carboni *et al.* 1990), although the origin of the released glutamate (glial/neuronal/vesicular) was not clear.

1.8.3 Oxidative stress in Parkinson's disease

Oxidative stress is thought to be central to the pathogenesis of Parkinson's disease (Jenner and Olanow 1998). Studies on post mortem homogenates from substantia nigra of Parkinson's disease brains have shown evidence of higher levels of oxidative damage than normal, including increased levels of lipid hydroperoxides (Dexter *et al.* 1994), 8-hydroxyguanine (Alam *et al.* 1997a) and protein carbonyls (Alam *et al.* 1997b). Oxidation of dopamine by monoamine oxidase results in H₂O₂ production, which can usually be neutralised by glutathione peroxidase or catalase (Fig. 1.6). However, levels of catalase in the brain are low (Chance *et al.* 1979) and glutathione has been shown to be reduced by 30 – 40% in the substantia nigra of Parkinson's disease brain (Sofic *et al.* 1992; Sian *et al.* 1994). Iron catalyses the conversion of H₂O₂ to the highly reactive OH[•] (Halliwell and Gutteridge 1984) and there is also evidence of elevated levels of iron in substantia nigra of Parkinson's disease brain (Dexter *et al.* 1989). Oxidative stress is also thought to damage the ubiquitin-proteasomal system during the pathogenesis of Parkinson's disease, leading to protein aggregation (Fig. 1.7, Schapira 2008). Recently, the monoamine oxidase inhibitor rasagiline has been used clinically to treat Parkinson's disease, and has shown to be effective in improving motor function in patients who are in the early stages of the syndrome (Chen *et al.* 2007).

1.8.4 Rotenone model of Parkinson's disease

Rotenone is lipophilic and readily crosses membranes independently of transporters, and therefore induces a systemic inhibition of complex I activity in animals (Greenamyre *et al.* 2003). Chronic infusion of rotenone at a concentration of 20 – 30 nM into rats was found to cause nigrostriatal degeneration of dopaminergic neurons over a period of days to weeks (Betarbet *et al.* 2000). Lewy body-type intracellular inclusions containing α -synuclein and ubiquitin were also identified. The rats also developed the behavioural patterns typically associated with Parkinsonism, including bradykinesia, rigidity, and flexed posture. However, the degree of inhibition of complex I activity was found to be uniform across brain regions, and other organs displayed similar levels of inhibition of complex I activity. The mechanism of nigrostriatal degeneration was proposed to involve oxidative damage and, in accordance with this interpretation, *in vitro* models showed that rotenone depletes the glutathione pool in dopaminergic neurons (Scherer *et al.* 2002).

1.9 Mitochondrial dysfunction in other chronic neurodegenerative disorders

1.9.1 Alzheimer's disease

Alzheimer's disease is recognised as the most common chronic neurodegenerative disorder of the developed world, affecting 5 – 15 % of the population over the age of 65 years (Katzman 1986). The initial pathophysiology involves degeneration of synapses in the cerebral cortex, followed by neuronal cell death and atrophy of regions including the temporal and parietal lobes and frontal cortex. The central histopathological features include extracellular amyloid plaques and intracellular neurofibrillary tangles made up of β -amyloid and hyperphosphorylated tau protein. Memory loss is an early characteristic symptom of the disease, followed by cognitive decline and eventual complete dependency. Most cases of Alzheimer's disease are regarded as being sporadic, with a common initial aetiology yet to be elucidated. Rare cases of genetic causes for the onset have been discovered, which were shown to involve mutations in either the amyloid precursor protein (Goate *et al.* 1991; Goate 2006) or presenilin (Levy-Lahad *et al.* 1995a; Levy-Lahad *et al.* 1995b) genes.

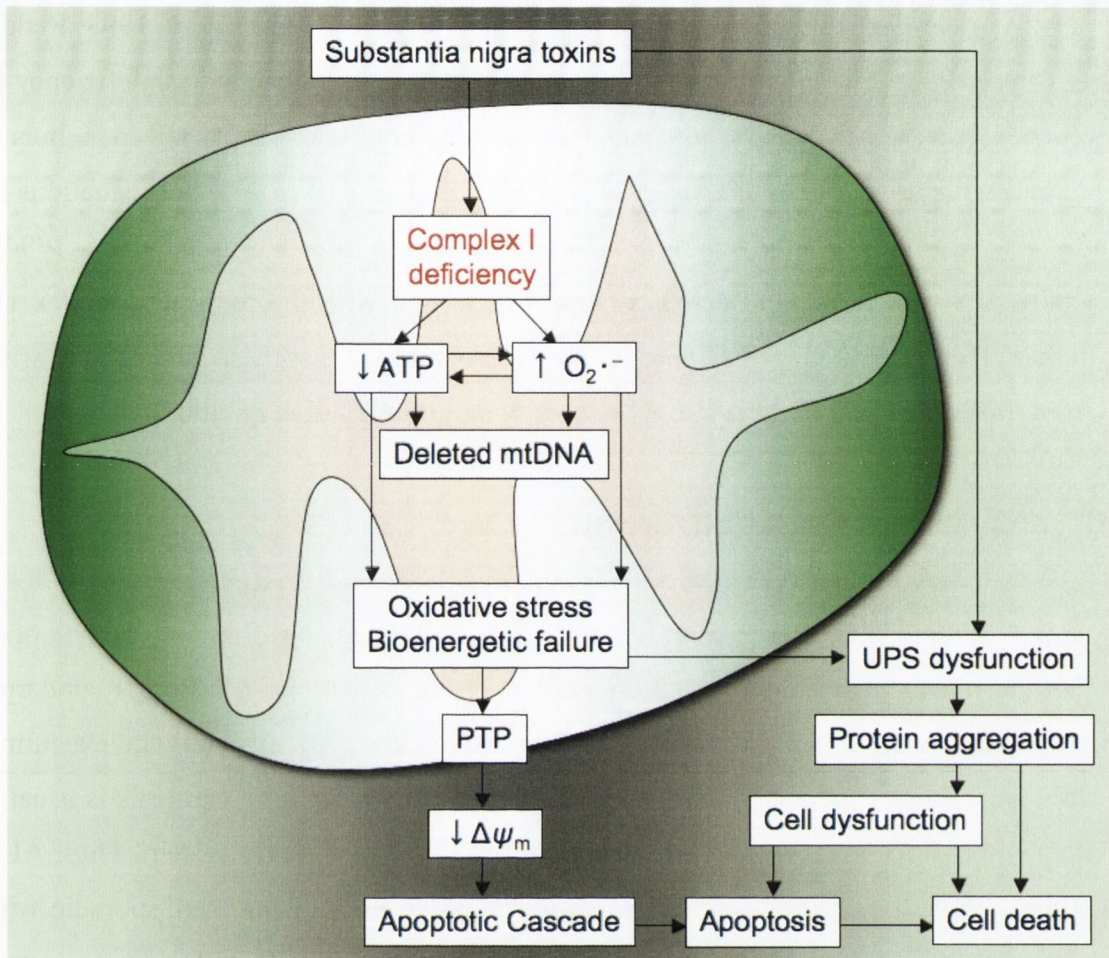


Fig. 1.7 The pathogenic pathways to cell death in models of Parkinson's disease

Complex I inhibitors such as rotenone and MPTP inhibit complex I activity, leading to decreased ATP production and increased free-radical generation. Adapted from Fig. 2 in Schapira (2008).

There is increasing evidence to support the hypothesis that mitochondrial dysfunction plays a role in the pathophysiology of Alzheimer's disease (Bonilla *et al.* 1999; Reddy and Beal 2008). The most frequently reported finding of a mitochondrial deficiency is reduced complex IV activity in the post mortem brain samples from Alzheimer's disease patients by up to 50% (Kish *et al.* 1992; Mutisya *et al.* 1994) but reductions in the activities of the other complexes have also been found (Parker *et al.* 1994). Defects in mitochondrial DNA have been associated with the reduced complex IV activity (Lin *et al.* 2002; Coskun *et al.* 2004). It is thought that these defects may arise from the burden of oxidative stress (Maurer *et al.* 2000; Hirai *et al.* 2001).

1.9.2 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is characterised by loss of both upper (corticospinal) and lower (spinomuscular) motor neurons. Sufferers undergo progressive weakening of muscle and increasing levels of difficulty in executing voluntary actions such as walking and breathing. The progressive paralysis is usually fatal within 2 to 5 years, is usually due to failure of the respiratory system. Thus, ALS is the most lethal chronic neurodegenerative disorder. 90% of cases are sporadic with unknown initial aetiology, while 10% are dominantly inherited. Oxidative stress, excitotoxicity and mitochondrial dysfunction have all been implicated in the pathogenesis of ALS (Lin and Beal 2006).

Complex I activity has been reported to be reduced in skeletal muscle of sporadic ALS patients (Wiedemann *et al.* 1998). Complex IV activity was shown to be reduced in anterior horn motor neurons in post mortem samples from sporadic ALS sufferers (Borthwick *et al.* 1999). The reduction was proposed to be related to damaged mitochondrial DNA, and another study demonstrated significant deletion and depletion of intact mitochondrial DNA in ALS skeletal muscle (Vielhaber *et al.* 2000). ALS cybrids have indicated that reductions in complex I activity may be related to mitochondrial DNA abnormalities (Schapira and DiMauro 2002). A study on a single patient with sporadic ALS related reduced complex IV activity to a mutation in the mitochondrial DNA gene that codes for subunit I of the enzyme (Comi *et al.* 1998).

1.9.3 Huntington's disease

Huntington's disease is an autosomal-dominant chronic neurodegenerative disorder with distinctive pathological features including severe atrophy of the caudate nucleus and degeneration of other neural populations including the putamen, globus pallidus and substantia nigra pars reticulata (Schapira and DiMauro 2002). The resulting behavioural features include chorea, ataxia and dementia. Pathogenesis usually occurs in early to mid-adulthood, but juvenile and late-onset forms have also been characterised. The mutation responsible for causing Huntington's disease has been identified as an expanded CAG repeat of more than 36 times, of a gene on chromosome 4 coding for a protein named huntingtin (Huntington's Disease Collaborative Research Group. 1993). The function of the huntingtin protein is unknown, as is relation of the mutant huntingtin protein to the pathogenesis of Huntington's disease.

Complex II/III activity was found to be reduced by 77% in post mortem caudate nucleus homogenates from Huntington's disease sufferers (Mann *et al.* 1990). Other groups have reported similar findings (Browne *et al.* 1997; Tabrizi *et al.* 1999), and one study showed a decrease in complex IV activity by 33% in the caudate (Gu *et al.* 1998). Reduced aconitase activity, which suggests oxidative damage, (Castro *et al.* 1994; Hausladen and Fridovich 1994) was also shown to occur in the caudate, indicating mitochondrial enzymes may be subjected to increased levels of oxidative stress during Huntington's disease (Tabrizi *et al.* 1999). Inhibition of aconitase activity has also been observed in excitotoxicity models (Patel *et al.* 1996), and can be prevented by SOD. Chronic systemic infusion of the complex II inhibitor 3-nitropropionic acid into rats (Brouillet *et al.* 1993) and primates (Brouillet *et al.* 1995) results in lesions in the caudate nucleus similar to those seen in Huntington's disease. Removal of striatal glutamatergic input by prior decortication reduced the size of lesions in the rat model, suggesting a role for excitotoxicity in this model (Beal *et al.* 1993).

1.10 Aims of thesis

1. Complex I activity has been shown to be reduced in Parkinson's disease, and excitotoxicity has been implicated in the pathogenesis of the disease. The effects of such a reduction in complex I activity on glutamate release from nerve terminals were modelled under conditions of energy demand which mimic those expected to occur *in vivo*. The correlating ATP levels were measured.
2. Complex I of isolated synaptosomal mitochondria has been shown to have tighter control over oxygen consumption and ATP production than the other complexes of the electron transport chain. Experiments were performed to compare the control of complex III and complex IV over nerve terminal physiology, including glutamate release, $\Delta\psi_m$ and $\Delta\psi_p$ with that of complex I in *in situ* mitochondria.
3. The role of mitochondrial dysfunction in aging has been the subject of intense research in recent years. Much of this work has indicated that slow deterioration of specific mitochondrial components occurs progressively and suggests that these subtle reductions in mitochondrial capabilities may lead to chronic neurodegeneration. The work in this thesis aims to model the control of these age-related changes in *in situ* mitochondria on various aspects of nerve terminal physiology.

Chapter 2

General Methods

2.1 Materials

Female Wistar rats (aged 6 weeks, unless otherwise stated) were supplied by the Bioresources Unit, School of Biochemistry and Immunology, Trinity College, Dublin. Bovine serum albumen, Ficoll, FCCP, Triton X-100, antimycin A, rotenone, oligomycin, L-Glutamic acid, L-Glutamic dehydrogenase, tris, MgCl_2 , CaCl_2 , NaCl_2 , $\beta\text{-NADH}$, $\beta\text{-NADP}^+$, EDTA were supplied by Sigma Chemical Co., Poole, Dorset, England. BioRad dye was supplied by BIO-RAD Laboratories GmbH, Munich, Germany. JC-1 and 'PMPI' dyes were supplied by Molecular Probes, Eugene, Oregon, USA. ENLITEN® ATP assay system was supplied by Prmega, Madison, Wisconsin, USA. Ethanol was supplied by Merck, Darmstadt, Germany. Sucrose was supplied by BDH, Poole, Dorset, England.

2.2 Preparation of buffers and solutions

Buffers and reagents were prepared using water deionised with a Millipore – U10 deioniser, except rotenone and antimycin A, which were prepared in 100% ethanol. All reagents were weighed out on either a Mettler K7T model top loading balance (for weights > 5 g) or a Mettler College Model analytical balance (for weights < 5 g),

2.3 Measurement of pH

A Corning pH meter, which was calibrated daily using standard pH buffers of pH 4.0, 7.0 and 10.0, was used to measure pH.

2.4 Pipetting

A set of Gilson automatic pipettes was used throughout experiments to pipette volumes within the range of 2 μl – 5 ml. Pipette tips were cut to prevent damage to synaptosomes.

2.5 Centrifugation

Centrifugations were performed using either a Sorvall 'RC-5B' or 'RC-5C plus' centrifuge, with an SS-34 '8 × 50 ml' rotor. Ultracentrifugation was performed using a Sorvall OTD 50B ultracentrifuge. An Eppendorf 5415R bench centrifuge containing a '24 × 1.5 ml' rotor was used for volumes < 1.5 ml. All centrifuging was done at 4°C.

2.6 Luminescence, fluorimetry and spectrophotometry

A SpectraMAX GeminiXS well-plate reader was used to monitor glutamate release and results were monitored continuously as described by (Nicholls *et al.* 1987) using the accompanying 'SoftMax Pro' software package. Measurement in the well-plate reader allows 96 rates to be taken simultaneously. Cycle time was set to minimum (32 s for a 96-well read), and the well-plate set to shake for 3 s between reads to prevent settling of the synaptosomes. For measurement of ATP using luciferin/luciferase reagent, the 'Endpoint' luminescence-reading option was selected in the SoftMax Pro program. Spectrophotometry was performed on an Agilent 8453 spectrophotometer, and analysis done on UV-Visible ChemStation Software program.

2.7 Preparation of synaptosomes and nonsynaptic mitochondria

Rat brain synaptosomes and nonsynaptic mitochondria were isolated by the method of (Lai and Clark 1978) with some modifications. Before the preparations began, all buffers were placed on ice and all instruments and centrifuge tubes rinsed with the appropriate buffer. For each preparation, two rats were first stunned and then killed by cervical dislocation. They were quickly decapitated, their brains extracted and placed in ~ 40 ml STE buffer (320 mM sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4). The brains were chopped into small pieces using a scissors and allowed to settle to the bottom. The bloody buffer was decanted and replaced with fresh buffer, and the process was repeated 2 – 4 times. When the blood had been removed from solution and the brains were finely chopped, the pieces were poured into a glass Kontes homogenising tube with ~ 30 ml STE buffer and homogenised with at least 8 down

and up strokes of a tight-fitting glass pestle. The homogenate was poured equally into two 50 ml centrifuge tubes and topped up to 40 ml and balanced with STE buffer before centrifugation for 3,000 g for 3 min at 4°C to remove all unwanted material. The supernate was decanted and centrifuged at 10,000 g for 10 min. The pellet (which has been referred to both as the 'crude mitochondrial pellet' or the 'crude synaptosomal pellet') was carefully removed, leaving any traces of blood in the centrifuge tube, topped up to 9 ml with STE buffer and homogenised using the tight-fitting pestle. The homogenate was placed on top of a discontinuous Ficoll gradient (9 ml of 7.5% Ficoll w/v in dH₂O on top of 18ml of 10% Ficoll w/v in dH₂O, Fig. 2.1) and centrifuged for 100,000 g for 45 min at 4°C (Fig. 2.1).

The resulting uppermost layer of myelin was removed by aspiration and the first the synaptosomes were taken up by a Pasteur pipette and resuspended in 40 ml STE buffer and centrifuged for 12,000 g for 15 min at 4°C to wash off the Ficoll. Following this, the pellet of non-synaptic mitochondria were resuspended in 3 ml of STE buffer, homogenised and divided into 2 Eppendorf tubes, before being centrifuged for 5 min in the benchtop centrifuge at maximum speed (12,000 g). Following centrifugation, the synaptosomal and mitochondrial pellets were resuspended and homogenised separately in a 2 ml glass homogeniser. The concentration of protein in the sample was then determined. Synaptosomes then underwent a 10 min centrifuging at 12,000 g in 1 ml TES buffer (250 mM sucrose, 5 mM TES, pH 7.4) and stored in 1 mg pellets on ice until experiments were ready to be performed.

2.8 Determination of protein concentration

Synaptosomes are quantified by the concentration of protein/ml after membrane rupturing. BioRad dye contains a detergent to rupture membranes. Protein concentration was determined by the Bradford assay. A range of BSA concentrations were set up in Eppendorf tubes as shown in table 2.1. 10 µl of homogenate was diluted a hundredfold to 1 ml with dH₂O and 100 µl of this added to 700 µl dH₂O in triplicate. 200 µl of BioRad dye was added to each Eppendorf tube and the tubes were thoroughly vortexed before being incubated at room temperature for 30 min. The

BSA (μl)	0	2	5	10	15	20
dH ₂ O (μl)	800	780	750	700	650	600
BSA (0.1 mg/ml) added (μl)	0	20	50	100	150	200
BioRad dye added (μl)	200	200	200	200	200	200
Total vol. (ml)	1	1	1	1	1	1

Table 2.1 Contents of solutions used in the Bradford assay.

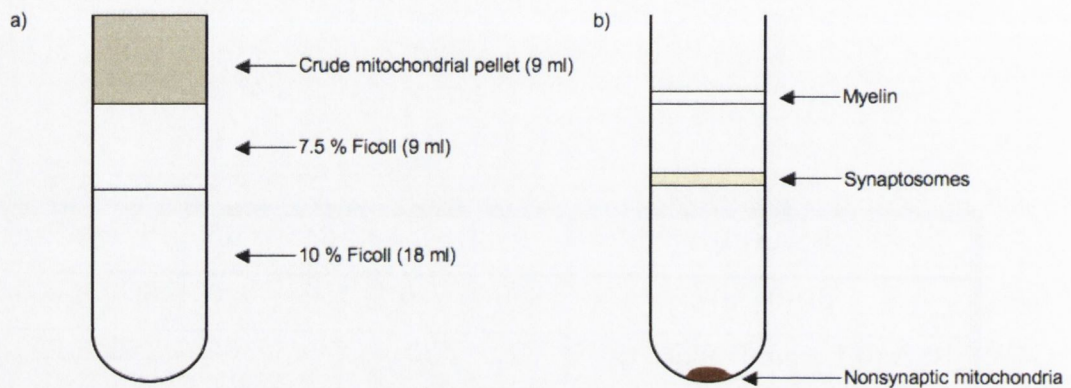


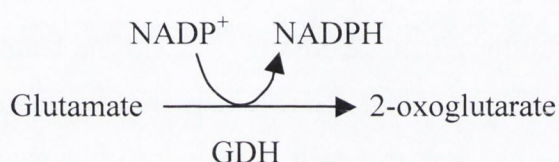
Fig. 2.1 Preparation of synaptosomes using a discontinuous Ficoll gradient

a) 9 ml of 7.5% (w/v) Ficoll was delicately layered on top of 18 ml of 10% (w/v) Ficoll, ensuring that an interface between the two layers could be seen clearly. The 9 ml of crude mitochondrial pellet in STE buffer 2 were layered on top before being centrifuged at 100,000 g for 45 min. b) The resulting layers after centrifugation: synaptosomes pass through the 7.5% (w/v) layer but not the denser 10% (w/v) layer and only mitochondria which are derived from areas other than the presynaptic nerve terminal ('nonsynaptic mitochondria') pass through, forming a pellet at the bottom.

contents of each Eppendorf were poured into 1 ml cuvettes and the absorbances read at 595 nm (blue) by a spectrophotometer.

2.9 Measurement of glutamate release

The release of glutamate from synaptosomes was monitored using a method based on the continuous coupled assay devised by Nicholls *et al.* (1987). Glutamate is converted to 2-oxoglutarate by L-glutamic dehydrogenase (GDH), which uses NADP^+ as a cofactor, converting it to NADPH simultaneously:



NADPH, when excited at $\lambda = 340$ nm, fluoresces at $\lambda = 460$ nm, and thus when fluorescence is monitored at this wavelength, the rate of change of concentration of NADPH in solution can be followed. The coupling of the reactions allows the released glutamate to be followed.

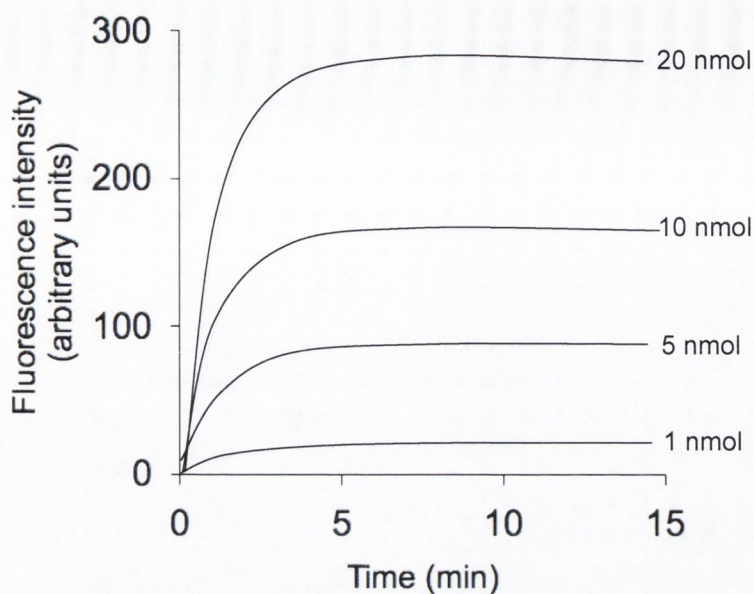
Following assay of protein concentration, synaptosomes were divided (1 mg into each Eppendorf tube) and topped up to 1 ml with TES buffer (250 mM sucrose, 5 mM TES, pH 7.4). The synaptosomes were then centrifuged at 12,000 g for 5 min and stored as pellets on ice until experiments were performed. For experiments performed in the PerkinElmer LS-55 Luminescence Spectrometer, synaptosomal pellets were resuspended in cuvettes with a final volume of 2 ml of Krebs buffer (3 mM KCl, 140 mM NaCl, 25 mM Tris HCl, 10 mM glucose, 2 mM MgCl_2 , pH 7.4 at 37°C). 1 mM NADP^+ , 31.6 U GDH and 1.4 mM CaCl_2 were added in volumes of 20 μl or less. Fluorescence at $\lambda = 460$ nm emission ($\lambda = 340$ nm excitation) was monitored continuously and simultaneously in 4 cuvettes using the Wavelength program of the FLWinLab software. For experiments performed on the SpectraMAX GeminiXS well-plate fluorimeter, synaptosomal pellets (1 mg) were resuspended in Krebs buffer (3 mM KCl, 140 mM NaCl, 25 mM Tris HCl, 10 mM glucose/2 mM deoxyglucose, 2 mM MgCl_2 , pH 7.4 at 37°C) and 100 μl added to wells containing this 100 μl of this

same medium with 2 mM NADP⁺, 6.32 U GDH, and 2.8 mM CaCl₂ previously added. Fluorescence at $\lambda = 460$ nm emission ($\lambda = 340$ nm excitation) was monitored continuously and simultaneously in 96 wells using the kinetic mode (cycle time of 32 s) in SoftMax Pro software.

2.10 Glutamate standard curve

To calibrate the amount of glutamate that correlated with the readings given in relative fluorescence units (RFU) on the fluorimeter, standard amounts of glutamate were added to medium and recording of the increase in fluorescence started immediately and followed for 25 min (Fig. 2.2). The blank rate was observed for 5 min, then GDH was added and the resulting shift in fluorescence noted. Change in fluorescence brought about by each amount of glutamate was plotted against the amount of glutamate added (Fig. 2.3). A standard curve was then drawn up from which rates of release could be estimated. The fluorimetric assay for glutamate has been shown to produce a non-linear response when standard amounts of glutamate are added (Nicholls *et al.* 1987) and this was found to be the case when experiments were performed in the well plate reader, where a second order equation was found to fit the data (Fig. 2.3).

a)



b)

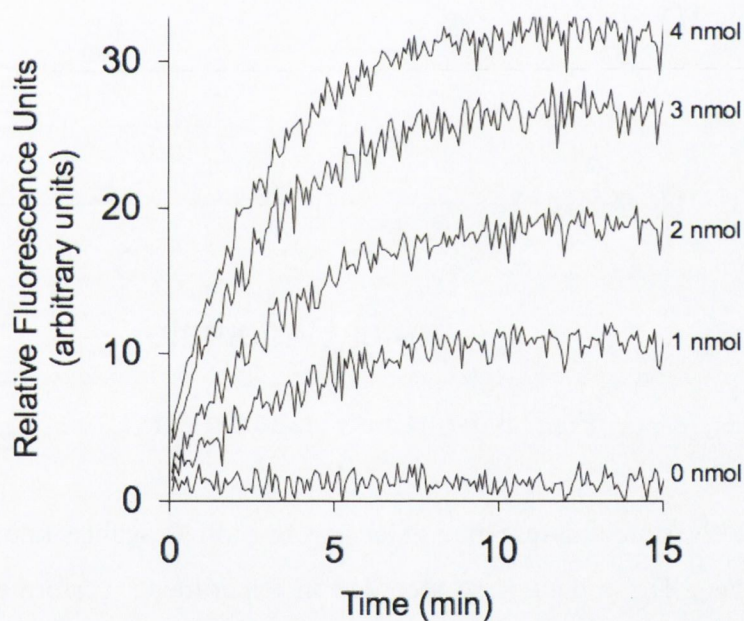


Fig. 2.2 Conversion of NADP^+ to NADPH coupled with that of glutamate to 2-oxoglutarate monitored fluorimetrically.

Standard amounts of glutamate were added to a) cuvettes or b) wells. Fluorescence was monitored at excitation $\lambda = 340 \text{ nm}$, emission $\lambda = 460 \text{ nm}$. Data represent traces from a single experiment.

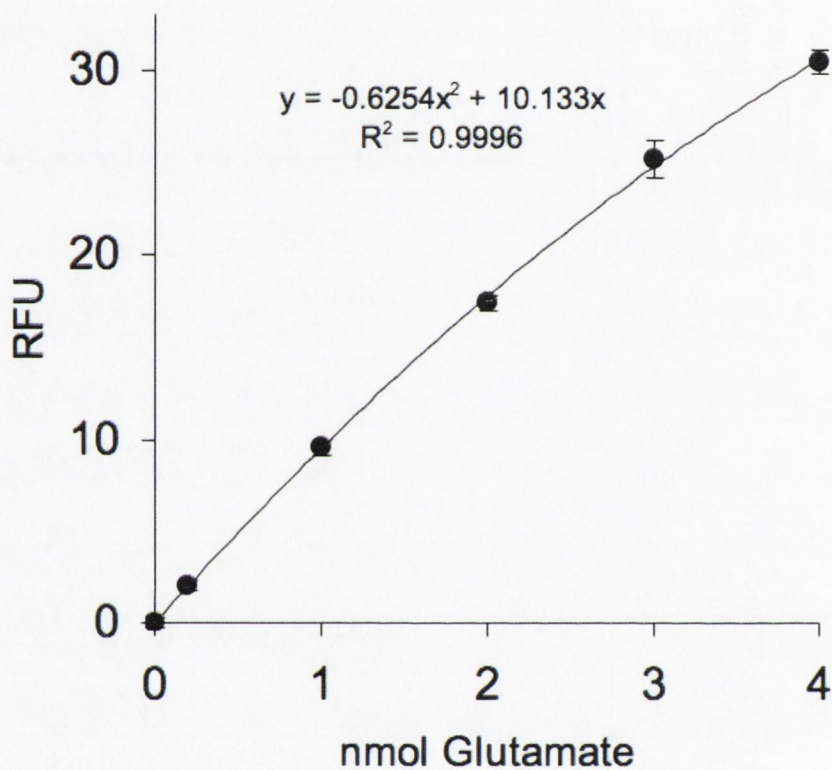


Fig. 2.3 Glutamate standard curve

The mean steady state fluorescence values were plotted against known amounts of added glutamate. These data were recorded in experiments performed in a 96-well plate. 100 μ l of glutamate in Kreb's buffer was added to 100 μ l of Kreb's containing 2 mM NADP^+ and 6.32 U glutamate dehydrogenase. Fluorescence was monitored at excitation $\lambda = 340$ nm, emission $\lambda = 460$ nm. Data represent the means of triplicate experiments $\pm$ SEM.

2.11 Correction for L-Glutamic dehydrogenase ‘enzyme lag’

In the presence of a relatively high concentration of L-Glutamic dehydrogenase (15.8 U/ml), not all of the glutamate was converted to 2-oxo-glutarate immediately (Fig. 2.3). Therefore, to calculate how much glutamate is in each well at any given time, the kinetics of enzyme activity must be accounted for. The rate enzymatic conversion of a standard amount of glutamate at any given point (i.e. the tangent to the curve at that point) plotted against the residual glutamate in the well at that point gives a straight line (Fig. 2.5). During the continuous assay the concentration of glutamate in the medium which has been *released but not yet assayed* for any rate given by the recorder can be deduced from this (Nicholls *et al.* 1987). To obtain these tangents, a mathematical program, ‘OriginPro 7.5’ was used to smooth the traces given by a standard addition of glutamate (Fig. 2.4) and the tangents taken from the 200 – 450 s time range in this data.

2.12 Continuous fluorimetric assay of glutamate release in a 96-well plate

Using a Perkin-Elmer fluorimeter entails considerable limitations, particularly the fact that only 4 cuvettes could be measured simultaneously. This gave rise to the suggestion that the differences in time between the preparation of synaptosomes and the carrying out of experiments would have an effect on the rates of glutamate release. As well as this, it was impossible to perform the required experimental procedures on a single synaptosomal preparation due to the large volume of sample required for use of cuvettes, which must contain a volume at least 2 ml for values to be recorded. These difficulties were overcome by using the fluorimeter function on the SpectraMAX GeminiXS well-plate reader, which allowed simultaneous continuous measurement of up to 96 samples at a time (Fig. 2.6), although temporal sensitivity is sacrificed using this method.

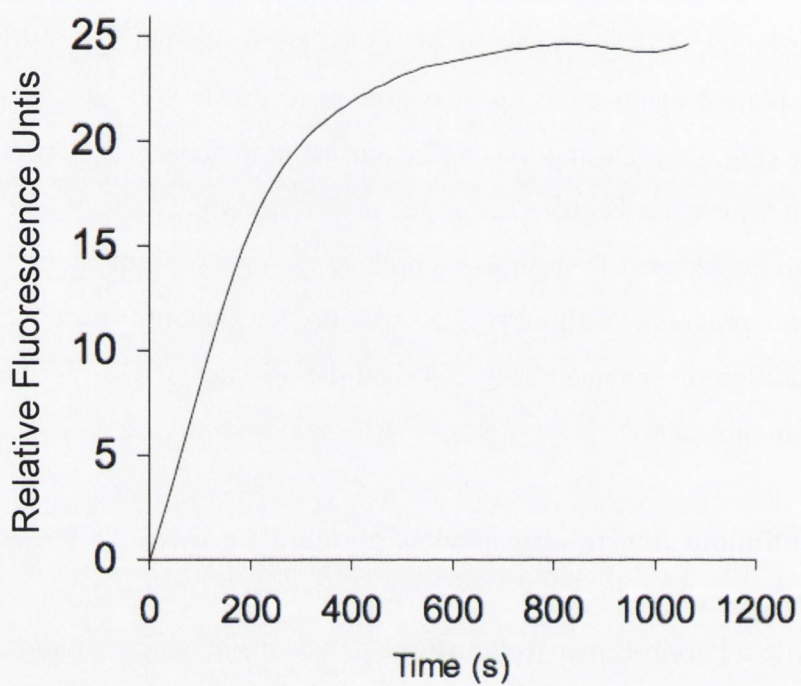


Fig. 2.4 Smoothed rate of conversion of 3 nmol glutamate

Smoothing raw data taken from addition of a standard amount of glutamate (such as that shown in Fig. 2.2b) allows for tangents to be calculated and enzyme lag to be accounted for.

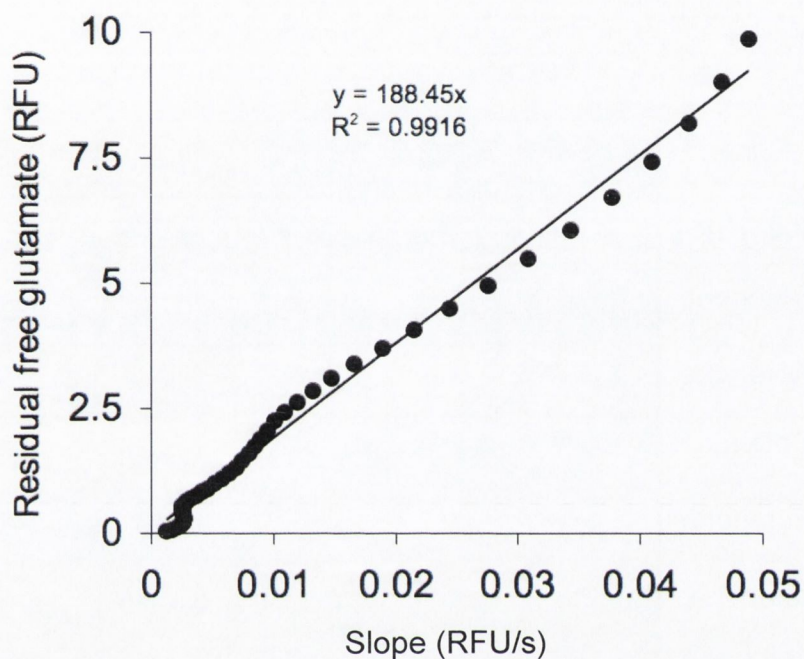


Fig. 2.5 Plotting residual free glutamate at any point against the tangent of the curve at that point gives a line through the origin.

For the liner rates calculated by ‘SoftMax Pro’ program connected to the well-plate reader, the residual free glutamate was calculated using the slope of this line, and this amount added to the final rate. Data shown is calculated from the trace of a single experiment, produced by the addition of 3 nmol glutamate.

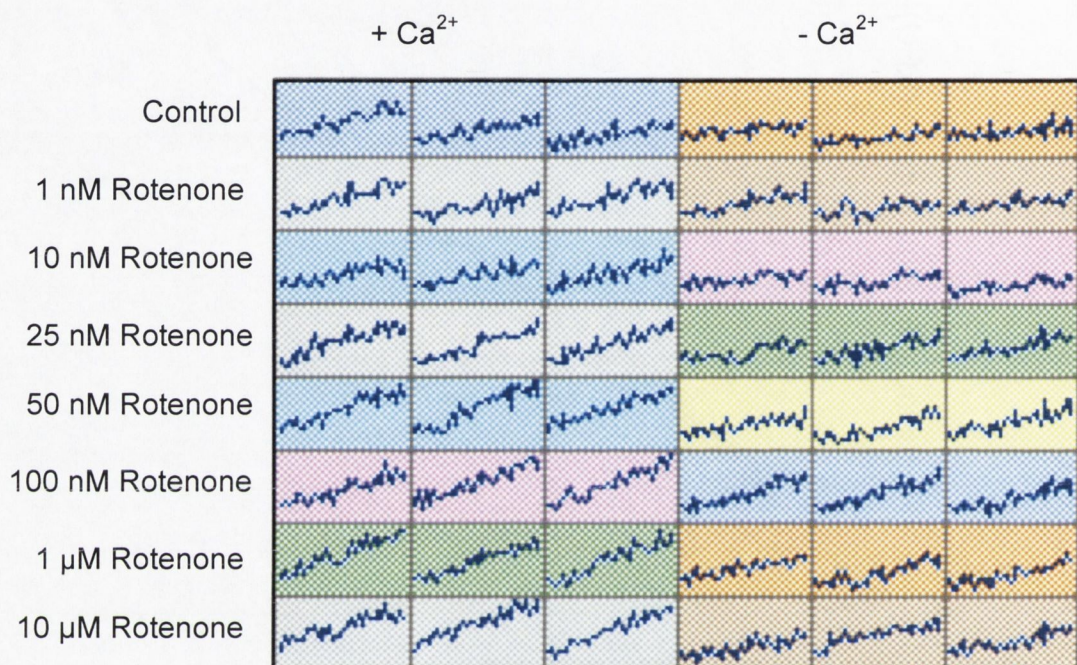


Fig. 2.6 Traces showing release of glutamate from synaptosomes depolarised with KCl monitored continuously using a well-plate fluorimetric detector.

Groups were incubated with 2 μ l of rotenone in ethanol so that final concentrations were as shown. 1.4 mM CaCl₂ was added to the medium of 1 group shown here (+ Ca²⁺), while the medium of the second group (- Ca²⁺) contained no CaCl₂. Rates for all groups were measured simultaneously over 20 min.

2.13 Enzyme activity assays

2.13.1 Complex I activity assay

Complex I activity was measured by following the oxidation of NADH to NAD^+ spectrophotometrically at absorbance $\lambda = 340$ nm. Samples were incubated for 20 min and immediately frozen in the -80°C freezer. The synaptosomes were thawed out in a 37°C incubator and freeze-thawed 3 times with liquid nitrogen to break up the membranes and allow easy passage of substrate to complex I. CoQ is reduced to ubiquinol during the oxidation of NADH by complex I. In this assay, decylubiquinol was used instead of CoQ to initiate the reaction. The absorbance at 340 nm was followed and the rates recorded. $10\ \mu\text{M}$ rotenone was added after 5 min to measure the rotenone-insensitive rate and the insensitive rates subtracted from the rotenone-sensitive rates observed at the various concentrations of rotenone.

2.13.2 Complex III activity assay

Complex III activity was measured by following the reduction of cytochrome *c* spectrophotometrically at absorbance $\lambda = 550$ nm. Samples were incubated for 20 min and immediately frozen in the -80°C freezer. Prior to carrying out the assay, ubiquinol₂ was prepared for the reaction mixture from ubiquinone. This involved acidifying $10\ \mu\text{M}$ decylubiquinone (in ethanol) to pH 2 with 6 M HCl, adding NaBH_4 and extracting with diethylether : cyclohexane (2 : 1 v/v). The upper phase was collected, dried under N_2 and the resulting powder dissolved in EtOH. This solution was acidified to pH 2.0 with HCl before being stored at -80°C . The concentration of ubiquinol was evaluated by recording the change in absorbance at $\lambda = 275$ nm after adding KOH and using the extinction coefficient at this wavelength, $\epsilon = 12.25\ \text{mM}^{-1}\text{cm}^{-1}$.

After subjecting the samples to 3 freeze-thaw cycles, the reaction was initiated by addition of sample to the reaction mixture and the rate of cytochrome *c* reduction followed at 30°C . This time point was called $t = 0$ and the first-order decay rate constant (K) was calculated as from the difference between the natural logarithms of

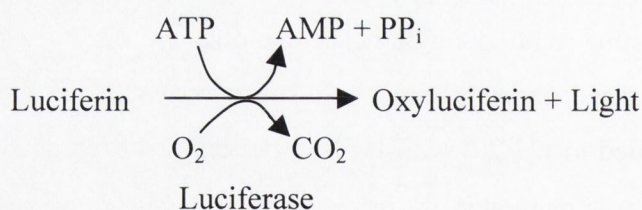
the absorbance at this point and three time points, 1, 2 and 3 min after time 0. The mean of the calculated values was taken to be (K) and the activity was expressed as a percentage of control.

2.13.3 Complex IV activity assay

Complex IV activity was measured by following oxidation of cytochrome *c* at 550 nm and 30°C (Wharton and Tzagoloff, 1967). The reference cuvette contained 10 mM potassium phosphate pH 7.0, 50 µM reduced cytochrome *c* and 1 mM reduced ferricyanide in 1 ml final volume. The test cuvettes contained 10 mM potassium phosphate pH 7.0 and 50 µM reduced cytochrome *c*. The reaction was initiated by the addition of 10 µg synaptosomal protein to the test cuvettes. This time point was called $t = 0$ and the first-order decay rate constant (K) was calculated as from the difference between the natural logarithms of the absorbance at this point and three time points 1, 2 and 3 min after. The mean of the calculated values was taken to be (K) and the activity was expressed as a percentage of control.

2.14 ATP determinations

ATP levels were determined using a luciferase coupled assay a modification of the method of Kauppinen and Nicholls 1986.



Emission $\lambda = 560$ nm. Reactions were stopped by adding 200 µl of 1 M perchloric acid/50 mM EDTA (at 0°C) to samples of 200 µl in volume, centrifuged and supernate was neutralised with 375 µl 3 M KOH/1.5 M triethanol amine and stored at -80°C. All samples were read within 1 week of preparation. To calibrate the readings, standard amounts of ATP were added to the Enliten® ATP assay kit and endpoint readings taken immediately (Fig. 2.7).

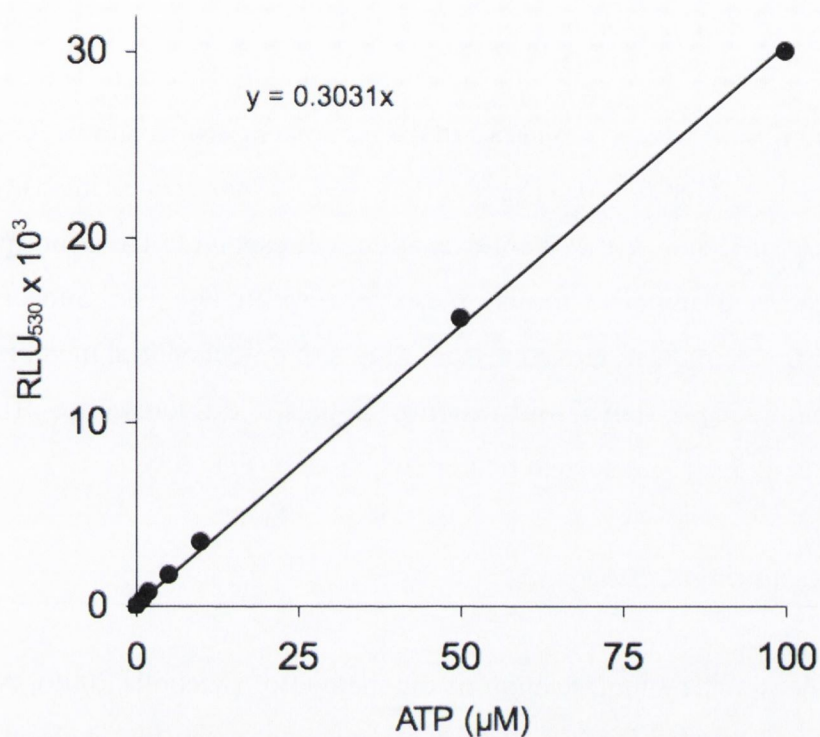


Fig. 2.7 ATP standard curve

10 μl ATP in H₂O was added in known amounts to the 100 μl of reconstituted luciferin/luciferase and the luminescence at 560 nm recorded immediately. This procedure was performed daily. Results show the means of data produced in triplicate, with SEM falling within the size of the data points shown.

2.15 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.* 1999). JC-1 is a charged carbocyanine that aggregates after diffusion into the dense mitochondrial matrix. The 'J-aggregates' have different fluorescence properties to the monomers, which allows JC-1 to be used as a fluorescent probe for $\Delta\psi_m$ (Reers *et al.* 1991). Fluorescence intensity at $\lambda = 590$ nm and at $\lambda = 535$ nm ($\lambda = 490$ nm excitation) correspond to the fluorescent peaks of the aggregate and monomer forms of the dye respectively. The ratio of emission at 590 to 535 is a semiquantitative measure of in situ mitochondrial membrane potential (Reers *et al.* 1995). Using K^+ diffusion potentials, the fluorescence of J-aggregates was calibrated to $\Delta\psi_m$ in isolated brain mitochondria (Fig. 2.8).

2.16 Measurement of $\Delta\psi_p$

$\Delta\psi_p$ was measured by modification of the method of (Nicholls 2006). An individual vial from a Molecular Devices 'membrane potential assay kit, explorer format' (R-8042) containing was reconstituted in 1 ml of distilled water, dispensed into 50 μ l aliquots, and frozen. The assay was optimised for sensitivity so that this 50 μ l of proprietary plasma membrane potential indicator (PMPI) stock was diluted to 10 ml with Krebs's buffer, and 100 μ l added to 100 μ l of Krebs's with 0.1 mg of sample per well to give a final concentration of 0.5 mg/ml of sample. The assay mixture was allowed a 45 min pre-incubation time before experiments were initiated. Stock inhibitors were diluted in Krebs's to allow addition with a multi-channel pipette of a volume of not more than 10 μ l. Spectral analysis was performed and excitation $\lambda = 530$ nm and emission $\lambda = 565$ nm were selected for reading. The difference in signal after 15 min was divided by the initial signal, which was later calibrated to $\Delta\psi_p$ by adding a range of KCl concentrations (0 – 100 mM) and using data from (Hare and Atchison 1992). This paper reported a logarithmic relationship between the external [KCl] and $\Delta\psi_p$ in synaptomes (Fig. 2.9).

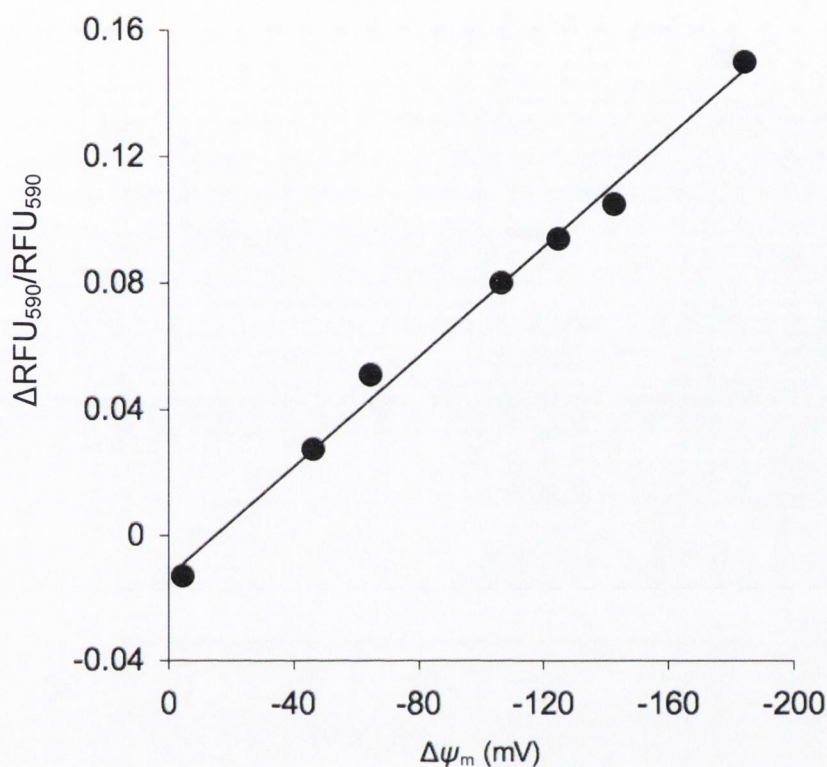


Fig. 2.8 The fluorescence of J-aggregates increases linearly with $\Delta\psi_m$ in isolated brain mitochondria

Isolated non-synaptic brain mitochondria (0.5 mg/ml) were incubated in a buffer consisting of 200 mM sucrose, 20 mM mannitol, 20 mM NaMops, 1 mM NaEDTA, and 2.5 μ M oligomycin (pH 7.2) with 300 nM JC-1 and 20 μ M valinomycin and a range of concentrations of KCl (100 μ M – 100 mM). The change in relative fluorescence units at emission $\lambda = 590$ nm (ΔRFU_{590}) after addition of 1 μ M FCCP was divided by the initial fluorescence at each respective concentration of KCl. The K^+ diffusion potentials were calibrated to $\Delta\psi_m$ using the Nernst equation and taking a value of $[K^+]$ in the mitochondrial matrix of 120 mM.

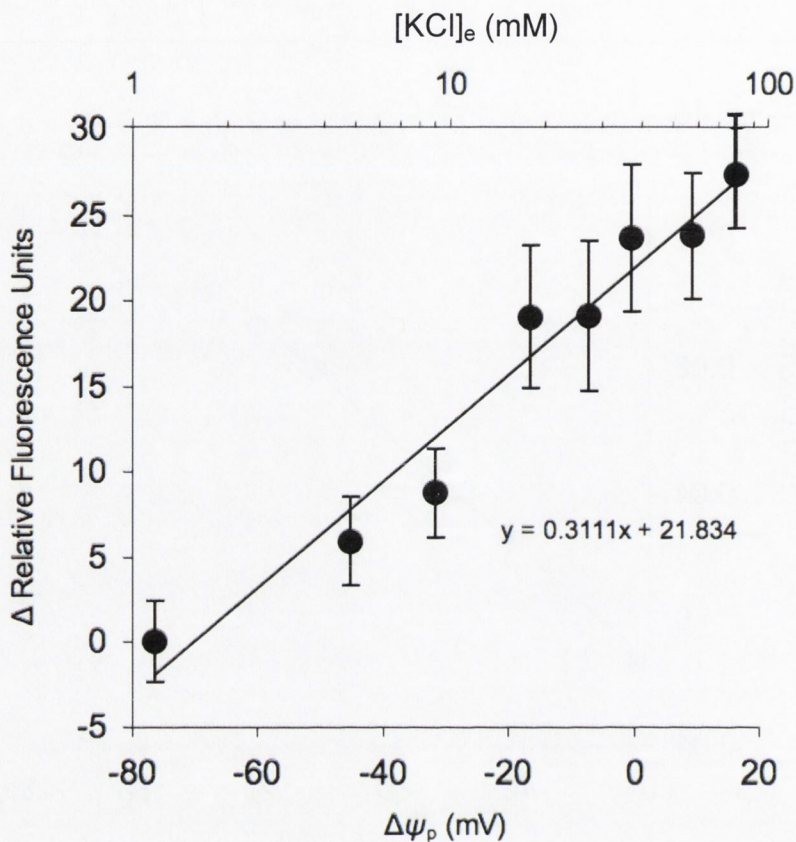
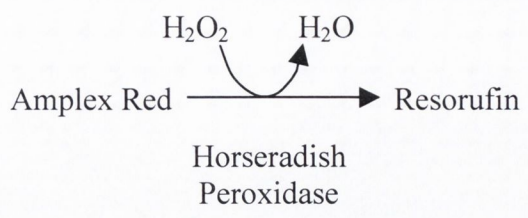


Fig. 2.9 Change in fluorescence intensity brought about by addition of known amounts of KCl and relationship to $\Delta\psi_p$

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 45 min in the presence of PMPI before addition of known amounts of KCl. The change in fluorescence intensity was monitored continuously and the value at 15 min shown here. Data represent mean $\pm$ SEM of experiments done in quadruplicate.

2.17 Measurement of hydrogen peroxide production

H₂O₂ production was followed using the Amplex Red assay system (Mohanty *et al.* 1997).



Assay sensitivity was optimised such that 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 $\lambda = 585 \text{ nm}$ ($\lambda = 550 \text{ nm}$ emission). The fluorescence units were calibrated using a standard curve of known amounts of H₂O₂ (Fig. 2.10).

2.16 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 on the *InStat 2.0* program. Values of $p < 0.05$ were taken to be significant.

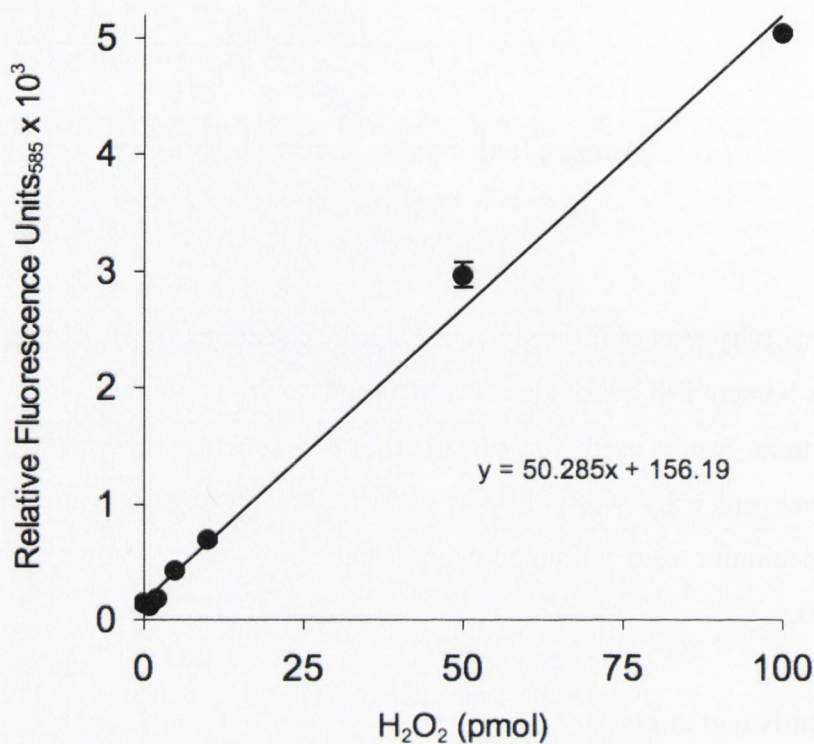


Fig. 2.10 H₂O₂ standard curve

H₂O₂ was diluted with H₂O before addition of 10 μ l of standard amounts to 90 μ l Kreb's buffer. Another 100 μ l Kreb's containing amplex red and horseradish peroxidase was added and fluorescence at $\lambda = 585$ nm ($\lambda = 550$ nm emission) was read immediately. Data represents mean $\pm$ of experiments done in triplicate. Where no error bars are shown the SEM lies within the size of the symbol.

Chapter 3

Complex I and control of glutamate release from synaptosomes

3.1 Introduction

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 1992b; Mattson 2003) and mitochondrial dysfunction is thought to be involved in this process (Doble 1999; Beal 2005; 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 homogenates in postmortem studies of Parkinson's disease patients (Schapira *et al.* 1989; Schapira *et al.* 1990a; Schapira *et al.* 1990b). 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 depolarisation on glutamate release from nerve terminals with compromised complex I activity.

Previous studies have examined the effects of metabolic inhibitors on glutamate release from synaptosomes (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 polarised 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 previously been reported in the literature, and increases in Ca^{2+} -independent glutamate release rates after depolarisation 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.* 1997) compared to nonsynaptic mitochondria (Davey and Clark 1996; Davey *et al.* 1998). 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 may have consequences for ATP production and subsequent release of glutamate from the nerve terminal.

3.2 Materials & methods

3.2.1 Monitoring glutamate release

Following assay of protein concentration, synaptosomes were divided (1 mg into each Eppendorf tube) and topped up to 1 ml with TES buffer (250 mM sucrose, 5 mM TES, pH 7.4). The synaptosomes were then centrifuged at 12,000 g for 5 min and stored as pellets on ice until experiments were performed. For experiments performed in the PerkinElmer LS-55 Luminescence Spectrometer, synaptosomal pellets were resuspended in cuvettes with a final volume of 2 ml of Krebs buffer (3 mM KCl, 140 mM NaCl, 25 mM Tris HCl, 10 mM glucose, 2 mM MgCl_2 , pH 7.4 at 37°C). 1 mM NADP^+ , 31.6 U GDH, 1.4 mM CaCl_2 and rotenone (1 nM – 10 μM final concentrations) where appropriate were added in volumes of 20 μl or less. Glutamate release was monitored continuously and simultaneously in 4 cuvettes using the Wavelength program of the FLWinLab software. Synaptosomes were depolarised after 5 min by addition of 80 μl of 1 mM KCl to give a final concentration of 40 mM and linear rates over the 2 – 3 min were taken using the 'Arithmetic' function of the

software. Calibration and enzyme lag (Nicholls *et al.* 1987) were accounted for when converting rates to pmol/min/mg.

For experiments performed on the SpectraMAX GeminiXS well-plate fluorimeter, synaptosomal pellets (1 mg) were resuspended in Krebs buffer (3 mM KCl, 140 mM NaCl, 25 mM Tris HCl, 10 mM glucose/2 mM deoxyglucose, 2 mM MgCl₂, pH 7.4 at 37°C) and 100 µl added to wells containing this 100 µl of this same medium with 2 mM NADP⁺, 6.32 U GDH, (2.8 mM CaCl₂ and 2 µl rotenone in ethanol where appropriate) previously added. 40 mM KCl or 1 mM 4-aminopyridine (in 8 µl H₂O) were used to depolarise synaptosomes after 5 min and rates were taken over a 20 min time period after depolarization using the kinetic mode (cycle time of 32 s) in SoftMax Pro software. Calibration and enzyme lag (Nicholls *et al.* 1987) were accounted for when converting rates to pmol/min/mg (described in detail in Section 2.10). Rates of glutamate release were later plotted against % inhibition of complex I activity brought about by each concentration of rotenone, measured in a separate group of experiments.

3.2.2 Complex I activity assay

Complex I activity was measured using an Agilent ChemStation 8453 spectrophotometer or a SpectraMAX 340PC well-plate spectrophotometer. The titration of complex I activity with rotenone was performed identically to the glutamate release experiments – in well plates with final concentration of 0.5 mg/ml synaptosomes in a final volume of 200 µl. After freezing at -80°C and undergoing 3 rapid freeze-thaw cycles, 20 µg synaptosomal sample was added to 1 ml plastic cuvette containing 25 mM potassium phosphate buffer (pH 7.2 at 30°C), 0.2 mM NADH, 10 mM MgCl₂, 1 mM KCN and 2.5 mg BSA, final volume 1 ml in the spectrophotometer. 8 cuvettes were read simultaneously with cycle time of 18 s, allowing measurement activity of control and 7 concentrations of inhibitor. 10 µl decylubiquinol (50 µM final concentration) was added to initiate the reaction. Linear rates were taken over 5 min before addition of 10 µl of 1 mM rotenone in ethanol (final concentration 10 µM) to each cuvette for calculation of the rotenone-insensitive

rate. This rate was subtracted from the initial rates and the resulting rates expressed as a percentage of the control rate.

For experiments performed in the well-plate spectrophotometer, 4 µg of synaptosomal protein was added to buffer containing the concentrations of reagents present in the spectrophotometer experiments, with final volume of 200 µl in each well. Linear rates were taken over 10 min and the rotenone-insensitive rate subtracted.

3.2.3 ATP measurements

ATP levels were determined using a luciferase-coupled assay by modification of the method of Kauppinen and Nicholls 1986a. Two groups of experiments were performed; In the first group of experiments, synaptosomes (0.5 mg/ml) were resuspended in 700 µl Kreb's buffer with the full range of rotenone concentrations used in the glutamate release experiments (1 nM – 10 µM). 200 µl of samples were extracted and added to 200 µl of 1 M perchloric acid, 50 mM EDTA (at 0°C) at 3 time points: immediately upon addition of synaptosomes to the rotenone-containing medium ('- 20 min' time point) at the point of depolarisation ('0 min' time point) and 20 minutes after depolarisation ('+ 20 min' time point). Samples centrifuged for 1 min at 10,000 rpm and were 200 µl of supernatant was neutralised with 375 µl of 3 M KOH, 1.5 M triethanol amine and stored at -80°C (Fig. 3.1A). In the second, only 2 concentrations of rotenone were used so that time points would correlate with the glutamate release experiments. In these experiments, synaptosomes were resuspended in 200 µl of Kreb's buffer and 200 µl of 1 M perchloric acid, 50 mM EDTA (at 0°C) added at 2 time points: immediately upon addition of synaptosomes to the rotenone-containing medium and 15 minutes later (Fig. 3.1B). (Where indicated, synaptosomes were depolarised with either 4-aminopyridine or KCl after 5 min incubation). 200 µl of supernate was neutralised with 375 µl of 3 M KOH, 1.5 M triethanol amine and stored at -80°C. All samples were assayed within 1 week of preparation. 10 µl of extract was added to 100 µl reconstituted ENLITEN® luciferin/luciferase reagent and luminescence intensity (emission λ = 560 nm) recorded immediately in a SpectraMAX GeminiXS in Endpoint mode.

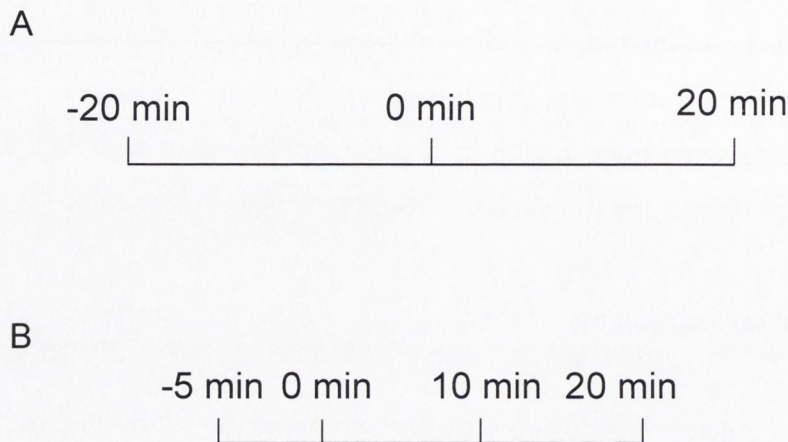


Fig. 3.1 Experimental time points used for ATP extraction, complex I activity and glutamate release assays.

Rotenone was added to the medium before addition of synaptosomes, and depolarising agents were added at the '0 min' time point. **A:** ATP was PCA extracted from samples containing 1 nM – 10 μ M rotenone at 3 time points: immediately upon addition of synaptosomes to the medium ('- 20 min' time point) at the point of depolarisation ('0 min' time point) and 20 minutes after depolarisation ('+ 20 min' time point). **B:** ATP was PCA extracted from samples containing 25 nM and 10 μ M rotenone at 2 time points: immediately upon addition of synaptosomes to the medium ('-5 min' time point) and 15 min later ('10 min' time point). For complex I activity assays, synaptosomes were incubated at 37°C for 15 min and were then removed and frozen (also at the '10 min' time point represented here). Glutamate release rates were taken over 20 min (from the '0 min' time point to the '20 min' time point represented here).

3.3 Results

3.3.1 Rotenone increases the rate of Ca^{2+} -independent glutamate release from depolarised synaptosomes

Using the PerkinElmer fluorimeter, Ca^{2+} -independent release was found to occur at a rate of 100.1 ± 4.6 pmol/min/mg protein after depolarising with 40 mM KCl (Fig. 3.2). In the presence of 1.4 mM CaCl_2 , the release rate was 175.4 ± 8.6 pmol/min/mg, suggesting a Ca^{2+} -dependent release rate of ~ 75 pmol/min/mg. This rate was not significantly affected by any concentration of rotenone. However, 50 nM rotenone significantly increased Ca^{2+} -independent release to 143.6 ± 8.2 pmol/min/mg ($p < 0.05$, Fig. 3.2). 1 μM rotenone brought about the highest release rate of 145.3 ± 7.5 pmol/min/mg (Fig. 3.2). A similar pattern of release following rotenone titration was observed in the SpectraMAX well plate reader following depolarisation by both 4-aminopyridine (Fig. 3.3) and KCl (Fig. 3.4). 1 mM 4-aminopyridine induces repetitive action potential-like depolarisations of synaptosomes (Tibbs *et al.* 1989; Tibbs *et al.* 1996), which cause a Ca^{2+} -dependent release of glutamate from the vesicular pool. When depolarised with 4-aminopyridine in the presence of 25 nM rotenone, the rate of release was increased to 101.6 ± 15.5 pmol/min/mg ($p < 0.05$, Fig. 3.3). The highest release rate was 217.7 ± 30.8 pmol/min/mg, brought about by 1 μM rotenone. This corresponds to a release of 21% of the initial total glutamate pool (over the 20 min period after depolarisation), whereas 2.6% is released in the absence of inhibitor. This total pool was evaluated by addition of 0.5% vol/vol Triton X100 and was found to be 20.7 ± 0.14 nmol/mg. In the presence of 1.4 mM CaCl_2 , synaptosomes released 107.8 ± 16.1 pmol glutamate/min/mg when depolarised 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 vesicular glutamate pool was evident as rotenone concentrations approached 1 μM .

Fig. 3.4 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 depolarised with 40 mM KCl. The Ca^{2+} -independent release rate was also higher after depolarisation with 40 mM KCl than 1 mM 4-aminopyridine at all concentrations of

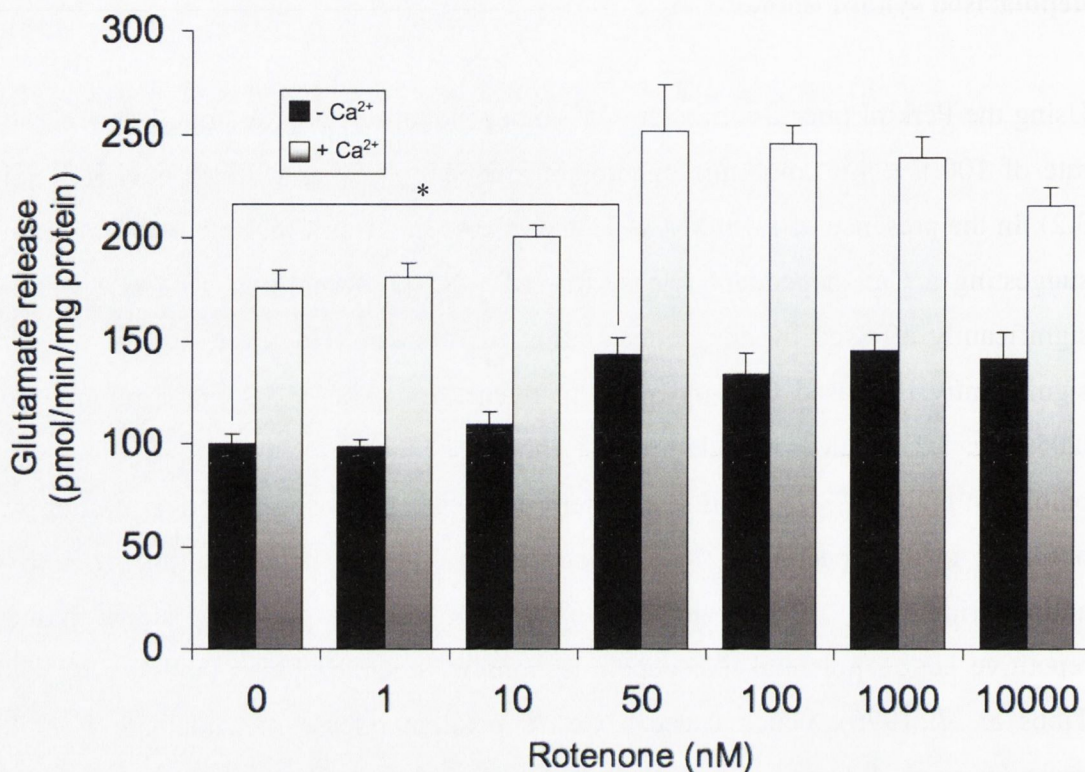


Fig. 3.2 Rotenone increases Ca²⁺-independent glutamate release from synaptosomes depolarised with KCl

Rotenone (1 nM – 10 μ M) was added to cuvettes containing 0.5 mg/ml synaptosomes in Kreb's buffer, with 1 mM NADP⁺ and 31.6 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 40 mM KCl and glutamate release rates were taken over 2 – 3 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (* = $p < 0.05$).

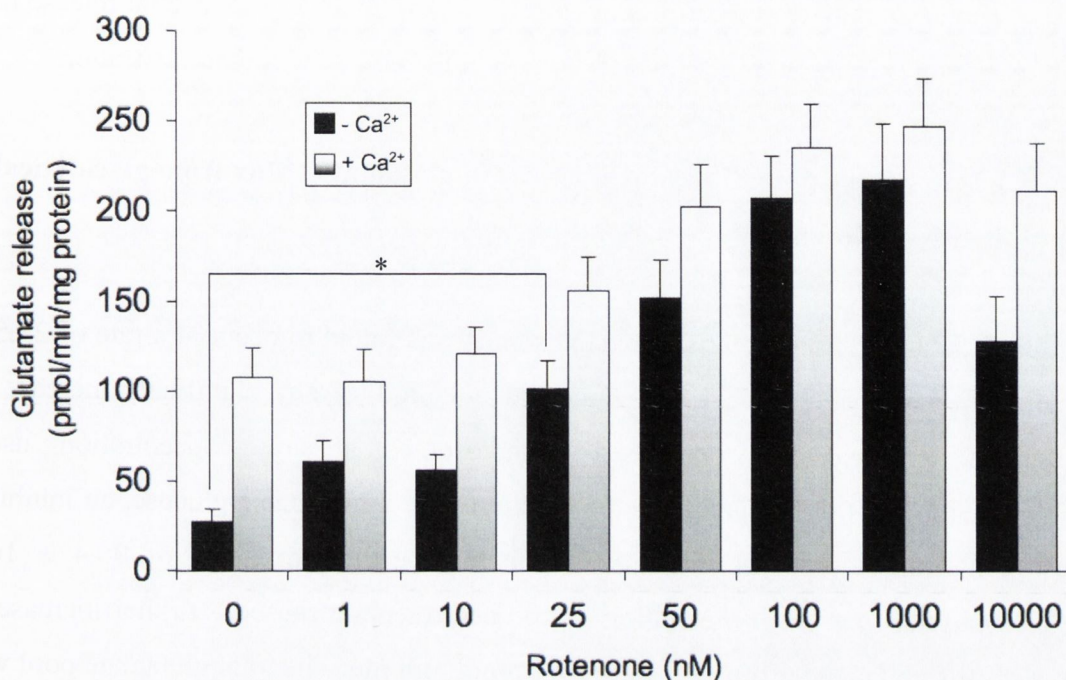


Fig. 3.3 Rotenone increases Ca²⁺-independent glutamate release from synaptosomes depolarised with 4-aminopyridine

Rotenone (1 nM – 10 μ M) was added to wells containing 0.5 mg/ml synaptosomes in Krebs's buffer, with 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 1 mM 4-aminopyridine and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 6 synaptosomal preparations. (* = $p < 0.05$).

rotenone. In the presence of 25 nM rotenone, the rate was increased to 192.5 ± 14.7 pmol/min/mg ($p < 0.001$). When depolarised with KCl, 100 nM rotenone 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 KCl which was not affected by the rotenone titration.

3.3.2 Rotenone potentiates the increase in glutamate efflux from glycolytically inhibited synaptosomes

Glutamate efflux from polarised synaptosomes was found to occur at a rate of 25.5 ± 6.5 pmol/min/mg (Fig. 3.5). Rotenone did not produce any significant increase in glutamate release from polarised synaptosomes at any concentration used. 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 100 nM rotenone caused a further increase in the rate of glutamate efflux to 248.6 ± 9.1 pmol/min/mg. The total glutamate pool was found to be reduced by deoxyglucose from 20.7 ± 0.14 pmol/min/mg to 16.8 ± 0.15 pmol/min/mg ($p < 0.01$) after 5 min and to 13.9 ± 0.14 after another 15 min ($p < 0.01$). 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.

3.3.3 Rotenone combined with inhibition of glycolysis blocks exocytotic release of glutamate from depolarised synaptosomes

The presence of the glycolytic inhibitor deoxyglucose increased Ca^{2+} -independent release of glutamate to 60.0 ± 14.8 pmol/min/mg (Fig. 3.6, '0 nM Rotenone – Ca^{2+} ') from 27.1 ± 7.2 pmol/min/mg (Fig. 3.3, '0 nM Rotenone - Ca^{2+} ') and decreased release in the presence of 1.4 mM CaCl_2 from 107.8 ± 16.1 (Fig. 3.3, 0 nM Rotenone + Ca^{2+}) to 84 ± 8.2 pmol/min/mg (Fig. 3.6, '0 nM Rotenone + Ca^{2+} '), when depolarised with 4-aminopyridine. This suggests that Ca^{2+} -dependent release was blocked under these conditions. Similarly, when depolarised with KCl, exocytotic release of glutamate from synaptosomes was blocked (Fig. 3.7).

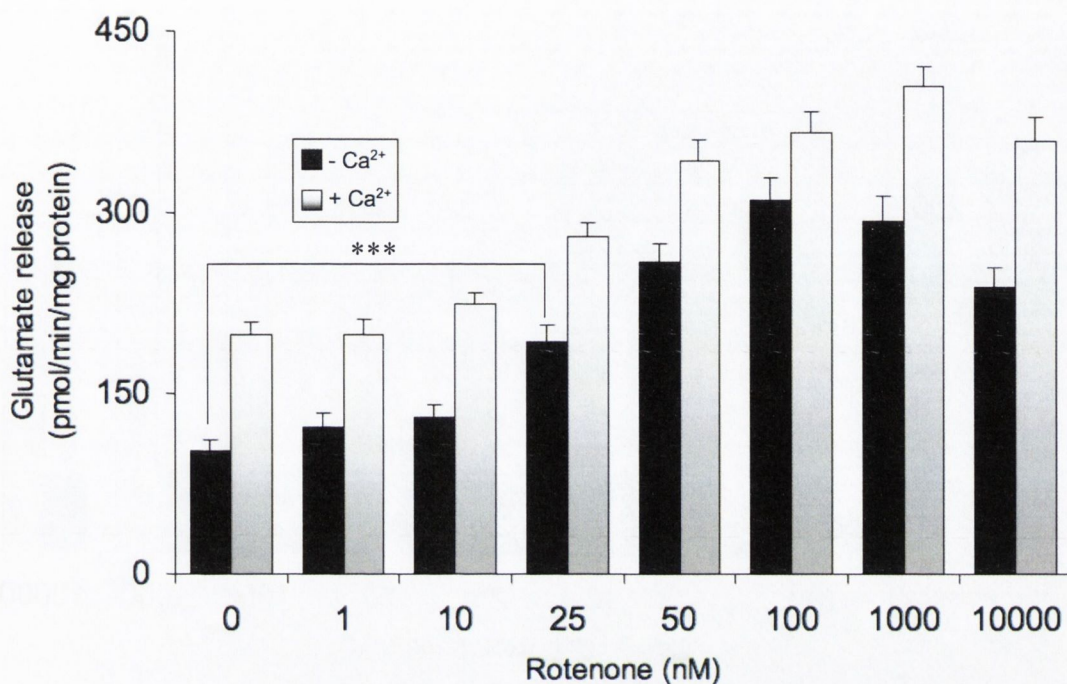


Fig. 3.4 Rotenone increases Ca²⁺-independent glutamate release from synaptosomes depolarised with KCl

Rotenone (1 nM – 10 μ M) was added to wells containing 0.5 mg/ml synaptosomes in Krebs's buffer, with 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 40 mM KCl and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 6 synaptosomal preparations. (***) = $p < 0.001$).

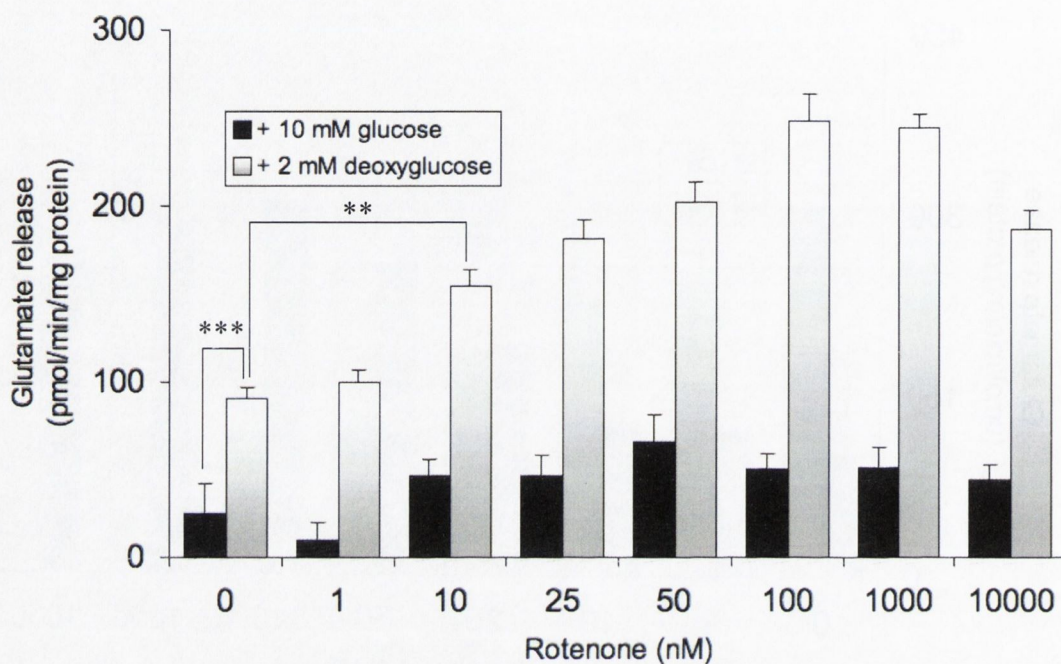


Fig. 3.5 Rotenone and deoxyglucose increase glutamate release from synaptosomes

Rotenone (1 nM – 10 μ M) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer (with either 10 mM glucose or 2 mM deoxyglucose), 1 mM NADP⁺ and 3.16 U GDH and 1.4 mM CaCl₂. Synaptosomes were incubated for 5 min before glutamate efflux rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 6 synaptosomal preparations. (** = $p < 0.01$, *** = $p < 0.001$).

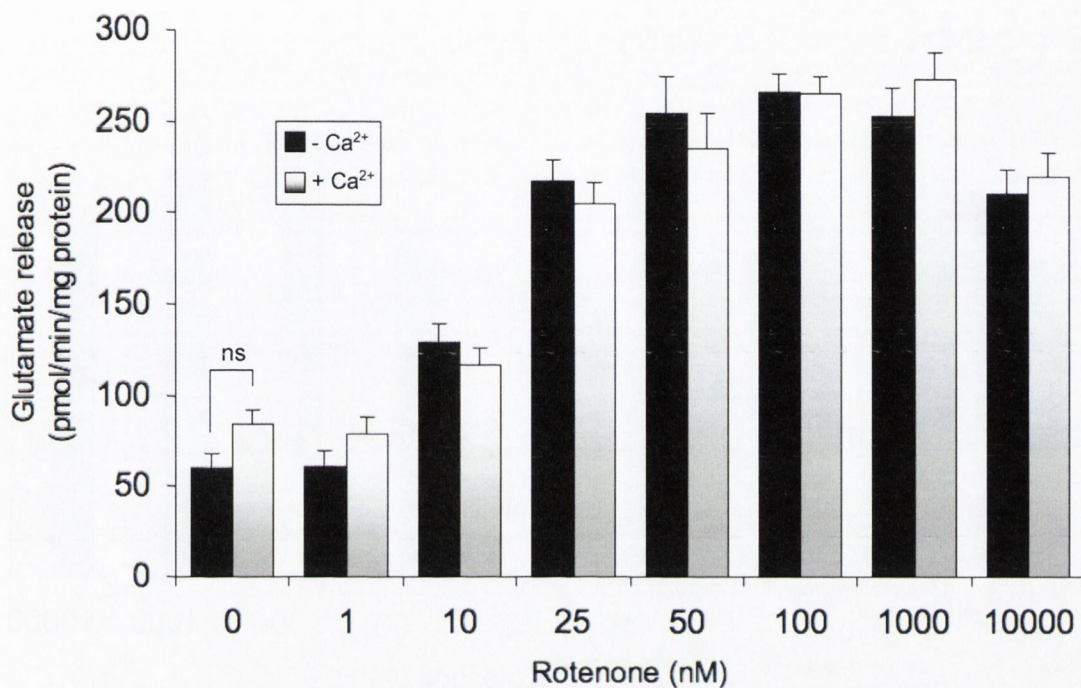


Fig. 3.6 Inhibition of mitochondrial complex I and glycolysis blocks Ca²⁺-dependent glutamate release from synaptosomes depolarised with 4-aminopyridine

Rotenone (1 nM – 10 μ M) was added to wells containing 0.5 mg/ml synaptosomes in Krebs's buffer with 2 mM deoxyglucose, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 1 mM 4-aminopyridine and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 4 synaptosomal preparations. (ns = not significant).

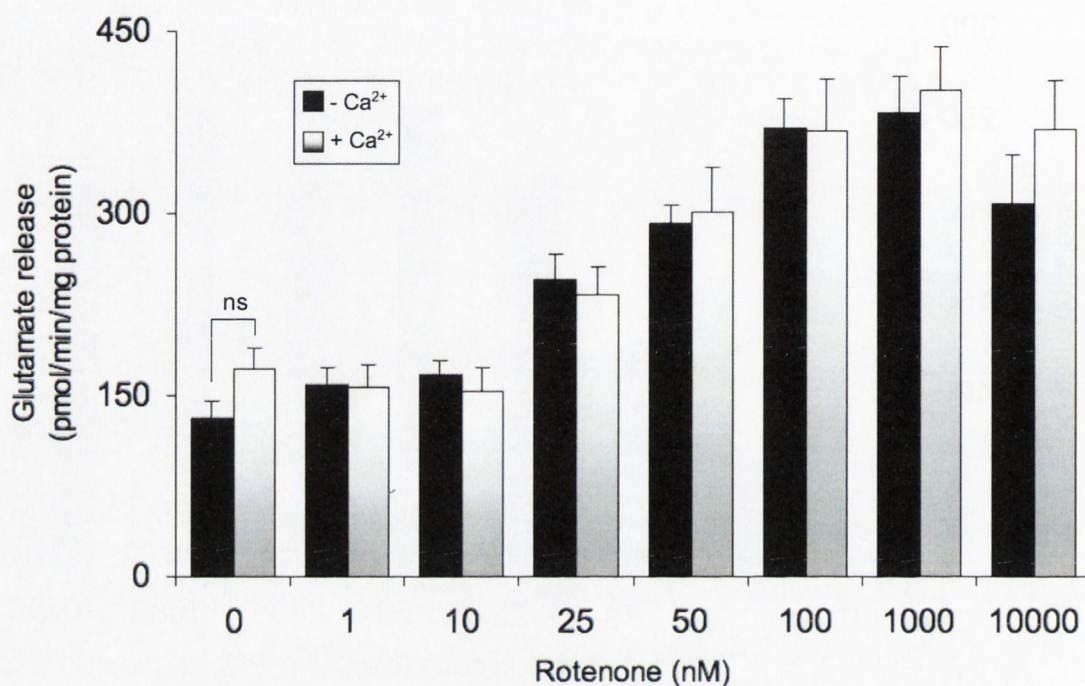


Fig. 3.7 Inhibition of mitochondrial complex I and glycolysis blocks Ca²⁺-dependent glutamate release from synaptosomes depolarised with KCl

Rotenone (1 nM – 10 μ M) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer with 2 mM deoxyglucose, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 1 mM 4-aminopyridine and glutamate release rates were taken over 20 min. Data represents the mean + SEM of experiments done in triplicate on 4 synaptosomal preparations. (ns = not significant).

3.3.4 Antioxidant enzymes do not prevent rotenone-induced increase in glutamate release from depolarised synaptosomes

Rotenone increased glutamate release from synaptosomes depolarised with KCl (in the presence of 1.4 mM CaCl_2) when assayed in the presence of superoxide dismutase, horseradish peroxidase or catalase (Fig. 3.8). In the presence of superoxide dismutase, glutamate was released at a rate of 164.0 ± 20.8 pmol/min/mg, which was not significantly different from control (204.0 ± 9.1 pmol/min/mg). The rate of release was found to be increased to 290.5 ± 15.7 pmol/min/mg by horseradish peroxidase in the absence of inhibitor, and to 277.4 ± 43.3 pmol/min/mg by catalase. 100 nM rotenone elicited the fastest rates of release with superoxide dismutase and peroxidase, at 427.8 ± 54.8 pmol/min/mg and 583.6 ± 35.2 respectively. This rate with peroxidase was significantly faster than control at this concentration of rotenone (397.7 ± 21.3 pmol/min/mg). The maximum release rate in the presence of catalase was found to occur at 1 μM rotenone, with release of 523.0 ± 78.1 pmol/min/mg at this concentration.

Preincubation for 30 min with the antioxidant enzymes also failed to prevent the rotenone-induced increase in glutamate release. Under these conditions, 119.4 ± 7.1 pmol/min/mg was released by depolarisation with KCl in the presence of CaCl_2 (Fig. 3.8). In accordance with the effects of preincubation of synaptosomes in (Sanchez-Prieto *et al.* 1987) this rate was significantly lower than the rate brought about by depolarisation after 5 min. Nonetheless, titration with rotenone increased the release in a similar pattern, and a maximum release rate of 266.5 ± 17.2 pmol/min/mg was recorded in the presence of 100 μM rotenone. The release rates were unchanged by superoxide dismutase and peroxidase in the absence of inhibitor (108.2 ± 9.7 pmol/min/mg and 132.7 ± 11.7 pmol/min/mg, respectively) but increased by catalase (153.9 ± 15.4 pmol/min/mg). This increase was evident across the titration, and 308 ± 24.7 pmol/min/mg was recorded in the presence of catalase and 100 μM rotenone. The maximum rate of release (brought about by 100 μM rotenone) was unchanged after preincubation with superoxide dismutase (258.2 ± 16.4 pmol/min/mg) or peroxidase (265.4 ± 21.1 pmol/min/mg).

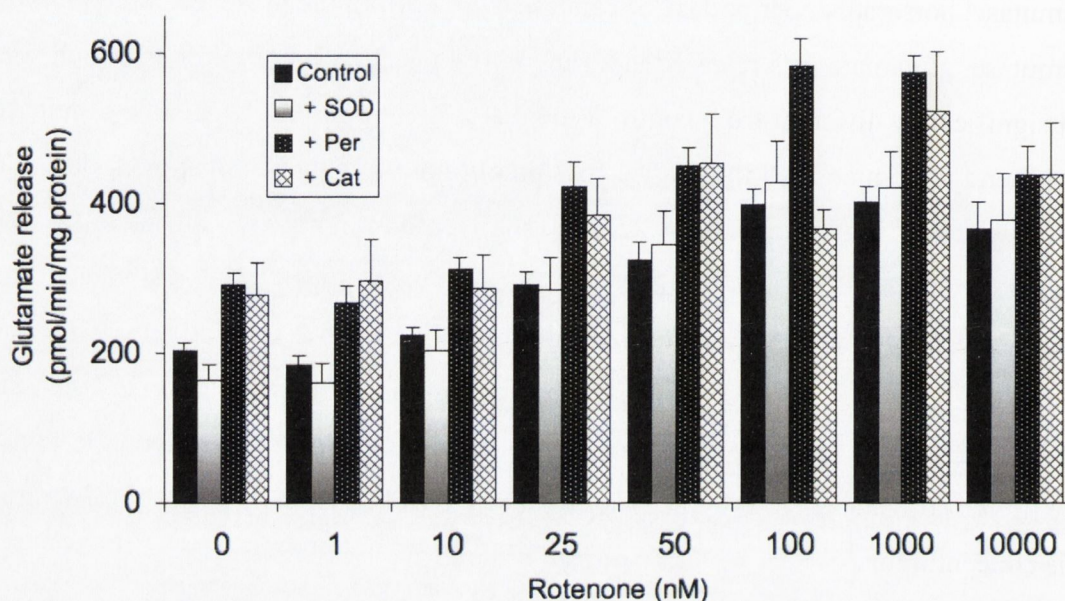


Fig. 3.8 Peroxidase and catalase potentiate glutamate release from synaptosomes depolarised with KCl

Rotenone (1 nM – 10 μ M) was added to wells containing 0.5 mg/ml synaptosomes in Krebs's buffer, 1 mM NADP^+ , 3.16 U GDH and 1.4 mM CaCl_2 . 1 U superoxide dismutase was added to one group (+ SOD), 1 U peroxidase added to a second (+ Per) and 0.5 U catalase to the third (+ Cat), Synaptosomes were incubated for 5 min before being depolarised with 40 mM KCl and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations.

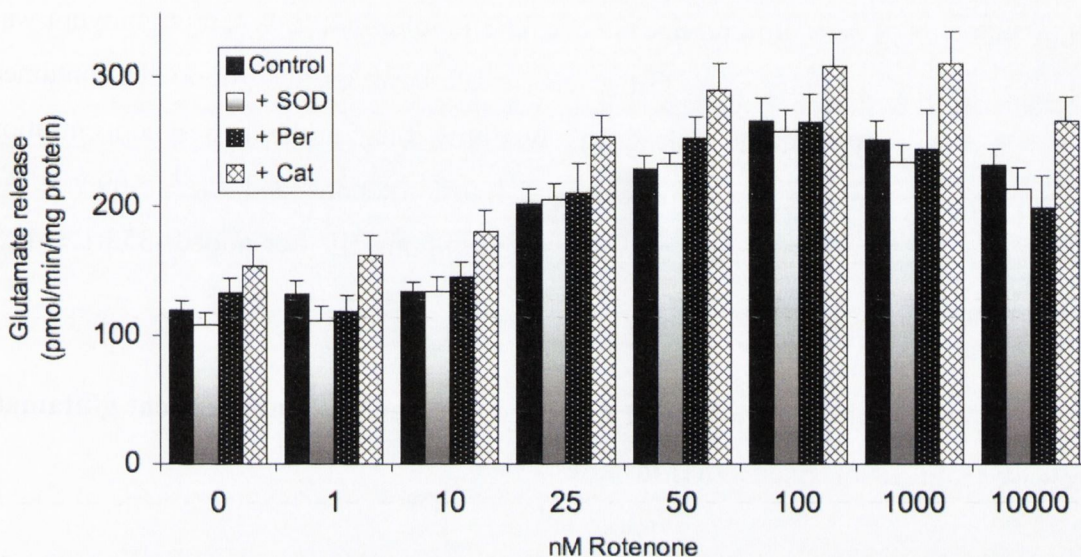


Fig. 3.9 Catalase, but not peroxidase or superoxide dismutase, potentiates glutamate release from synaptosomes after 30 min preincubation

Rotenone (1 nM – 10 μ M) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP^+ , 3.16 U GDH and 1.4 mM CaCl_2 . 1 U superoxide dismutase was added to one group (+ SOD), 1 U peroxidase added to a second (+ Per) and 0.5 U catalase to the third (+ Cat), Synaptosomes were incubated for 30 min before being depolarised with 40 mM KCl and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations.

3.3.5 Rotenone and oligomycin increase glutamate release from synaptosomes depolarised with KCl

In the presence of the complex V inhibitor oligomycin (20 $\mu\text{g/ml}$), KCl-induced Ca^{2+} -independent glutamate release was increased from 72.5 ± 22.7 pmol/min/mg to 341.0 ± 26.4 pmol/min/mg (Fig. 3.10). The rate of in the presence of CaCl_2 was increased from 147.4 ± 3.6 pmol/min/mg to 386.2 ± 42.5 pmol/min/mg. When oligomycin was present, a significant difference in the release rate between the group with contained CaCl_2 and the group with no added Ca^{2+} was only found at the highest concentration of rotenone. The rate in the presence of 1 μM rotenone and Ca^{2+} (409.7 ± 7.4 pmol/min/mg) was significantly lower in when oligomycin was added (353.1 ± 11.6 pmol/min/mg).

3.3.6 Partial inhibition complex I activity increases Ca^{2+} -independent glutamate release from depolarised synaptosomes

Rotenone attenuated the rate of reduction of NADH by synaptosomal complex I at the concentrations used in the glutamate release assays (Fig. 3.11). These assays were performed on a number of the various conditions matching those of the glutamate release experiments and the rate of glutamate release was plotted against % inhibition of complex I activity brought about by the corresponding concentration of rotenone (Figs 3.12 – 3.16).

Glutamate release from synaptosomes depolarised with 4-aminopyridine was significantly increased by 25 nM rotenone, and complex I activity was inhibited by $37.8 \pm 6.8\%$ at this concentration (Fig. 3.12). Highest Ca^{2+} -independent glutamate release rates of were observed between 60 and 90% inhibition of complex I activity. A similar pattern of release was seen after depolarisation with 40 mM KCl (Fig. 3.13), and 25 nM rotenone brought about $42.7 \pm 5.6\%$ inhibition of complex I activity under these conditions. Inhibition of glycolysis combined with partial inhibition of complex I activity increased glutamate release rates from polarised synaptosomes (Fig. 3.14) and 4-aminopyridine had minimal influence on release under these conditions, either in the absence or presence of Ca^{2+} (Fig. 3.15). Depolarisation with KCl increased Ca^{2+} -independent release, while Ca^{2+} -dependent release was blocked (Fig. 3.16).

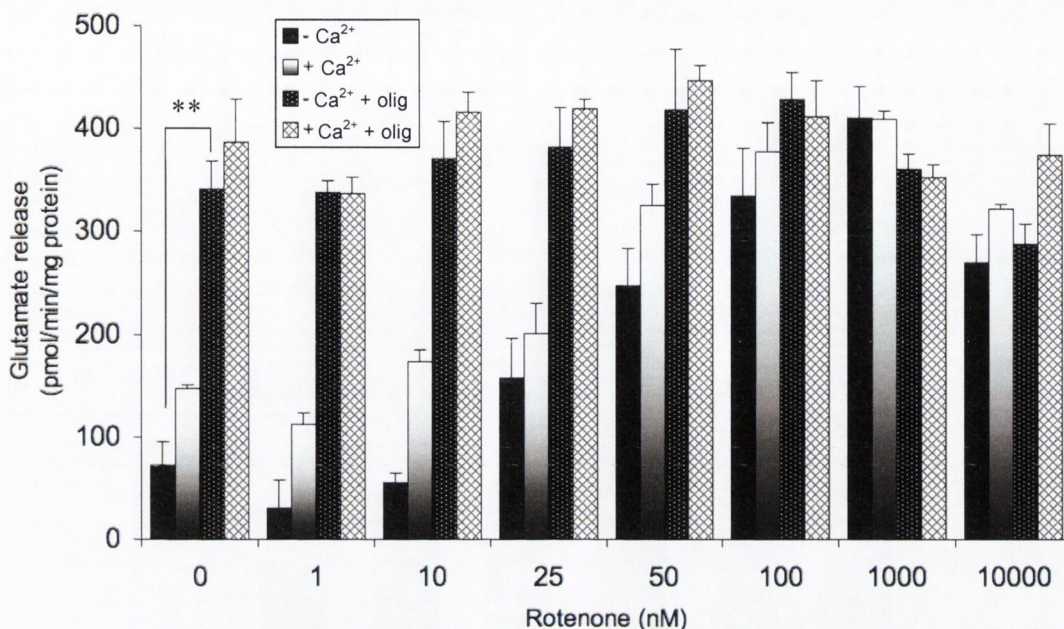


Fig. 3.10 Rotenone and oligomycin increase glutamate release from synaptosomes depolarised with KCl

Rotenone (1 nM – 10 μ M) and oligomycin (20 μ g/ml) were added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺) as indicated. Synaptosomes were incubated for 5 min before being depolarised with 40 mM KCl and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (** = $p < 0.01$).

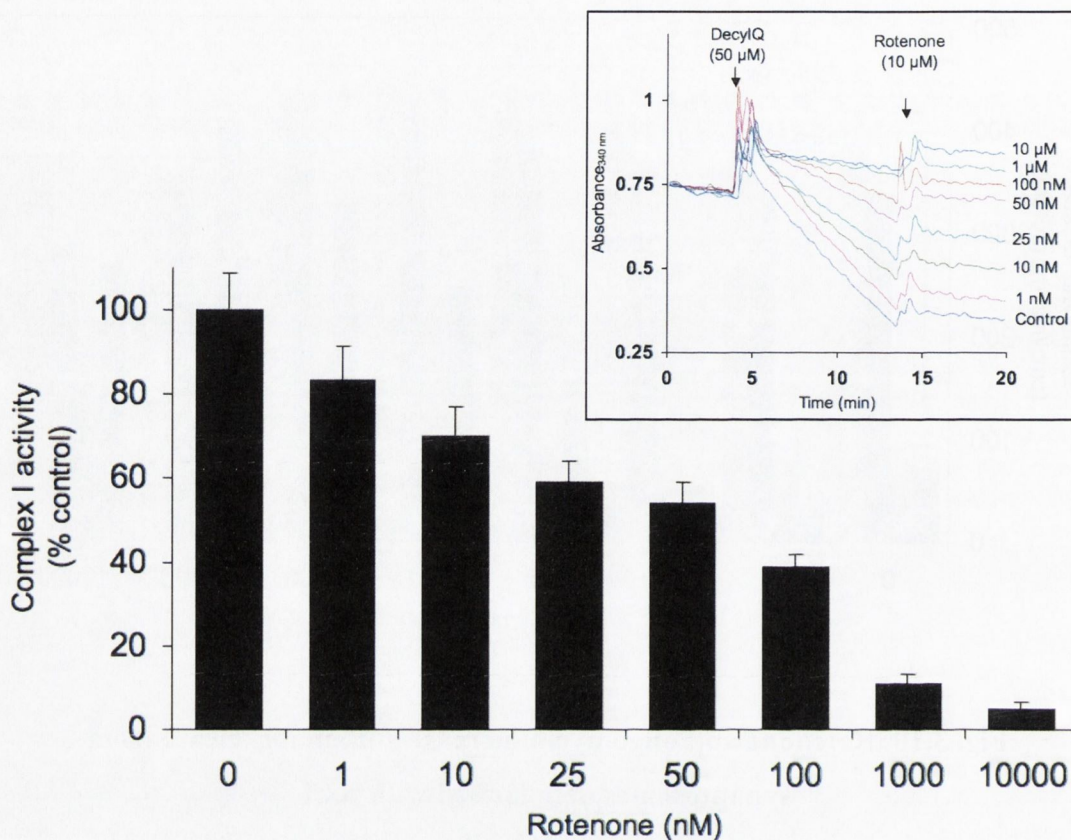


Fig. 3.11 Rotenone titration inhibits complex I activity in a concentration dependent manner

Rates were taken during the calculated at the linear section and the 'rotenone-insensitive rate' subtracted for each sample. The resulting data were expressed as a percentage of the control which contained no rotenone. Data shown are results from triplicate experiments on 4 separate synaptosomal preps. **Inset:** Typical absorbance traces of oxidation of NADH by samples that had been incubated with the concentrations of rotenone shown.

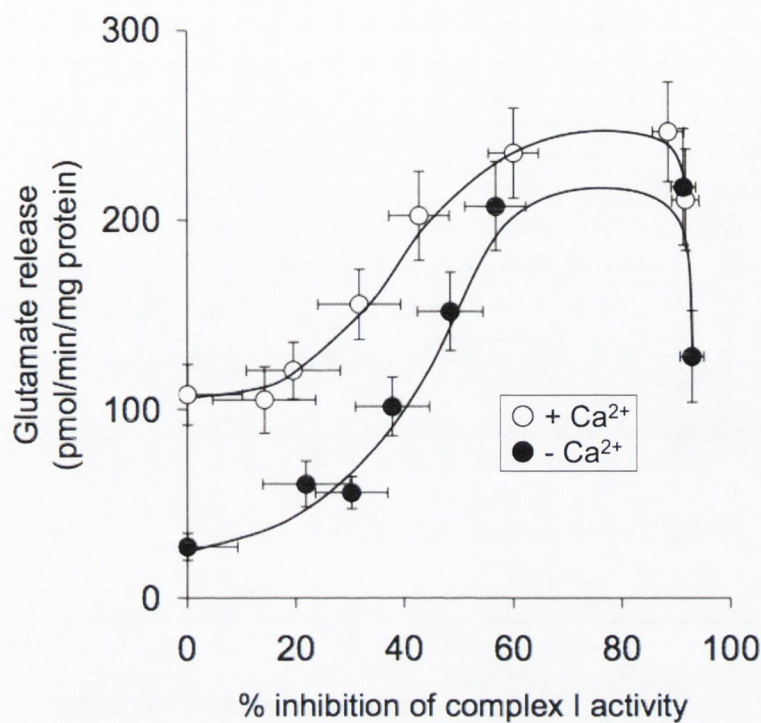


Fig. 3.12 Partial inhibition of complex I activity increases release of non-vesicular glutamate stores from synaptosomes depolarised with 4-aminopyridine

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarised with 1 mM 4-aminopyridine. Rates of glutamate release at each concentration of rotenone (1 nM – 10 μM) were plotted against percent inhibition of complex I activity brought about by that concentration of rotenone. Freehand curves were drawn through the results. Points represent mean ± SEM of experiments done in triplicate on at least 4 separate synaptosomal preparations.

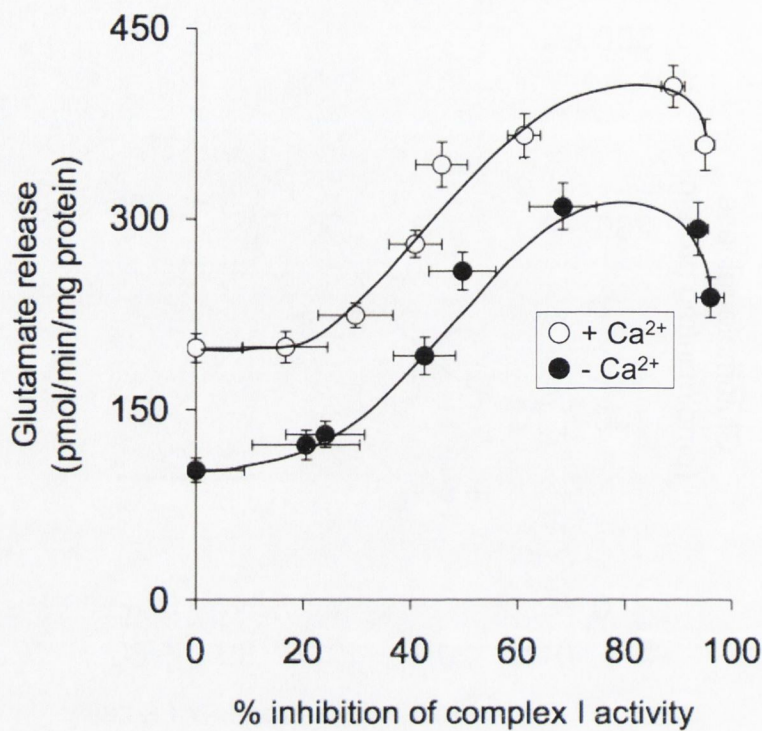


Fig. 3.13 Partial inhibition of complex I activity increases release of non-vesicular glutamate stores from synaptosomes depolarised with KCl

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarized with 40 mM KCl. Rates of glutamate release at each concentration of rotenone (1 nM – 10 μM) were plotted against percent inhibition of complex I activity brought about by that concentration of rotenone. Freehand curves were drawn through the results. Points represent mean ± SEM of experiments done in triplicate on at least 4 separate synaptosomal preparations.

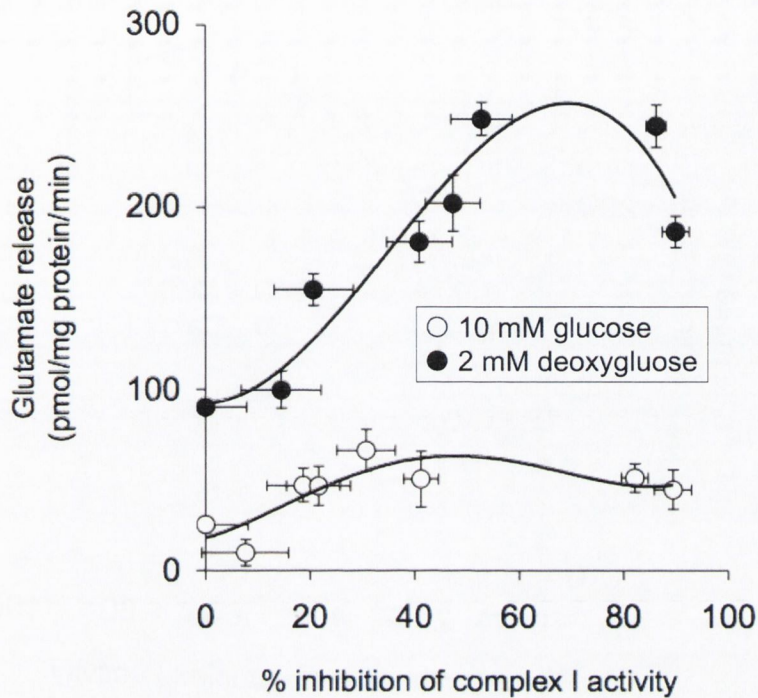


Fig. 3.14 Inhibition of complex I potentiates the increase in glutamate efflux from glycolytically inhibited synaptosomes.

Synaptosomes (0.5 mg/ml) were incubated with 10 mM glucose (○) or 2 mM deoxyglucose (●) and rotenone (1 nM – 10 μ M) and in the presence of 1.4 mM CaCl_2 . Rates of glutamate release at each concentration of rotenone (1 nM – 10 μ M) 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 $\pm$ SEM for experiments performed in triplicate on 3 separate synaptosomal preparations.

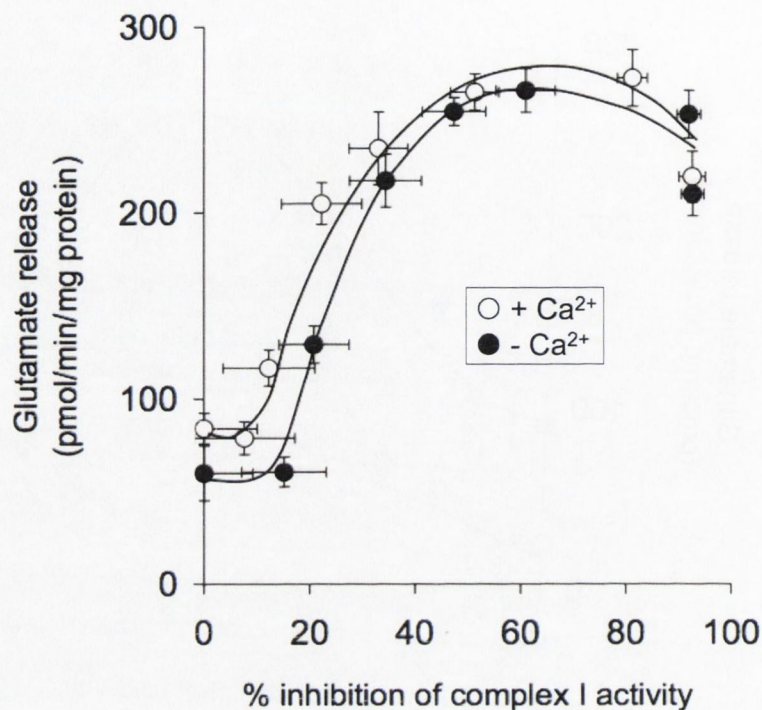


Fig. 3.15 Inhibition of complex I activity and glycolysis blocks the Ca²⁺-dependent component of glutamate release from synaptosomes depolarised with 4-aminopyridine

Synaptosomes (0.5 mg/ml) were preincubated for 5 min with 2 mM deoxyglucose in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarised with 1 mM 4-aminopyridine. Rates of glutamate release at each concentration of rotenone (1 nM – 10 μM) 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 performed in triplicate on at least 3 separate synaptosomal preparations.

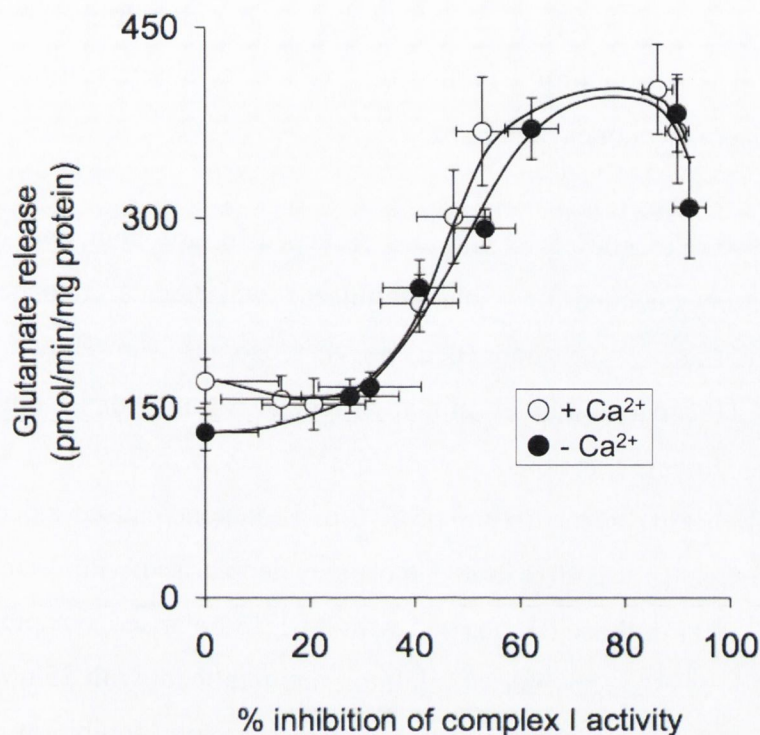


Fig. 3.16 Inhibition of complex I activity and glycolysis blocks the Ca^{2+} -dependent component of glutamate release from synaptosomes depolarised with KCl

Synaptosomes (0.5 mg/ml) were preincubated for 5 min with 2 mM deoxyglucose in the presence (○) or absence (●) of 1.4 mM CaCl_2 before being depolarised with 40 mM KCl. Rates of glutamate release at each concentration of rotenone (1 nM – 10 μM) 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 $\pm$ SEM for experiments performed in triplicate on at least 3 separate synaptosomal preparations.

3.3.7 Rotenone and deoxyglucose deplete ATP levels in synaptosomes

After 20 min incubation in the absence of Ca^{2+} , ATP levels were significantly reduced by 100 nM rotenone and above (Fig. 3.17). Depolarisation with KCl for 20 min significantly reduced ATP levels at 25 nM rotenone and above. In the presence of 1.4 mM CaCl_2 , depolarisation with KCl reduced [ATP] in the absence of inhibitor and at all concentrations of rotenone (Fig. 3.18).

The ATP levels were examined 15 min after resuspension to obtain data in which the timing more closely matched the glutamate release experiments. 2 concentrations of rotenone were chosen, 25 nM and 10 μM . Synaptosomes were shown to maintain 90% of initial ATP concentration 10 min after depolarisation with 4-aminopyridine or KCl (Fig. 3.18). However, incubation with 25 nM rotenone reduced the level to 60% with both depolarising agents ($p < 0.05$). 10 μM rotenone caused a decrease in the ATP content to 40% ($p < 0.001$) in synaptosomes depolarised with 4-aminopyridine, and 47% ($p < 0.001$) in those depolarised with KCl. In polarised 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 polarised synaptosomes and those depolarised 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.

3.3.8 Synaptosomal ATP levels as a function of inhibition of complex I activity

Incubation of synaptosomes for 20 min with 1 nM – 10 μM rotenone resulted in a decrease in the level of ATP by ~ 10 % at ~ 55% inhibition of complex I activity, and higher levels of inhibition reduced the level of ATP to 50% of control (Fig. 3.20). In the presence of 1.4 mM CaCl_2 , [ATP] was reduced at ~ 40% inhibition of complex I activity and above (Fig. 3.21), suggesting a lower threshold brought about by the presence of Ca^{2+} . At highest levels of inhibition of complex I activity (90 – 100%) ATP The level was reduced by ~ 40% in this group. (Fig. 3.21).

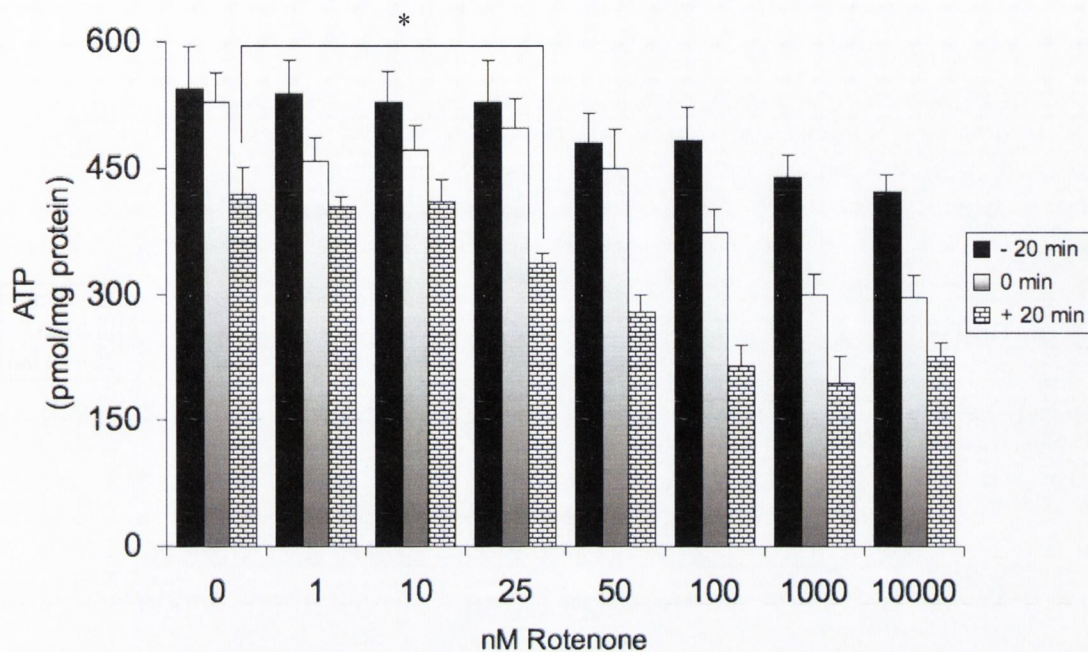


Fig. 3.17 Rotenone reduces synaptosomal ATP levels in synaptosomes depolarised with KCl in the absence of Ca^{2+}

Samples were PCA extracted immediately after resuspension (- 20 min), at the point of depolarisation (0 min) and 20 min after depolarisation (+ 20 min) with 40 mM KCl, neutralised and ATP measured using the luciferin/luciferase luminescence assay system. Results represent the mean + SEM of 3 experiments. . (* = $p < 0.05$).

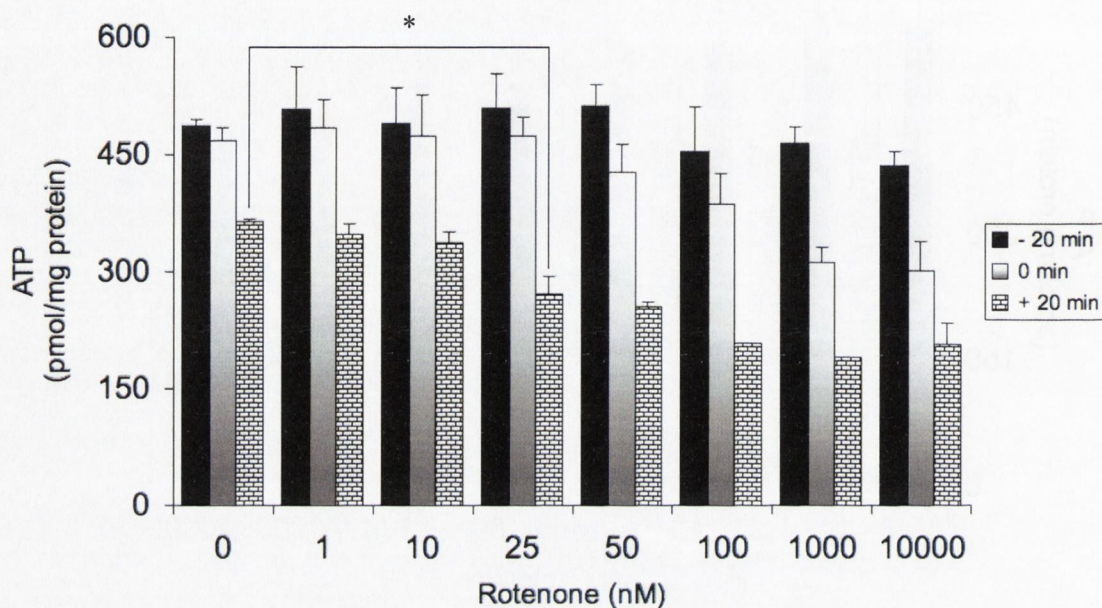


Fig. 3.18 Rotenone reduces synaptosomal ATP levels in synaptosomes depolarised with KCl in the presence of Ca^{2+}

Synaptosomes (0.5 mg/ml) were PCA extracted immediately after resuspension (- 20 min), at the point of depolarisation (0 min) and 20 min after depolarisation (+ 20 min) with 1 mM 4-aminopyridine, neutralised and ATP measured using the luciferin/luciferase luminescence assay system. Results represent the mean + SEM of 3 experiments. (* = $p < 0.05$).

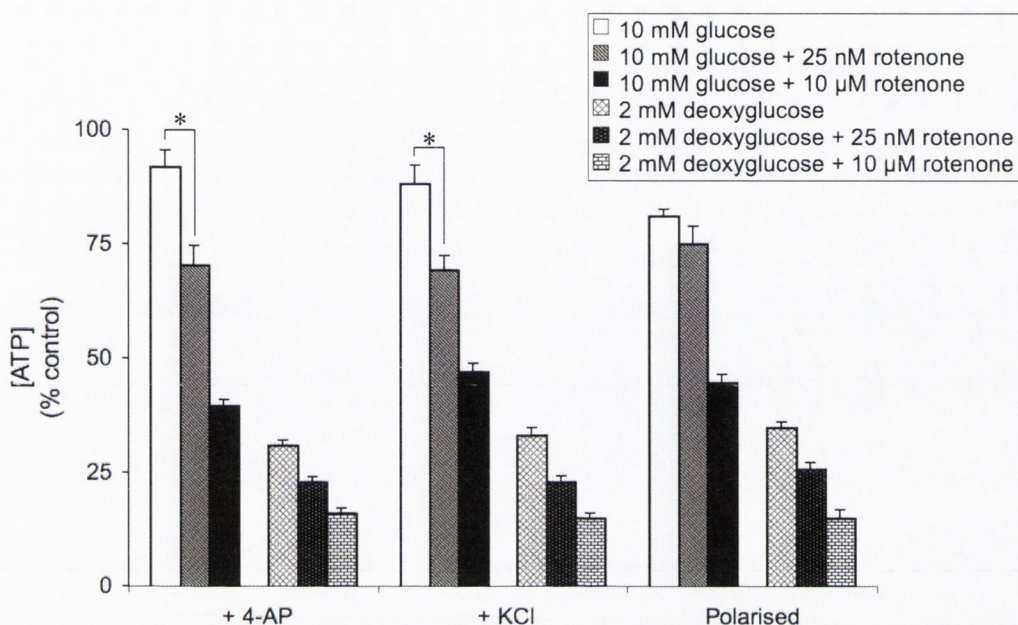


Fig. 3.19 Reduction in ATP levels in synaptosomes by rotenone is potentiated by inhibition of glycolysis

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 depolarised 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 $\pm$ SEM for experiments performed on 3 separate synaptosomal preparations. (* = $p < 0.05$).

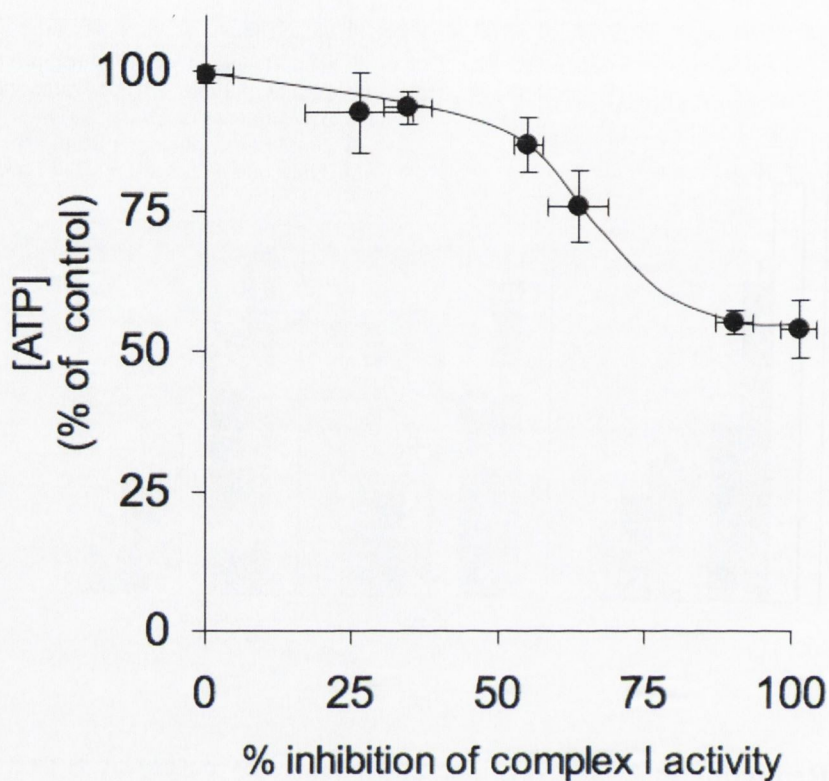


Fig. 3.20 Inhibition of complex I activity with rotenone decreases ATP levels in synaptosomes

Synaptosomes were incubated in Kreb's buffer for 20 min with 1 nM – 10 μ M rotenone. ATP was PCA extracted and samples were frozen at -80°C . Synaptosomes incubated with the same concentrations of rotenone in an experiment done in parallel and assayed for complex I activity. All ATP levels were read within a week of preparation, and results represent the mean $\pm$ SEM of 3 experiments. All complex I activities were read within 2 weeks of preparation, and results represent the mean $\pm$ SEM of 3 separate synaptosomal preparations.

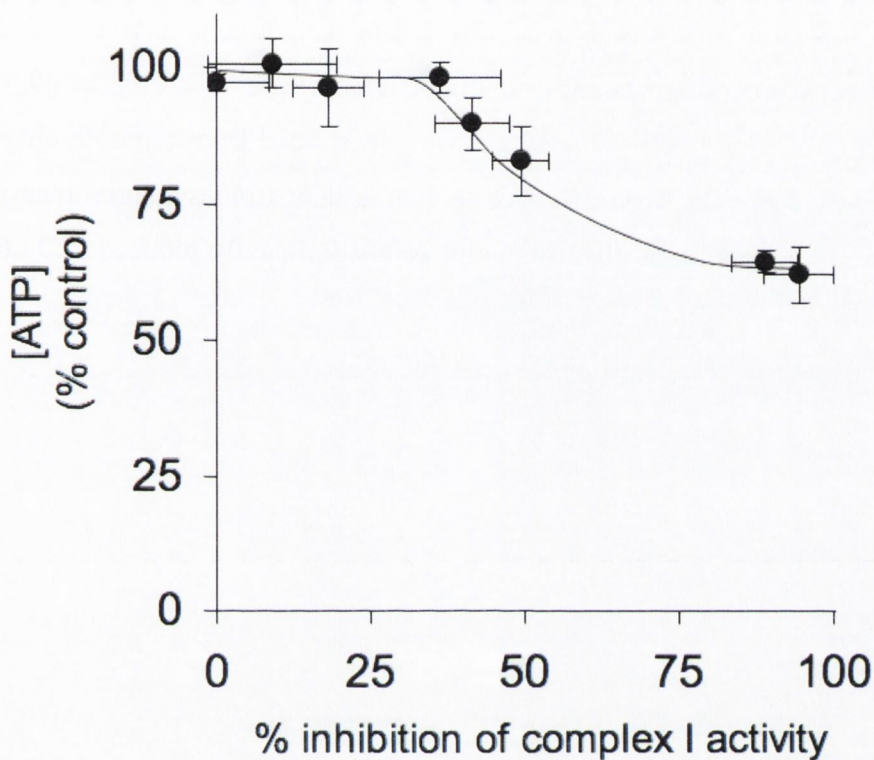


Fig. 3.21 Inhibition of complex I activity with rotenone decreases ATP levels in synaptosomes in the presence of Ca^{2+}

Synaptosomes were incubated in Kreb's buffer for 20 min with 1 nM – 10 μM rotenone and 1.4 mM CaCl_2 . ATP was PCA extracted and samples were frozen at -80°C . Synaptosomes incubated with the same concentrations of rotenone in an experiment done in parallel and assayed for complex I activity. All ATP levels were read within a week of preparation, and results represent the mean $\pm$ SEM of 3 experiments. All complex I activities were read within 2 weeks of preparation, and results represent the mean $\pm$ SEM of 3 separate synaptosomal preparations.

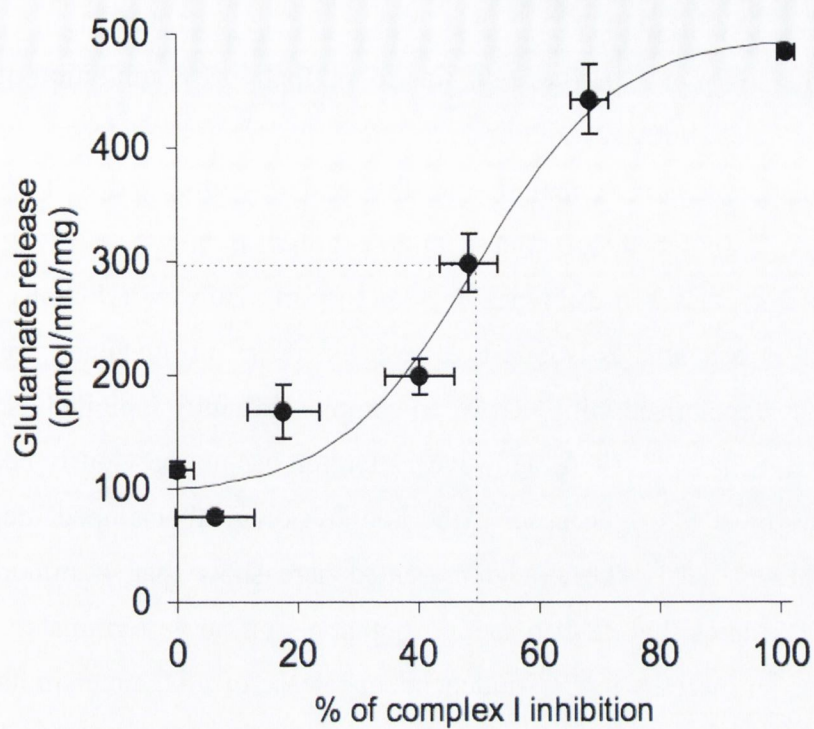
3.3.9 Age-related changes in control of complex I control of Ca^{2+} -independent glutamate release from depolarised synaptosomes

Partial inhibition of complex I activity using rotenone (1 nM – 1 μM) increased Ca^{2+} -independent glutamate release from synaptosomes isolated from 6-month old (Fig. 3.22A) and 18-month old rats (Fig. 3.22B). Fitting a sigmoidal curve revealed that the mid-point between minimum and maximum release rates occurred at $49.6 \pm 7.0\%$ inhibition of complex I activity in synaptosomes isolated from 6-month old rats (Fig. 3.22A). Using the same type of curve to fit the data from synaptosomes from 18-month old rats it was found that this point was significantly reduced to $37.6 \pm 1.4\%$ (Fig. 3.22B).

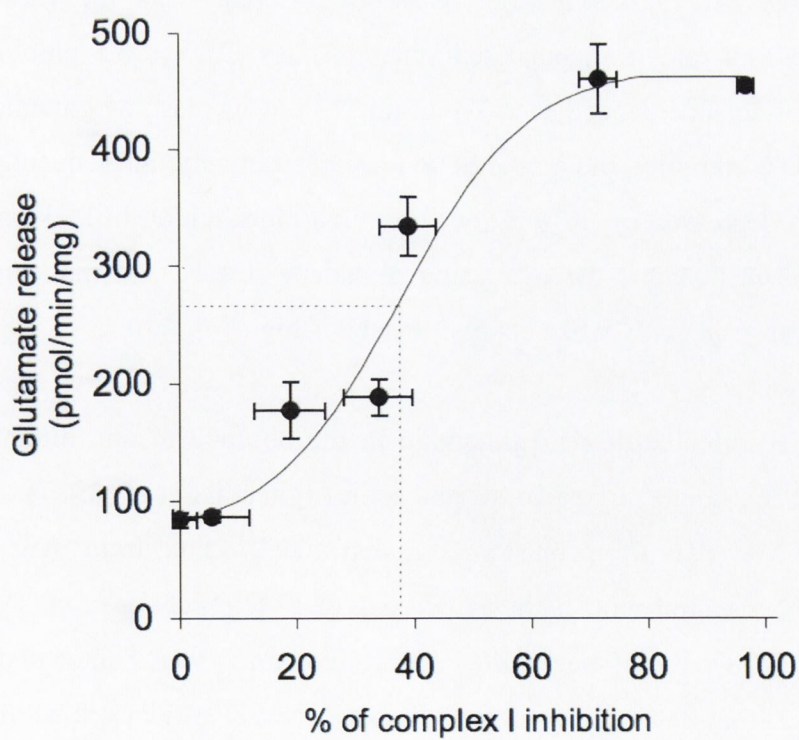
Fig. 3.22 Age-related differences in complex I activity control of Ca^{2+} -independent glutamate release from depolarised synaptosomes.

Synaptosomes (0.5 mg/ml) were preincubated with rotenone (1 nM – 1 μ M) at 37°C for 5 min before being depolarised with 1 mM 4-aminopyridine. Rates of glutamate release at each concentration of rotenone (1 nM – 10 μ M) 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 maximal release rate calculated using a statistical fit. The 50% of maximal release rate was found to correspond to $49.6 \pm 7.0\%$ in synaptosomes from 6-month old rats (**A**) and $37.6 \pm 1.4\%$ in synaptosomes from 18-month old rats (**B**). Points shown represent the mean $\pm$ SEM of experiments performed in triplicate on 4 separate synaptosomal preparations.

A



B



3.4 Discussion

In this group of experiments it was found that ~ 40% inhibition of complex I activity with 25 nM rotenone significantly increased the Ca^{2+} -independent glutamate release from synaptosomes depolarised with 4-aminopyridine and KCl (Figs 3.3, 3.4, 3.12, 3.13). Ca^{2+} -independent release after depolarisation 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 depolarised with KCl when complex I is fully inhibited (Kauppinen *et al.* 1988; Erecinska *et al.* 1996). However, attention has not previously been drawn to this increase, most likely because of the nonphysiological ‘clamped’ depolarisation brought about by KCl. The results presented here show that 4-aminopyridine, an agent which mimics the effect of action potentials on nerve terminals (Tibbs *et al.* 1989) causes an increase in Ca^{2+} -independent release of glutamate similar in pattern to the KCl-induced increase when complex I activity is partially inhibited (Figs 3.12, 3.13). In synaptosomes depolarised 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, Fig. 3.3). This rate was not different from the Ca^{2+} -independent glutamate efflux rate from polarised synaptosomes (25.5 ± 6.5 pmol/min/mg, Fig. 3.5). This effect is extended to a 3-fold and 8-fold increase of glutamate release at 40% and 60% inhibition of complex I activity, respectively. Subsequently, 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 depolarising 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 a reduced ATP level impedes Na^+/K^+ ATPase function resulting in a collapse of plasma membrane potential. Glutamate uptake is thought to occur with cotransport of 3 Na^+ in and one K^+ out and thus relies on the maintenance of the ionic gradients across the membrane (Nicholls 1993). The results from resting

synaptosomes (i.e. not depolarised with either 4-aminopyridine or KCl, Figs 3.5, 3.14) suggest that the increase in glutamate release via reversal of uptake may result from a drop below a threshold ATP level (Fig. 3.19). The 25% reduction in ATP level 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 μ M rotenone, which causes 97% inhibition of complex I activity) did not result in glutamate release being significantly increased from polarised synaptosomes. 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 μ M 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 was inhibited with deoxyglucose, ATP was reduced by 65% in the absence of rotenone (Fig. 3.18) and glutamate release was increased more than 3-fold (Fig. 3.5, 0 nM rotenone). Combining inhibition of glycolysis with inhibition of complex I activity resulted in an even greater increase in glutamate release. 25 nM rotenone (41% inhibition of complex I activity) with deoxyglucose doubles the rate brought about by deoxyglucose alone (Figs 3.5, 3.14). ATP is reduced by 75% after 15 min under these conditions (Fig. 3.19). The highest rates of glutamate release in the absence of 4-aminopyridine or KCl occurred when deoxyglucose and 100 nM or 1 μ M rotenone are added, and 10 μ M 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 increases dramatically. Indeed, the highest rate of release in the presence of deoxyglucose after depolarisation with 4-aminopyridine was found in the presence of 100 nM rotenone (Fig. 3.6) and this rate was not significantly different from that induced by deoxyglucose and 100 nM rotenone (Fig. 3.5), suggesting that glutamate release is minimally influenced by 4-aminopyridine at this concentration of rotenone. Also, 25 nM rotenone is the decrease in ATP level brought about by 70 – 75% in both polarised and depolarised groups (Fig. 3.19), yet the glutamate release rates are increased by this concentration of rotenone in depolarised synaptosomes (Figs 3.3, 3.4) but not in polarised (Fig. 3.5).

Rotenone has been shown to increase synaptosomal reactive oxygen species (ROS) production in a concentration-dependent manner (Sipos *et al.* 2003). High levels of H_2O_2 production have been implicated in stimulating release of glutamate from synaptosomes (Gilman *et al.* 1992; Tretter and Adam-Vizi 2002). Addition of superoxide dismutase, peroxidase or catalase did not prevent the rotenone-induced increase glutamate release from depolarised synaptosomes (Figs 3.8, 3.9). Other reports suggest Ca^{2+} -dependent release of glutamate is reduced by ROS (Zoccarato *et al.* 1995; Zoccarato and Alexandre 1996). Peroxidase and catalase increased glutamate release from synaptosomes depolarised by KCl in the presence of CaCl_2 (Fig. 3.8). However, only catalase potentiated glutamate release rates after 30 min preincubation (Fig. 3.9). However, these increases were evident in the absence of rotenone, and are therefore unlikely to be due to the prevention of the effects rotenone-induced ROS production within the synaptosomes.

In each of the glutamate release experiments peak levels of glutamate release occurred at concentrations of $< 10 \mu\text{M}$ rotenone. Nonetheless, $10 \mu\text{M}$ rotenone was the concentration that consistently brought about the highest levels of inhibition of complex I activity. 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 hyperpolarisation 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 hyperpolarisation effect which lasted over 20 min after application of $10 \mu\text{M}$ rotenone, but show shorter hyperpolarisation phases at $3 \mu\text{M}$ and $1 \mu\text{M}$ rotenone (Bonsi *et al.* 2004). The duration of hyperpolarisation 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.

Ca^{2+} -dependent release of glutamate from synaptosomes depolarised with KCl was not significantly affected by titration of complex I activity (Fig. 3.13). Inhibition of complex I activity with 10 μM rotenone has previously been shown to reduce KCl-induced Ca^{2+} -dependent release from guinea-pig synaptosomes a time-dependent manner, with a Ca^{2+} -dependent rate still evident 15 min after incubation with the inhibitor (Kauppinen *et al.* 1988). In the present study synaptosomes were incubated with rotenone for 5 min prior to depolarisation and no effect on Ca^{2+} -dependent glutamate release was observed following KCl-induced depolarisation. Ca^{2+} -dependent release was decreased at higher levels of complex I activity from synaptosomes depolarised with 4-aminopyridine (Fig. 3.12) which may be due to the level of reduction of ATP in the presence of 4-aminopyridine. Fig. 3.19 shows that with 10 μM rotenone ATP was reduced to 40% following 10 min depolarisation with 4-aminopyridine compared to 47% with KCl. The extra strain of 4-aminopyridine on ATP levels may partially inhibit Ca^{2+} -dependent exocytotic release, with a threshold of normal Ca^{2+} -dependent release of below 47%, a figure similar to ATP threshold level for glutamate exocytosis that has been observed previously (Sanchez-Prieto *et al.* 1987). Indeed, further reduction of ATP by deoxyglucose in combination with rotenone completely abolishes the Ca^{2+} -dependent component of glutamate release (Figs 3.15, 3.16), in accordance with a result previously demonstrated using iodoacetate as an inhibitor of glycolysis in combination with excess rotenone (Kauppinen *et al.* 1988).

ATP is required for accumulation of glutamate into synaptic vesicles, and uptake into vesicles is inhibited at low concentrations of ATP (Naito and Ueda 1985). Vesicular ATPases pump protons into vesicles generating an electrochemical gradient, which drives glutamate uptake (Cidon and Sihra 1989). Glutamate is concentrated in vesicles at 100 mM while in the cytoplasm it is present at 1 – 10 mM (Nicholls 1993). Therefore, at low concentrations of ATP it would be expected that the vATPase-driven H^+ potential would fail, resulting in leakage of vesicular glutamate into the cytoplasmic pool. Comparison of the results of experiments done in the presence of deoxyglucose with those done with glucose supports this hypothesis, as the reduction in Ca^{2+} -dependent release in the presence of deoxyglucose correlates with increased Ca^{2+} -independent release at each concentration of rotenone.

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; Davey *et al.* 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). Fig. 3.20 shows that in *in situ* synaptosomal mitochondria, ~ 60% inhibition of complex I activity must occur before ATP production is significantly lowered. When Ca^{2+} is present, the threshold is reduced to 35 – 40% (Fig. 3.21). The higher thresholds of *in situ* synaptosomal mitochondria compared to isolated synaptosomal mitochondria are likely to occur as a result of ATP production through the glycolytic pathway, which has been shown to accelerate 8-fold in the presence of 1 μM rotenone in synaptosomes (Kauppinen and Nicholls 1986b). Inhibition of glycolysis combined with partial inhibition complex I by 25 nM rotenone reduces ATP to < 40% (Fig. 3.19).

The results in this chapter demonstrate that the low threshold of synaptosomal complex I on ATP production has consequences on glutamate release from isolated nerve terminals. The implications of this result for acute neurodegeneration via excitotoxic cell death occurs are most obvious. During ischemia, oxygen and glucose supply to the affected area is interrupted. Fig. 3.14 models the effect of inhibition of glycolysis on glutamate release from resting nerve terminals, and shows that glutamate release from isolated nerve terminals is increased approximately 3-fold under these conditions. Complex I activity has been shown to be reduced during ischemia by 25% in brain mitochondria (Almeida *et al.* 1995) and 40 – 50% in synaptosomes (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 of glutamate efflux brought about by inhibition of glycolysis alone (Fig. 3.14). Due to the lack of availability of ATP to drive Na^+/K^+ pump, neurons are more likely to depolarise after stimulation during ischemia (Hossmann 2006). Using 4-aminopyridine as a depolarising 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. 3.12).

Increased glutamate release from the nerve terminal caused by reduced complex I activity may also have ramifications for the pathogenesis of chronic neurodegenerative disorders. As detailed in Sections 1.6 and 1.7, 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 aetiology is not fully understood, however reduced complex I activity is thought to play central role in the pathogenesis of sporadic Parkinson's disease (Greenamyre *et al.* 2001; Dawson and Dawson 2003).

Glutamatergic projections from the subthalamic nucleus to the substantia nigra are thought to become overactive during Parkinson's disease due to the loss of inhibitory input of dopamine (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 result Fig. 3.13 is 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 combined with repetitive firing extrapolates to a substantial increase in release of cytoplasmic glutamate, which could contribute to excitotoxicity. In addition, the results in Fig. 3.22 suggest that a lower threshold of inhibition of complex I activity exists in the nerve terminals of older rats compared to younger rats, which 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 and other brain regions during Parkinson's disease may lead to higher rates of glutamate release with age.

Chapter 4

Complexes III, IV and V and control of glutamate release from synaptosomes

4.1 Introduction

A mechanism of slow excitotoxicity has been suggested to occur in chronic neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Beal 1992; Mattson 2003). Dysfunction of enzymes of the mitochondrial ETC have been implicated in the pathogenesis of these chronic neurodegenerative (Schapira and DiMauro 2002; Beal 2005; Lin and Beal 2006). Complex IV activity in the post mortem brain samples from Alzheimer's disease patients has frequently shown to be reduced by up to 50% (Kish *et al.* 1992; Mutisya *et al.* 1994) and reductions in the activities of the other complexes, including complex III, have also been found (Parker *et al.* 1994). Complex IV activity has also been shown to decrease with age in rats and mice (Benzi *et al.* 1992; Lenaz *et al.* 1997; Kwong and Sohal 2000; Navarro *et al.* 2002). Reductions in complex II/III activity have been shown to be specific to the brain areas affected by the pathogenesis of Huntington's disease (Mann *et al.* 1990; Browne *et al.* 1997; Tabrizi *et al.* 1999).

Previous studies have used the synaptosomal model to examine 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 such studies have indicated that inhibition of Ca^{2+} -dependent glutamate release and an increase in Ca^{2+} -independent glutamate efflux from polarised synaptosomes occurs due to reduced ATP levels after total inhibition of complex IV activity with KCN (Sanchez-Prieto and Gonzalez 1988). Inhibition of complex V activity with oligomycin combined with collapse of $\Delta\psi_m$ using FCCP was shown to block Ca^{2+} -dependent glutamate release (Sanchez-Prieto *et al.* 1987). However, the effects of *partial* inhibition of oxidative phosphorylation on glutamate release have not previously been reported in the literature, and increases in Ca^{2+} -independent glutamate release rates after depolarisation have been largely overlooked. The effects of inhibition of complex III activity on glutamate release from synaptosomes not previously been reported.

Complexes III and IV have been shown to have high thresholds of inhibition before major changes in oxygen consumption and ATP production are affected in isolated

brain mitochondria (Rossignol *et al.* 1999). Comparison of such data obtained from experiments performed on isolated nerve terminal mitochondria (Davey *et al.* 1997) with nonsynaptic mitochondria (Davey and Clark 1996; Davey *et al.* 1998) indicate the threshold levels are higher in synaptosomal mitochondria for both of these enzymes respectively. This suggests that complexes III and IV have low levels of control over isolated synaptosomal mitochondria. To examine the control of complexes III and IV over glutamate release from nerve terminals, experiments using ranges of concentrations of the complex III inhibitors myxothiazol and antimycin A, which inhibit complex III activity upstream and downstream of the Q-cycle respectively (von Jagow *et al.* 1984), and a range of concentrations of the complex IV inhibitor KCN on glutamate release rates were performed. Such data may be relevant for the role of excitotoxicity in the pathogenesis of age-related neurodegenerative disorders.

4.2 Methods

4.2.1 Monitoring glutamate release

Glutamate release was measured in the well-plate fluorimeter as described in Section 3.2.1, except using myxothiazol (10 nM – 10 μ M), antimycin A (1 nM – 1 μ M) or KCN (1 μ M – 1 mM) instead of rotenone as the ETC enzyme inhibitors. 40 mM KCl or 1 mM 4-aminopyridine (in 8 μ l H₂O) were used to depolarise synaptosomes after 5 min and rates were taken over a 20 min time period after depolarisation using the kinetic mode (cycle time of 32 s) in SoftMax Pro software. Calibration and enzyme lag (Nicholls *et al.* 1987) were accounted for when converting rates to pmol/min/mg (detailed in Section 2.10). In a parallel series of experiments, synaptosomes were incubated with identical inhibitor concentrations and following 15 min incubation, samples were frozen at -80°C and stored. Complex III activity and complex IV activity were later assayed and the rate of glutamate release plotted against the % inhibition of ETC enzyme activity.

4.2.2 Complex III activity assay

Complex III activity was measured using a SpectraMAX 340PC well-plate spectrophotometer. The titration of complex III activity with myxothiazol (10 nM – 10 μ M) or antimycin A (1 nM – 1 μ M) was performed identically to the glutamate release experiments – in well plates with final concentration of 0.5 mg/ml synaptosomes in a final volume of 200 μ l. Samples were frozen at -80°C and subsequently underwent 3 rapid freeze-thaw cycles. Complex III activity was determined by following the reduction of cytochrome *c* at absorbance $\lambda = 550$ nm. Wells were prepared by addition of medium containing 100 mM potassium phosphate, 0.3 mM potassium-EDTA, (pH 7.4), 1 mM KCN and 100 μ M cytochrome *c* with a final volume of 200 μ l. The reaction was initiated by the addition of sample (10 μ g) and results were expressed as first order decay rate constants (k).

4.2.3 Complex IV activity assay

Complex IV activity was measured using an Agilent ChemStation 8453 spectrophotometer. The titration of complex IV activity with KCN was performed identically to the glutamate release experiments – in well plates with final concentration of 0.5 mg/ml synaptosomes in a final volume of 200 μ l. After freezing at -80°C and undergoing 3 rapid freeze-thaw cycles, the activity of complex IV was determined using the method of Wharton and Tzagoloff (1967): The oxidation of cytochrome *c* at absorbance $\lambda = 550$ nm was followed. Reduced cytochrome *c* was prepared by the addition 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 PD₁₀ 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 μ M reduced cytochrome *c* and 100 μ l buffer with a final volume of 1 ml made up with H₂O. The reaction was initiated by the addition of 50 μ g synaptosomal samples. The results were expressed as first order decay rate constants (k).

4.3 Results

4.3.1 Myxothiazol increases the rate of Ca^{2+} -independent glutamate release from depolarised synaptosomes

The rate of glutamate release from synaptosomes depolarised with 4-aminopyridine in the absence of calcium was increased from 30.7 ± 11.7 pmol/min/mg protein to 118.5 ± 26.2 pmol/min/mg protein by 75 nM myxothiazol and 230.0 ± 14.3 pmol/min/mg by 100 nM myxothiazol ($p < 0.05$, Fig. 4.1). The maximum rate of 242.1 ± 22.5 pmol/min/mg protein in the absence of calcium was achieved by 1 μM myxothiazol, and corresponded to release of 23% of the total glutamate pool over 20 min. When depolarised with KCl, the Ca^{2+} -independent rate of release was increased from 117.7 ± 21.5 pmol/min/mg to 228.1 ± 22.1 pmol/min/mg by 75 nM, and was found to occur at a maximum of 402.9 ± 35.0 pmol/min/mg in the presence of 100 nM myxothiazol (39% of the total pool, Fig. 4.2). The Ca^{2+} -independent rate of release was not affected by myxothiazol at concentrations of less than 75 nM in synaptosomes depolarised with either 4-aminopyridine or KCl (Figs 4.1, 4.2).

In the presence of 1.4 mM CaCl_2 , synaptosomes released 112.0 ± 10.0 pmol glutamate/min/mg protein when depolarised with 4-aminopyridine, suggesting a Ca^{2+} -dependent release rate of ~ 81 pmol/min/mg (Fig. 4.1). The difference in release in the presence of CaCl_2 was evident at all concentrations of myxothiazol, although was reduced at higher concentrations to a minimum release rate of ~ 37 pmol/min/mg in the presence of 100 nM (total release in the presence of 1.4 mM CaCl_2 was 267.2 ± 21.7 pmol/min/mg under these conditions). This concentration of myxothiazol was shown to block Ca^{2+} -dependent glutamate release from synaptosomes depolarised with KCl, which was found to occur at ~ 102 pmol/min/mg in the absence of inhibitor (Fig. 4.2, 0 nM myxothiazol, + Ca, total rate of 219.8 ± 30.6 pmol/min/mg). However, this full Ca^{2+} -dependent glutamate release rate was found to be unaffected by the presence of 1 and 10 μM myxothiazol after depolarisation with KCl.

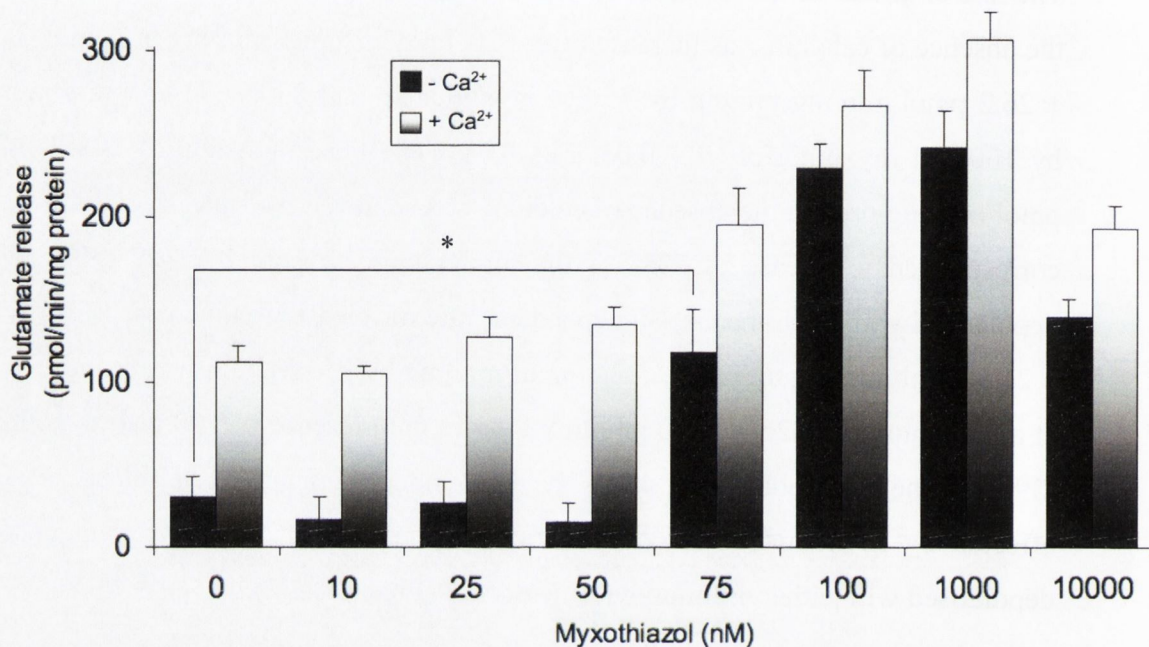


Fig. 4.1 Myxothiazol increases Ca²⁺-independent glutamate release from synaptosomes depolarised with 4-aminopyridine

Myxothiazol (10 nM – 10 μ M) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 1 mM 4-aminopyridine and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (* = $p < 0.05$).

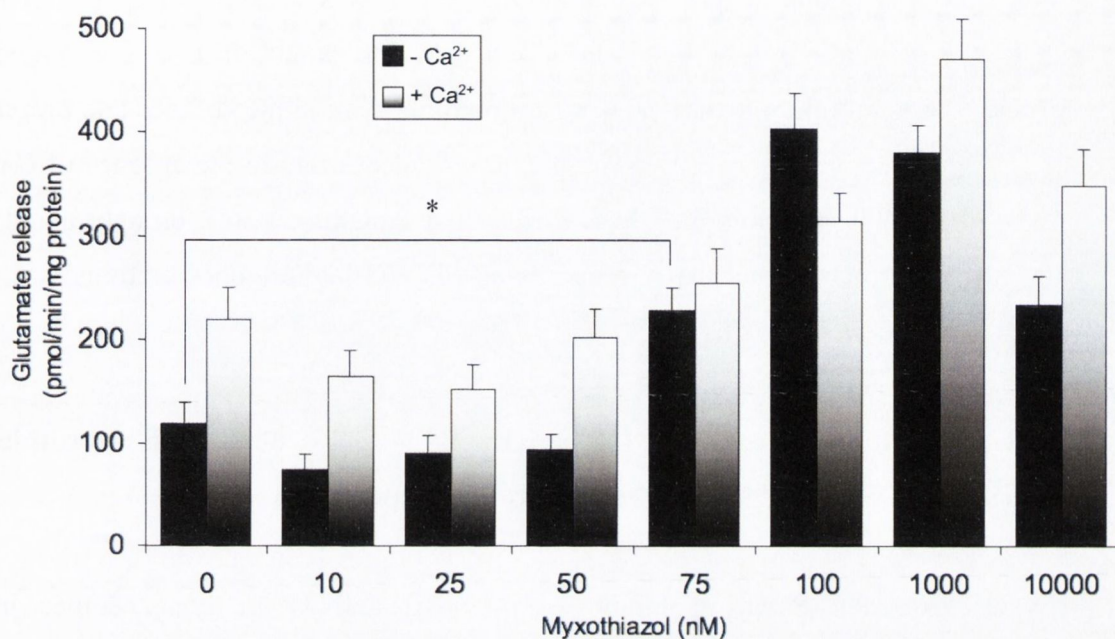


Fig. 4.2 Myxothiazol increases Ca²⁺-independent glutamate release from synaptosomes depolarised with KCl

Myxothiazol (10 nM – 10 μ M) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 40 mM KCl and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (* = $p < 0.05$).

4.3.2 Myxothiazol increases the rate of glutamate efflux from polarised synaptosomes

Glutamate efflux was found to occur at a rate of 12.6 ± 10.7 pmol/min/mg protein (Fig. 4.3). The rate in the presence of 1.4 mM CaCl_2 was not significantly different at 25.0 ± 9.0 pmol/min/mg. In the presence of 75 nM, the rate of efflux was increased to 91.8 ± 10.4 pmol/min/mg and 137.0 ± 12.0 pmol/min/mg in the absence and presence of CaCl_2 respectively ($p < 0.05$). The maximum release rate in the absence of CaCl_2 was 117.3 ± 8.9 pmol/min/mg (11% of the total glutamate pool), brought about by 100 nM myxothiazol, and the release rate at this and higher concentrations was not affected by the presence of

4.3.3 Inhibition of complex III activity by > 50 – 60% with myxothiazol increases the rate of glutamate release from synaptosomes

Myxothiazol was found to inhibit complex III activity in synaptosomes in a concentration dependent manner (Fig. 4.4). The increase in glutamate release brought about by 75 nM myxothiazol after depolarisation with 4-aminopyridine corresponded to $62.2 \pm 4.1\%$ inhibition of complex III activity (Fig. 4.5). The maximum Ca^{2+} -independent release rate was found to occur $72.0 \pm 2.4\%$ inhibition of complex III activity. In the presence of 1.4 mM CaCl_2 , the maximum release was brought about by $70.9 \pm 3.3\%$ inhibition of complex III activity. Inhibition by $99.9 \pm 3.71\%$ caused a release rate of 139.4 ± 11.0 pmol/min/mg protein in the absence of calcium, equivalent to $\sim 58\%$ of the maximum rate. When depolarised with KCl, the Ca^{2+} -independent release rate was not increased until $80.0 \pm 1.5\%$ inhibition of complex III activity, brought about by 75 nM myxothiazol (Fig. 4.6). Inhibition by $88.0 \pm 1.4\%$ caused the maximum rate of Ca^{2+} -independent release. Ca^{2+} -dependent release was blocked at this level of inhibition.

Glutamate efflux from polarised synaptosomes was increased by $60.2 \pm 3.0\%$ inhibition of complex III activity with myxothiazol (Fig. 4.6). The maximum efflux rate occurred at $71.6 \pm 2.7\%$ inhibition of complex III activity. Efflux rate was not significantly changed by higher levels of inhibition.

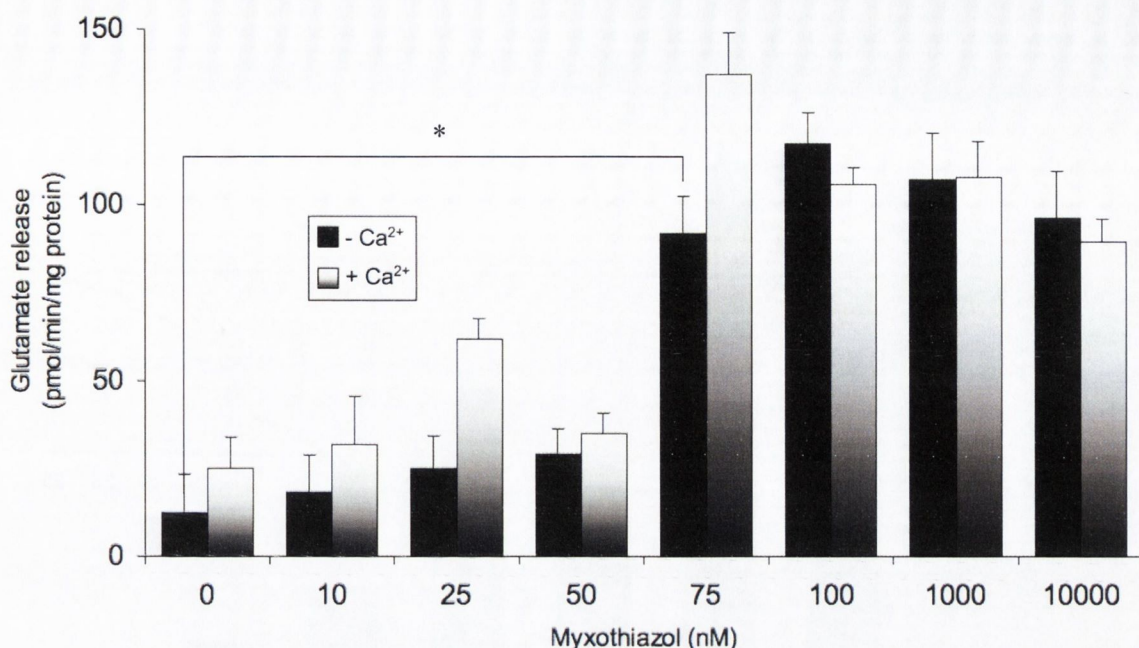


Fig. 4.3 Myxothiazol increases Ca²⁺-independent glutamate release from polarised synaptosomes

Myxothiazol (10 nM – 10 μ M) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for preincubated 5 min and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (* = $p < 0.05$).

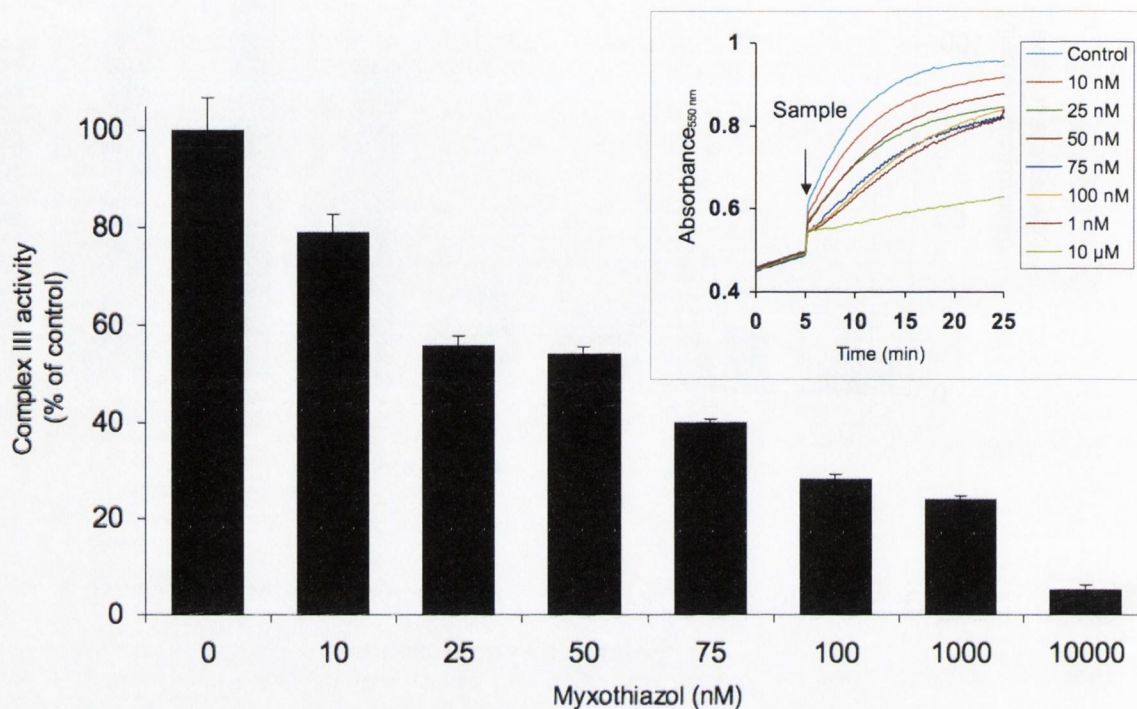


Fig. 4.4 Myxothiazol inhibits complex III activity in synaptosomes in a concentration dependent manner

Synaptosomes (0.5 mg/ml) were incubated for 15 min with myxothiazol (10 nM – 10 μ M) before being stored at -80°C and assayed for complex III activity. Reduction of cytochrome *c* was followed spectrophotometrically at absorbance $\lambda = 550$ nm as described in detail in Chapter 2, and expressed as a percentage of the control which contained no myxothiazol. Data shown are results from triplicate experiments on 3 separate synaptosomal preparations. **Inset:** Typical traces of reduction of cytochrome *c* by samples that had been incubated with the concentrations of myxothiazol shown.

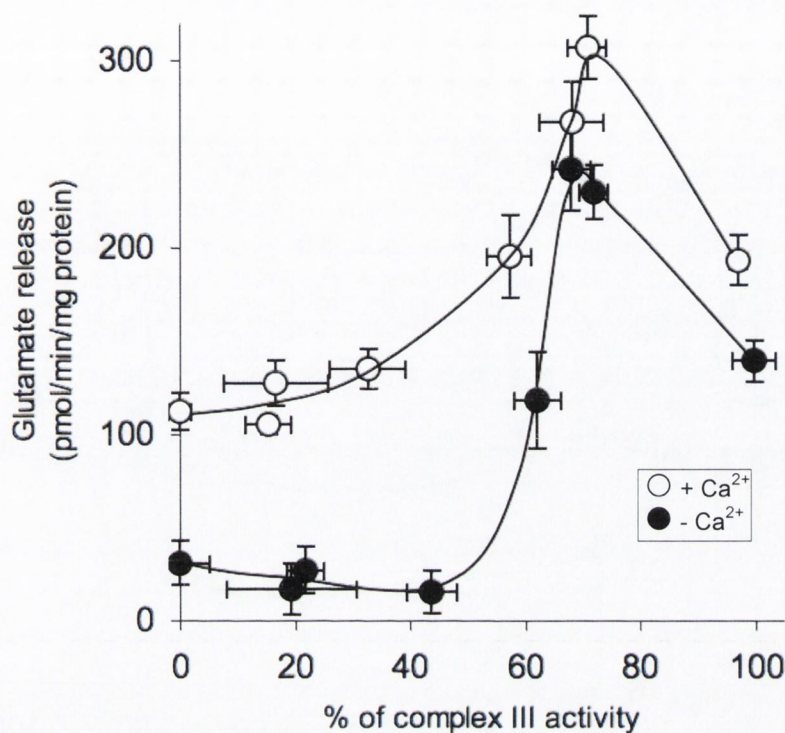


Fig. 4.5 Inhibition of complex III activity by > 50 – 60% with myxothiazol increases release of glutamate from synaptosomes depolarised with 4-aminopyridine

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarised with 1 mM 4-aminopyridine. Rates of glutamate release at each concentration of myxothiazol (10 nM – 10 μM) were plotted against percent inhibition of complex III activity brought about by that concentration of myxothiazol. Freehand curves were drawn through the results. Points represent mean ± SEM of experiments done in triplicate on 3 separate synaptosomal preparations.

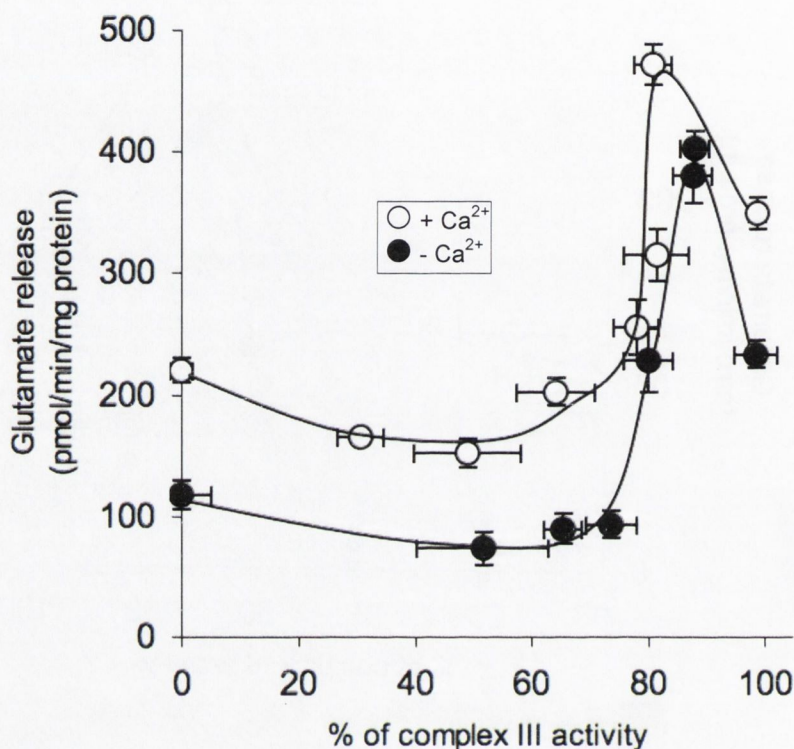


Fig. 4.6 Inhibition of complex III activity by > 80% with myxothiazol increases release of glutamate from synaptosomes depolarised with KCl

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarised with 40 mM KCl. Rates of glutamate release at each concentration of myxothiazol (10 nM – 10 μM) were plotted against percent inhibition of complex III activity brought about by that concentration of myxothiazol. Freehand curves were drawn through the results. Points represent mean ± SEM of experiments done in triplicate on 3 separate synaptosomal preparations.

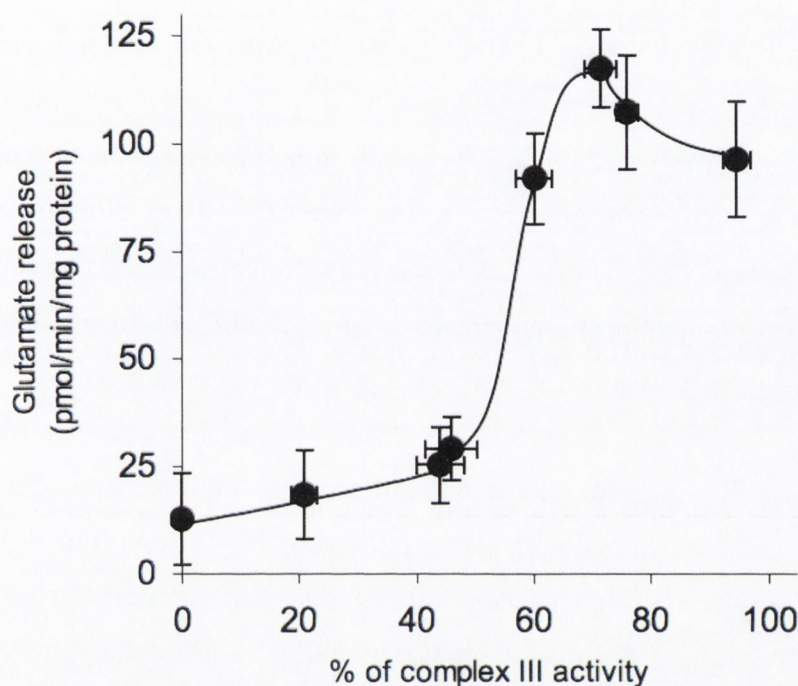


Fig. 4.7 Inhibition of complex III activity by > 50 – 60% with myxothiazol increases release of glutamate from polarised synaptosomes

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the absence of calcium. Rates of glutamate release at each concentration of myxothiazol (10 nM – 10 μ M) were plotted against percent inhibition of complex III activity brought about by that concentration of myxothiazol. Freehand curves were drawn through the results. Points represent mean $\pm$ SEM of experiments done in triplicate on 3 separate synaptosomal preparations.

4.3.4 Antimycin A increases Ca^{2+} -independent glutamate release and reduces Ca^{2+} -dependent glutamate release in depolarised synaptosomes

When depolarised with KCl, Ca^{2+} -independent glutamate release was increased from 115.3 ± 14.7 pmol/min/mg protein in the absence of inhibitor to 417.7 ± 17.8 pmol/min/mg protein in the presence of 50 nM antimycin A (40% of the total pool over 20 min, Fig. 4.7, $p < 0.001$). Incubation with higher concentrations did not increase the Ca^{2+} -independent release rate further. The rate in the presence of 1.4 mM CaCl_2 was found to be 222.8 ± 7.9 pmol/min/mg protein in the absence of inhibitor (giving a Ca^{2+} -dependent glutamate release rate of ~ 107 pmol/min/mg protein). The rate in the presence of 1.4 mM CaCl_2 was not different from the rate found to occur in the absence of calcium in the presence of 100 nM antimycin A (419.5 ± 25.6 pmol/min/mg with CaCl_2 vs. 429.6 ± 16.7 pmol/min/mg without).

The increase in glutamate release brought about by antimycin A after depolarisation with KCl was re-examined with concentrations of 10 – 50 nM (Fig. 4.9). In this set of experiments, the Ca^{2+} -independent release rate was increased from 116.4 ± 13.2 pmol/min/mg to 190.4 ± 14.1 pmol/min/mg by 15 nM antimycin A ($p < 0.05$). 40 nM antimycin A caused a maximum Ca^{2+} -independent release rate of 371.3 ± 20.7 pmol/min/mg (36% of the total glutamate pool over 20 min). The Ca^{2+} -dependent release rate was blocked by 10 nM antimycin A (167.5 ± 32.4 pmol/min/mg in the presence of CaCl_2 vs. 150.7 ± 14.2 pmol/min/mg in its absence) and at all concentrations of antimycin A (Fig. 4.9).

When depolarised with 4-aminopyridine, the Ca^{2+} -independent release rate was increased from 17.6 ± 14.1 pmol/min/mg to 208.0 ± 43.6 pmol/min/mg by 20 nM antimycin A ($p < 0.01$, Fig. 4.10). The Ca^{2+} -independent release rate in the presence of 15 nM antimycin A, at 15.7 ± 9.2 pmol/min/mg was not different from control. The maximum release rate in the absence of CaCl_2 after depolarisation with 4-aminopyridine was found in the highest concentration of antimycin A examined, 100 nM, and was measured to be 252.5 ± 25.3 pmol/min/mg protein (24% of the total glutamate pool). Ca^{2+} -dependent release was blocked by 40 nM (232.8 ± 17.9 pmol/min/mg in the presence of CaCl_2 vs. 231.3 ± 14.2 pmol/min/mg in its absence) and higher concentrations of antimycin A.

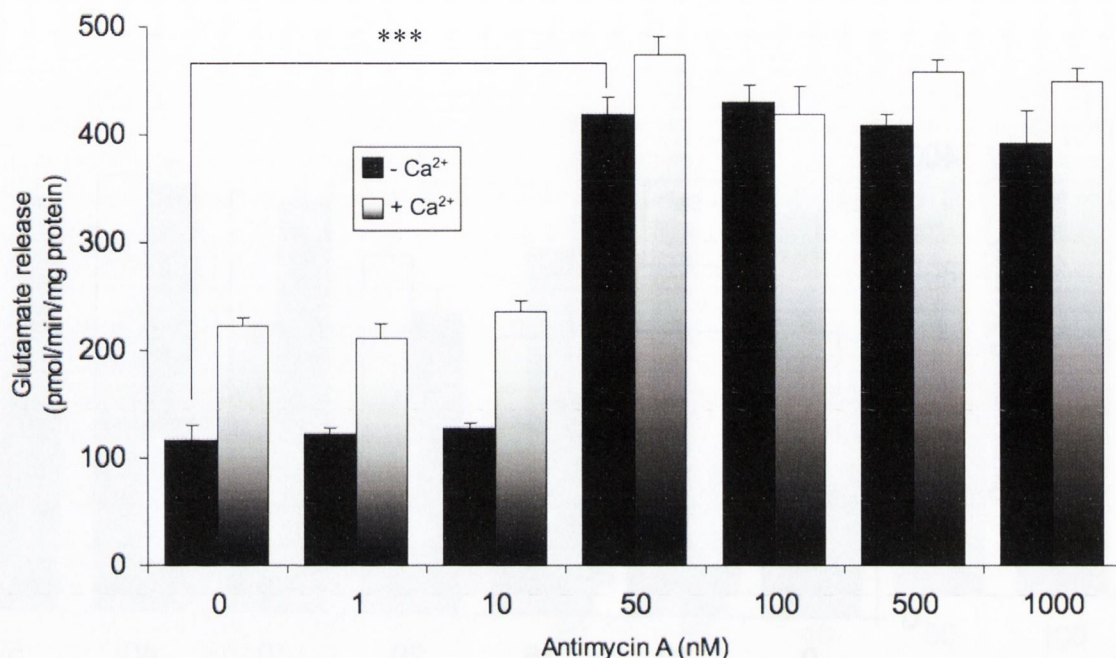


Fig. 4.8 Antimycin A increases Ca²⁺-independent glutamate release from synaptosomes depolarised with KCl at concentrations > 50 nM

Antimycin A (1 nM – 1 μ M) in ethanol was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 40 mM KCl and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (***) = $p < 0.001$).

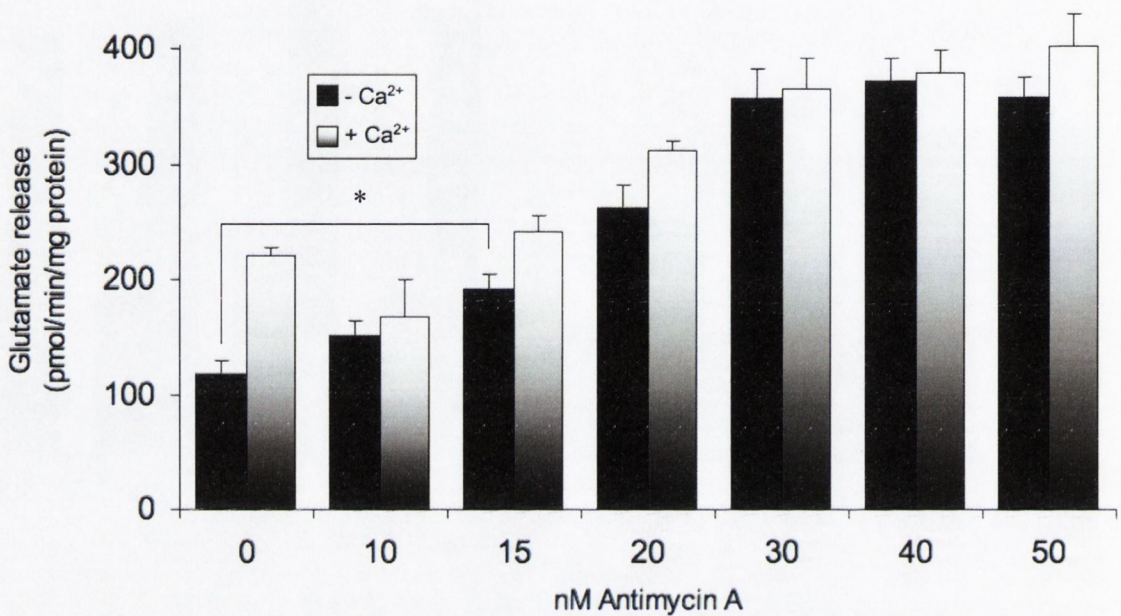


Fig. 4.9 Antimycin A increases Ca²⁺-independent glutamate release from synaptosomes depolarised with KCl

Antimycin A (10 nM – 50 nM) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 40 mM KCl and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (* = $p < 0.05$).

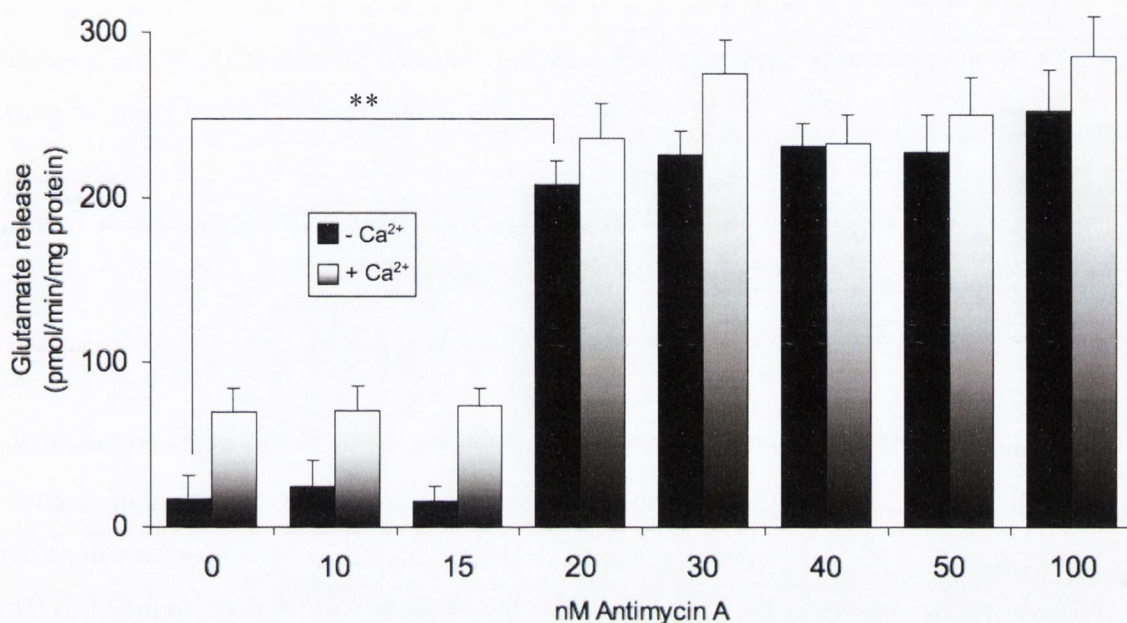


Fig. 4.10 Antimycin A increases Ca²⁺-independent glutamate release from synaptosomes depolarised with 4-aminopyridine at concentrations > 20 nM

Antimycin A (10 nM – 100 nM) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 1 mM 4-aminopyridine and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (** = $p < 0.01$).

4.3.5 Antimycin A increases the rate of glutamate efflux from polarised synaptosomes

20 nM antimycin A was found to increase the rate of glutamate efflux from 6.5 ± 7.8 pmol/min/mg to 88.1 ± 9.3 pmol/min/mg in the absence of Ca^{2+} and from 1.8 ± 6.1 pmol/min/mg to 64.8 ± 9.9 pmol/min/mg in the presence of Ca^{2+} ($p < 0.05$, Fig. 4.11). The maximum efflux rate was achieved by 100 nM antimycin A in the absence of Ca^{2+} and was found to be 117.2 ± 10.1 pmol/min/mg protein (11% of the total pool).

4.3.6 Inhibition of complex III activity by > 85% with antimycin A increases the rate of glutamate release from synaptosomes

20 nM antimycin A was found to inhibit complex III activity by $91.2 \pm 1.6\%$ in polarised synaptosomes in the absence of calcium (Fig. 4.12) and similar levels of inhibition were found for this concentration under all conditions examined. Hence the increase in Ca^{2+} -independent glutamate release rate from synaptosomes depolarised with 4-aminopyridine brought about by 20 nM antimycin A corresponded to $91.6 \pm 1.5\%$ inhibition of complex III activity (Fig. 4.13). 15 nM antimycin A brought about $90.4 \pm 1.7\%$ inhibition in the presence of KCl, and increased Ca^{2+} -independent glutamate release rate (Fig. 4.14). Inhibition of $94.8 \pm 1.1\%$ brought about the maximum release rate when depolarised with KCl. Glutamate efflux from polarised synaptosomes was not increased until $91.6 \pm 1.5\%$ inhibition was reached by 20 nM antimycin A (Fig. 4.15).

4.3.7 KCN increases Ca^{2+} -independent glutamate release rates and blocks Ca^{2+} -dependent glutamate release from depolarised synaptosomes

The rate of Ca^{2+} -independent glutamate release from synaptosomes depolarised with 4-aminopyridine was increased from 40.6 ± 13.4 pmol/min/mg to 264.3 ± 51.4 pmol/min/mg by 1 mM KCN ($p < 0.01$, Fig. 4.16). The rate in the presence of 1.4 mM CaCl_2 and 1 mM KCN, 259.2 ± 41.1 pmol/min/mg, was not different to that without calcium, and represents a release of approximately 25% of the total glutamate pool over 20 min. The rate in the absence of inhibitor was 110.0 ± 18.3 pmol/min/mg, implying a Ca^{2+} -dependent release rate of ~ 69 pmol/min/mg. The difference between

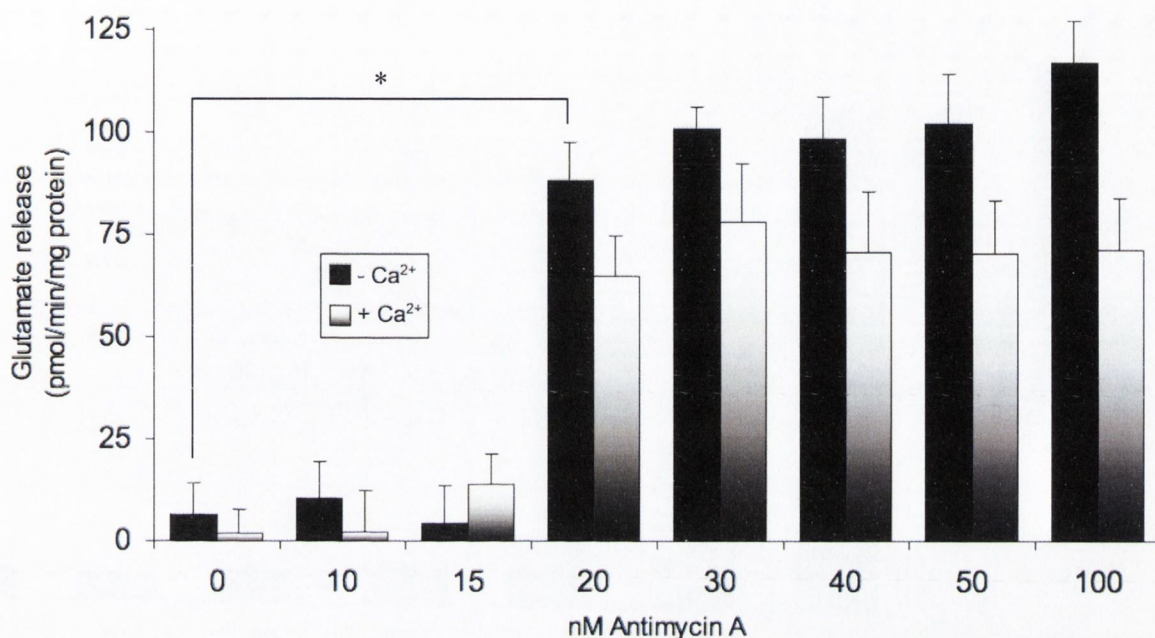


Fig. 4.11 Antimycin A increases glutamate release from polarised synaptosomes at concentrations ≥ 20 nM

Antimycin A (10 nM – 100 nM) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 1 mM 4-aminopyridine and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (* = $p < 0.05$).

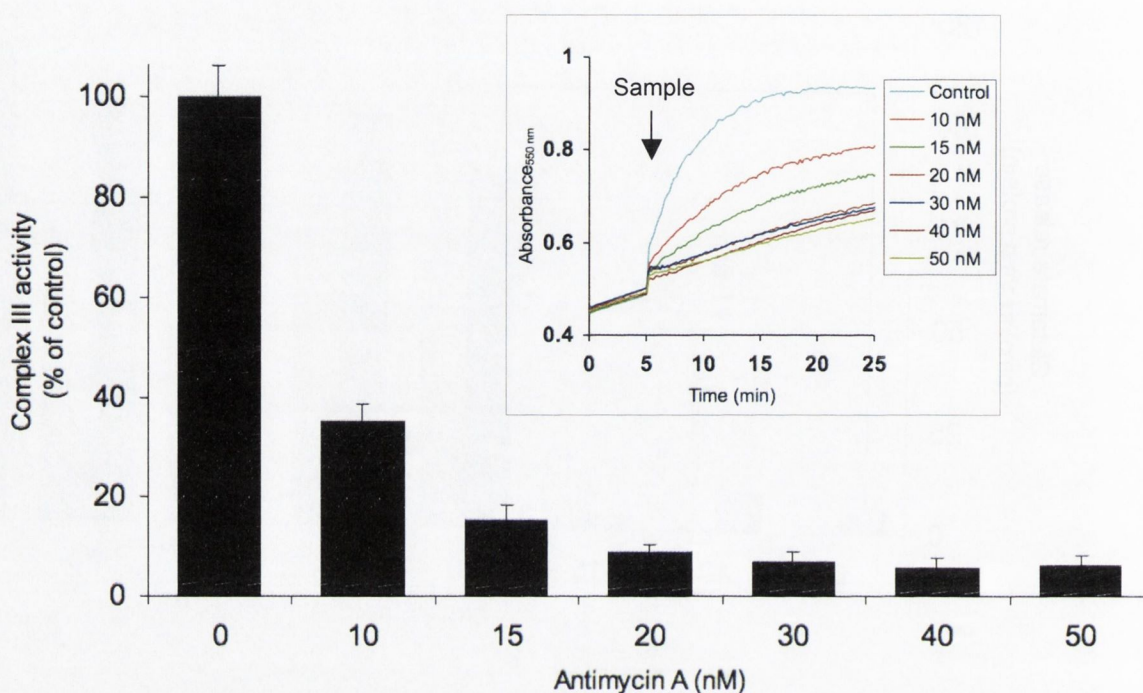


Fig. 4.12 Antimycin A inhibits complex III activity by > 90% at concentrations ≥ 20 nM

Synaptosomes (0.5 mg/ml) were incubated for 15 min with antimycin A (10 – 50 nM) before being stored at -80°C and assayed for complex III activity. Reduction of cytochrome *c* was followed spectrophotometrically at absorbance $\lambda = 550$ nm as described in detail in Chapter 2, and expressed as a percentage of the control which contained no antimycin A. Data shown are results from triplicate experiments on 3 separate synaptosomal preparations. **Inset:** Typical traces of reduction of cytochrome *c* by samples that had been incubated with the concentrations of antimycin A shown.

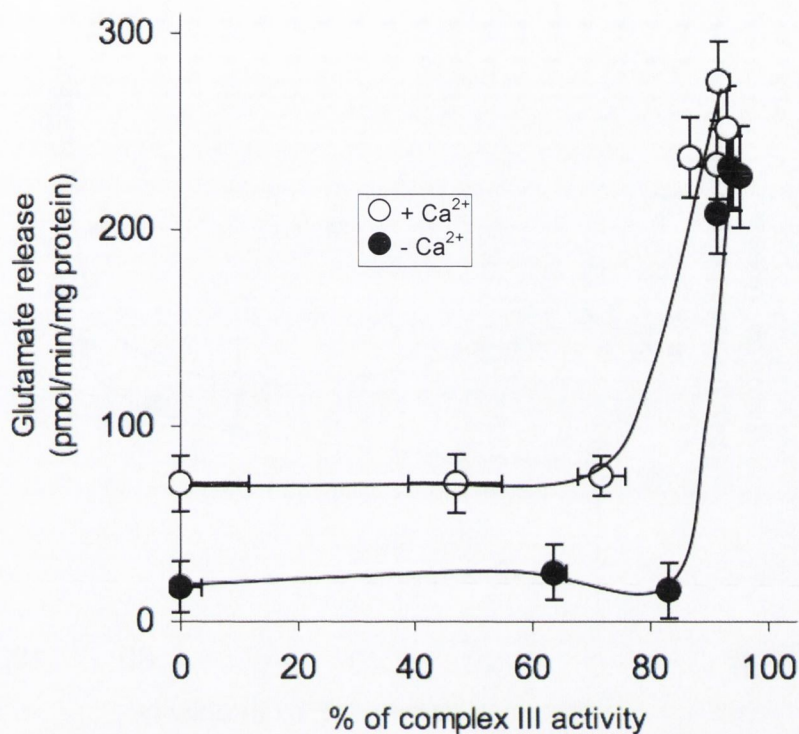


Fig. 4.13 Inhibition of complex III activity by > 85% with antimycin A increases release of glutamate from synaptosomes depolarised with 4-aminopyridine

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarised with 1 mM 4-aminopyridine. Rates of glutamate release at each concentration of antimycin A (10 – 50 nM) were plotted against percent inhibition of complex III activity brought about by that concentration of antimycin A. Freehand curves were drawn through the results. Points represent mean ± SEM of experiments done in triplicate on 3 separate synaptosomal preparations.

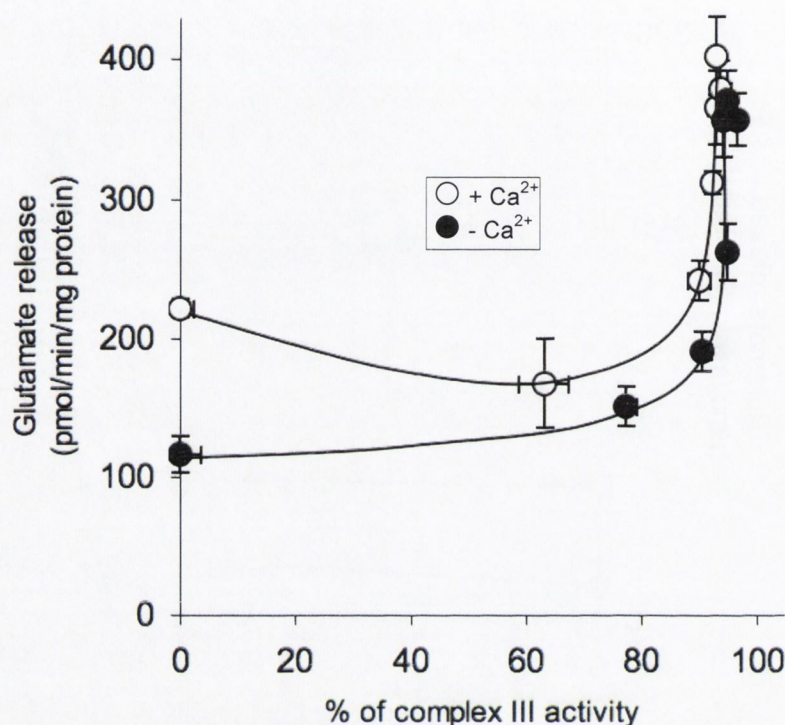


Fig. 4.14 Inhibition of complex III activity by > 90% with antimycin A increases release of glutamate from synaptosomes depolarised with KCl

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarised with 40 mM KCl. Rates of glutamate release at each concentration of antimycin A (10 – 50 nM) were plotted against percent inhibition of complex III activity brought about by that concentration of antimycin A. Freehand curves were drawn through the results. Points represent mean ± SEM of experiments done in triplicate on 3 separate synaptosomal preparations.

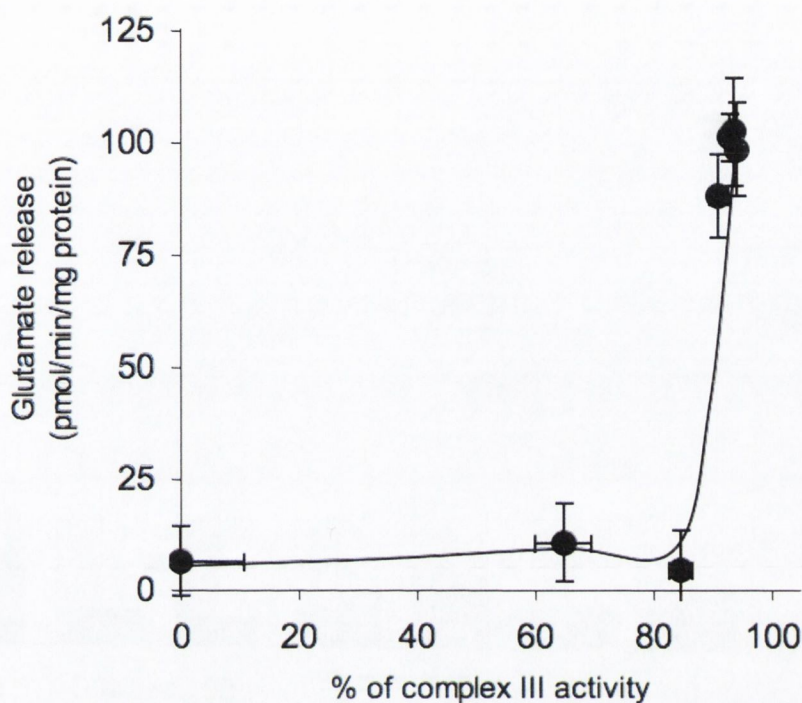


Fig. 4.15 Inhibition of complex III activity by > 85% with antimycin A increases release of glutamate from polarised synaptosomes

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the absence of calcium. Rates of glutamate release at each concentration of antimycin A (10 – 50 nM) were plotted against percent inhibition of complex III activity brought about by that concentration of antimycin A. Freehand curves were drawn through the results. Points represent mean $\pm$ SEM of experiments done in triplicate on 3 separate synaptosomal preparations.

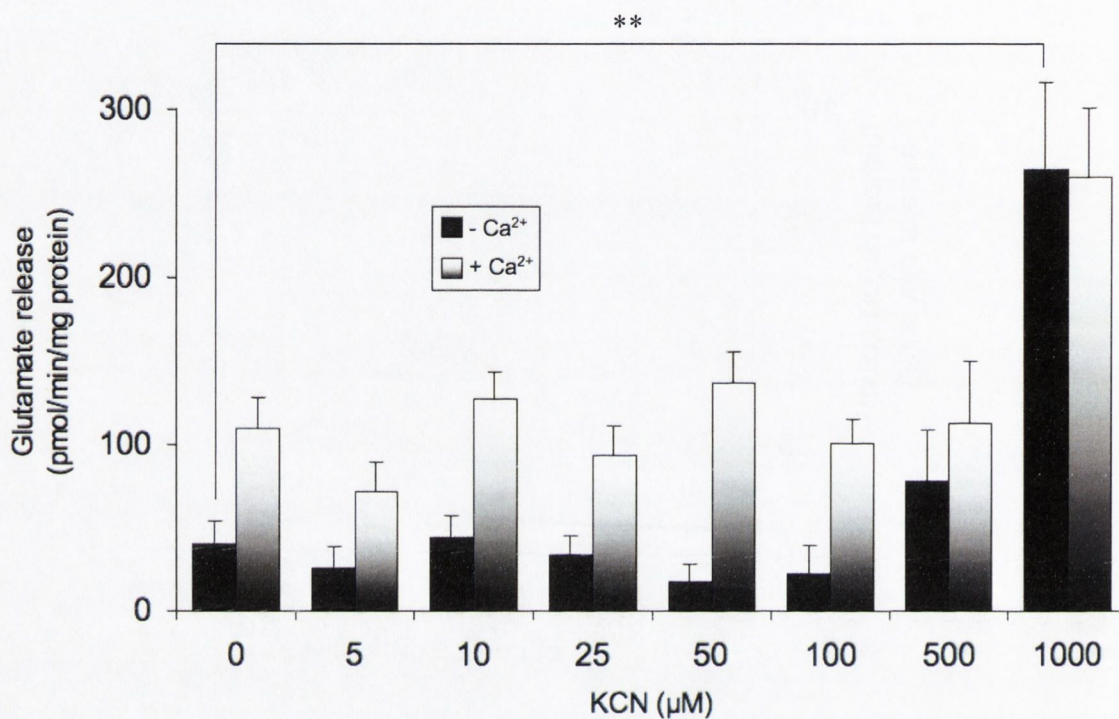


Fig. 4.16 KCN increases Ca^{2+} -independent glutamate release and blocks Ca^{2+} -dependent glutamate release from synaptosomes depolarised with 4-aminopyridine

KCN (5 μM – 1 mM) was added to wells containing 0.5 mg/ml synaptosomes in Krebs's buffer, 1 mM NADP^+ and 3.16 U GDH. 1.4 mM CaCl_2 was added to one group (+ Ca^{2+}) while the other group was Ca^{2+} -free (- Ca^{2+}). Synaptosomes were incubated for 5 min before being depolarised with 1 mM 4-aminopyridine and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (** = $p < 0.01$).

rates in the presence and absence of calcium remains significant at all concentrations under 500 μM KCN. At this concentration, 112.5 ± 37.3 pmol/min/mg was released in the presence of 1.4 mM CaCl_2 and 78.0 ± 31.1 pmol/min/mg in its absence. When depolarised with KCl, the Ca^{2+} -independent glutamate release rate was increased from 100.3 ± 14.3 pmol/min/mg to 201.4 ± 17.8 pmol/min/mg by 500 μM KCN ($p < 0.05$, Fig. 4.17). The maximum Ca^{2+} -independent release rate when depolarised with KCl was 264.1 ± 33.0 pmol/min/mg protein, 26% of the total glutamate pool over 20 min, in the presence of 1 mM KCN. The rate of release at this concentration in the presence of 1.4 mM CaCl_2 was not significantly different at 310.5 ± 15.7 pmol/min/mg.

4.3.8 KCN increases the rate of glutamate efflux from polarised synaptosomes

The rate of glutamate efflux from polarised synaptosomes was increased from 21.33 ± 8.7 pmol/min/mg to 176.2 ± 26.5 pmol/min/mg by 1 mM KCN in the absence of calcium ($p < 0.01$, Fig. 4.18). The rate was not affected by any other concentration of KCN. Nor was there a difference in efflux rate between groups with added calcium and those with no added calcium at any concentration of KCN.

4.3.9 Inhibition of complex IV activity by > 85% with KCN increases the rate of glutamate release from synaptosomes

KCN was found to inhibit complex IV activity in synaptosomes in a concentration dependent manner (Fig. 4.19). Inhibition of complex IV activity by $88.1 \pm 3.7\%$ increased Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with 4-aminopyridine to a maximum level recorded by inhibition at this point of the ETC (Fig. 4.20). Inhibition of $87.8 \pm 3.0\%$ in the presence of CaCl_2 was sufficient to block Ca^{2+} -dependent glutamate release. However, in synaptosomes depolarised with KCl, $94.0 \pm 2.0\%$ inhibition of complex IV activity did not increase the Ca^{2+} -independent glutamate release rate (Fig. 4.21). Also, the Ca^{2+} -dependent glutamate release rate remains unchanged until at least 85% inhibition of complex IV activity. Maximum Ca^{2+} -independent glutamate release was reached at $97.9 \pm 1.4\%$ inhibition of complex IV. The rate of glutamate efflux from polarised synaptosomes was increased by $87.9 \pm 2.4\%$ inhibition of complex IV activity (Fig. 4.22), but was not changed by any lower level of inhibition.

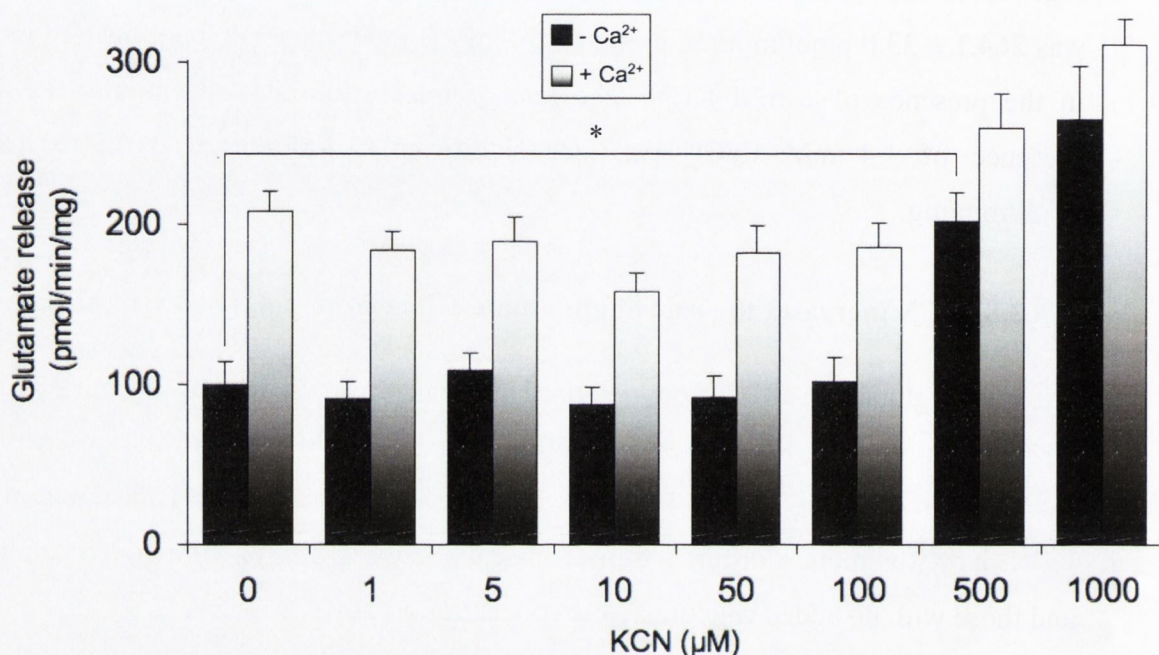


Fig. 4.17 KCN increases Ca²⁺-independent glutamate release and blocks Ca²⁺-dependent glutamate release from synaptosomes depolarised with KCl

KCN (1 μM – 1 mM) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min before being depolarised with 40 mM KCl and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (* = $p < 0.05$).

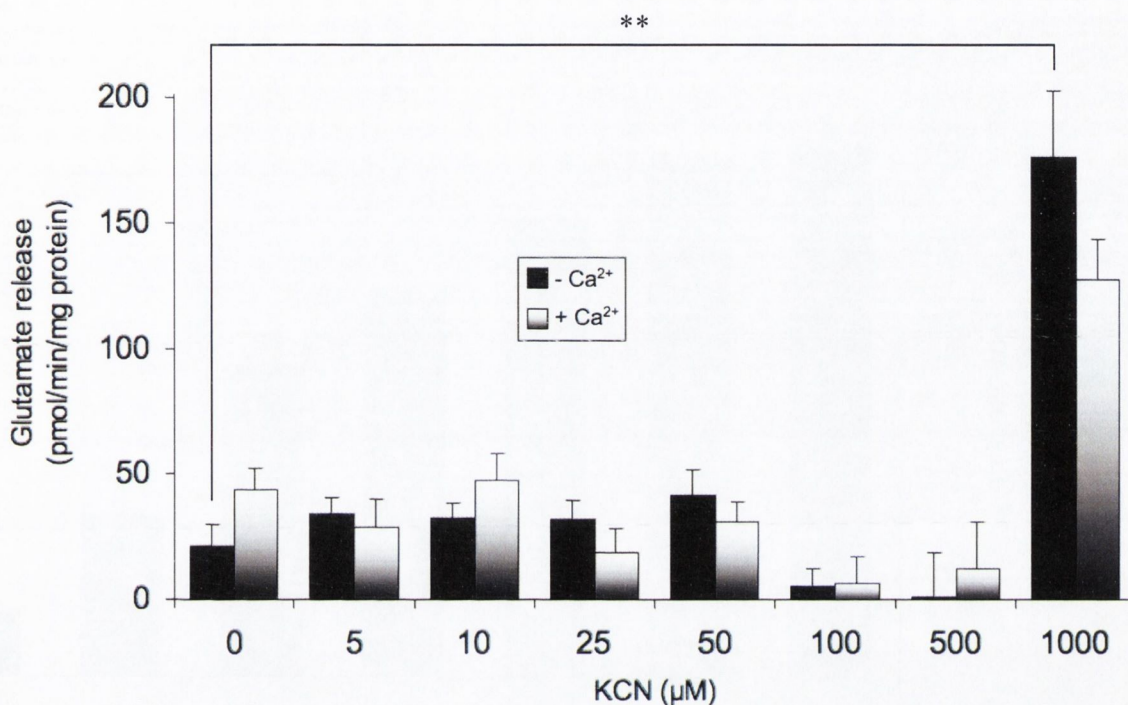


Fig. 4.18 KCN increases the rate of glutamate efflux from polarised synaptosomes

KCN (5 μM – 1 mM) was added to wells containing 0.5 mg/ml synaptosomes in Krebs's buffer, 1 mM NADP⁺ and 3.16 U GDH. 1.4 mM CaCl₂ was added to one group (+ Ca²⁺) while the other group was Ca²⁺-free (- Ca²⁺). Synaptosomes were incubated for 5 min and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations. (** = $p < 0.01$).

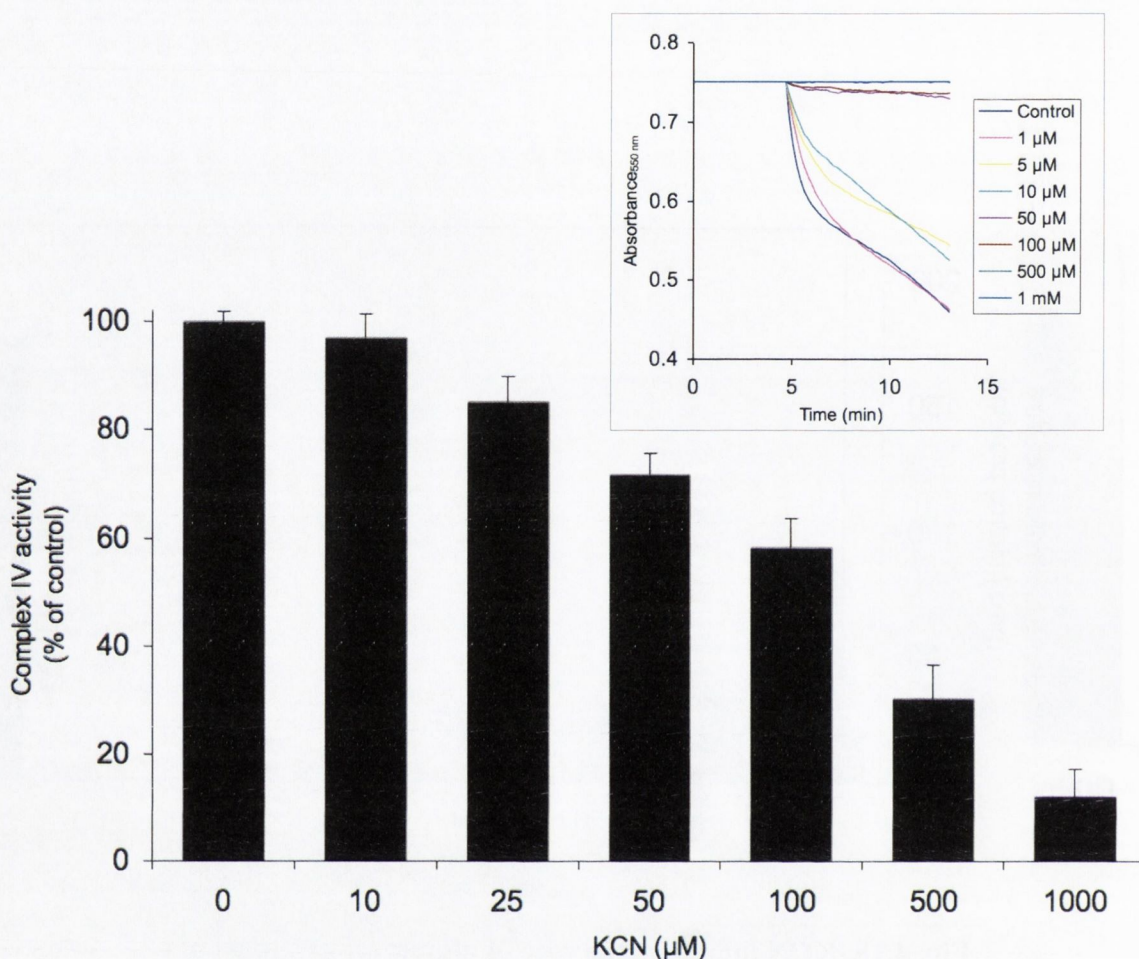


Fig. 4.19 KCN inhibits complex IV activity in synaptosomes in a concentration dependent manner

Synaptosomes (0.5 mg/ml) were incubated for 15 min with KCN (10 μM – 1 mM) before being stored at -80°C and assayed for complex III activity. Oxidation of cytochrome *c* was followed spectrophotometrically at absorbance $\lambda = 550$ nm as described in detail in Chapter 2, and expressed as a percentage of the control which contained no KCN. Data shown are results from triplicate experiments on 3 separate synaptosomal preparations. **Inset:** Typical traces of reduction of cytochrome *c* by samples that had been incubated with the concentrations of KCN shown.

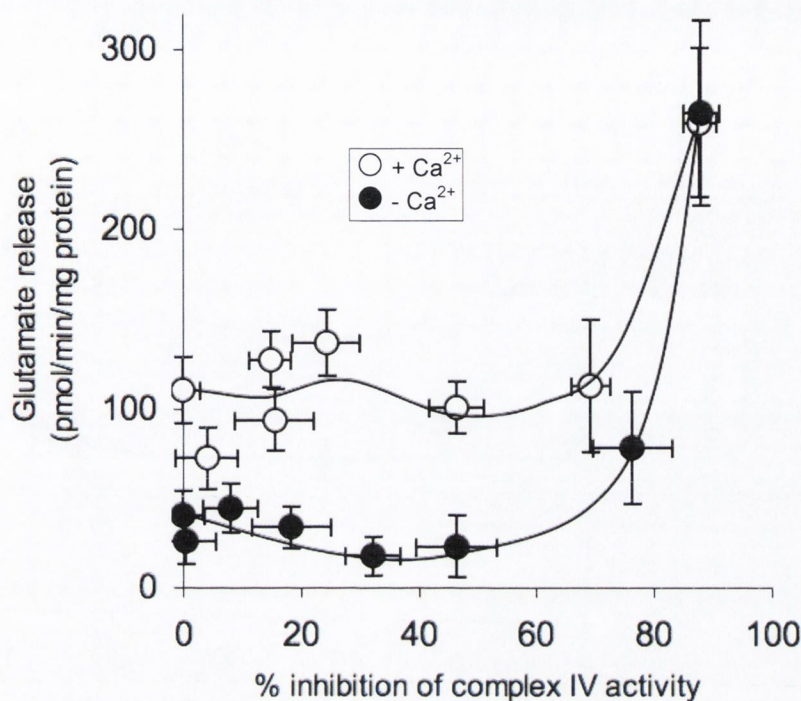


Fig. 4.20 Inhibition of complex IV activity by > 85% increases release of glutamate from synaptosomes depolarised with 4-aminopyridine

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarised with 1 mM 4-aminopyridine. Rates of glutamate release at each concentration of KCN (10 μM – 1 mM) were plotted against percent inhibition of complex IV activity brought about by that concentration of KCN. Freehand curves were drawn through the results. Points represent mean ± SEM of experiments done in triplicate on 3 separate synaptosomal preparations.

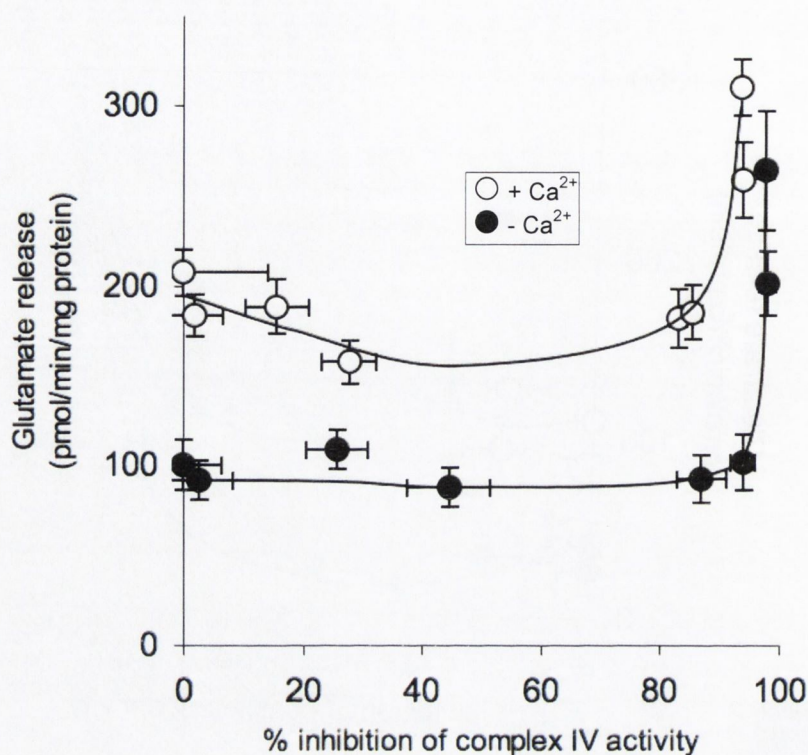


Fig. 4.21 Inhibition of complex IV activity by > 90% increases release of glutamate from synaptosomes depolarised with KCl

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarised with 40 mM KCl. Rates of glutamate release at each concentration of KCN (10 μM – 1 mM) were plotted against percent inhibition of complex IV activity brought about by that concentration of KCN. Freehand curves were drawn through the results. Points represent mean ± SEM of experiments done in triplicate on 3 separate synaptosomal preparations.

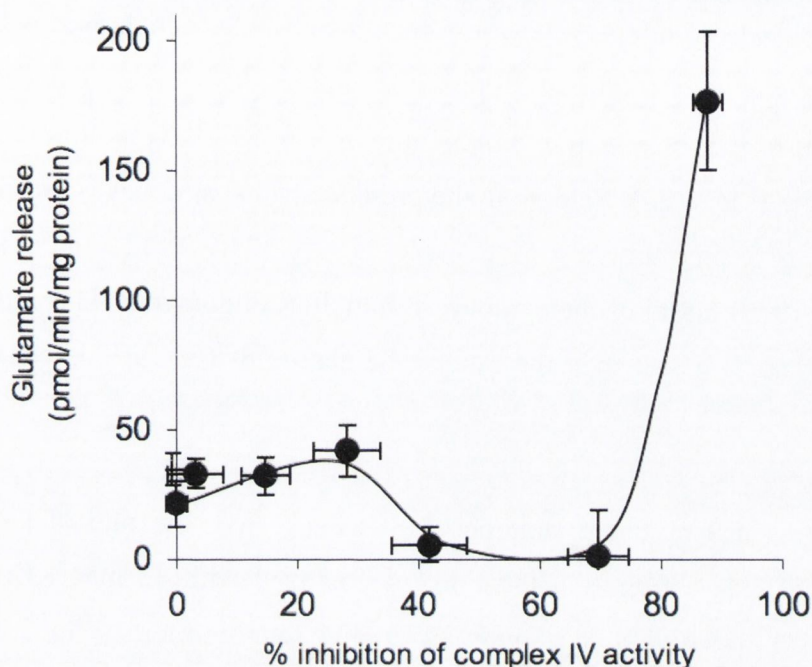


Fig. 4.22 Inhibition of complex IV activity by > 85% increases release of glutamate from polarised synaptosomes

Synaptosomes (0.5 mg/ml) were preincubated at 37°C for 5 min in the absence of Ca^{2+} . Rates of glutamate release at each concentration of KCN (10 μM – 1 mM) were plotted against percent inhibition of complex IV activity brought about by that concentration of KCN. Freehand curves were drawn through the results. Points represent mean $\pm$ SEM of experiments done in triplicate on 3 separate synaptosomal preparations.

4.3.10 Oligomycin and FCCP increase glutamate release from depolarised synaptosomes

Ca^{2+} -independent glutamate release from synaptosomes depolarised with 40 mM KCl was increased from 102.0 ± 16.6 pmol/min/mg to 195.4 ± 12.1 pmol/min/mg by 500 ng/ml and further increased to a maximum of 256.2 ± 19.6 pmol/min/mg by 10 $\mu\text{g/ml}$ oligomycin (Fig. 4.23). Release in the presence of 1.4 mM CaCl_2 was increased from 194.6 ± 14.2 pmol/min/mg to a maximum of 324.9 ± 12.7 pmol/min/mg in the presence of 10 $\mu\text{g/ml}$ of the complex V inhibitor, oligomycin. This rate was found to be significantly faster than the rate in the absence of Ca^{2+} at this concentration ($p < 0.05$).

In the presence of the protonophore FCCP (1 μM), the rate of Ca^{2+} -independent glutamate release from synaptosomes depolarised with 40 mM KCl was increased from 102.0 ± 16.6 pmol/min/mg to 394.9 ± 24.7 pmol/min/mg (Fig. 3.24). The release in the presence of Ca^{2+} was found to be 493.0 ± 22.5 pmol/min/mg at the same concentration of FCCP, which was significantly faster than the Ca^{2+} -independent glutamate release rate ($p < 0.05$). However, Ca^{2+} -independent release rate in the presence of 10 $\mu\text{g/ml}$ combined with 1 μM FCCP was increased to 439.7 ± 35.0 pmol/min/mg, which was not significantly different from the rate in the presence of Ca^{2+} under these conditions (493.8 ± 24.8 pmol/min/mg).

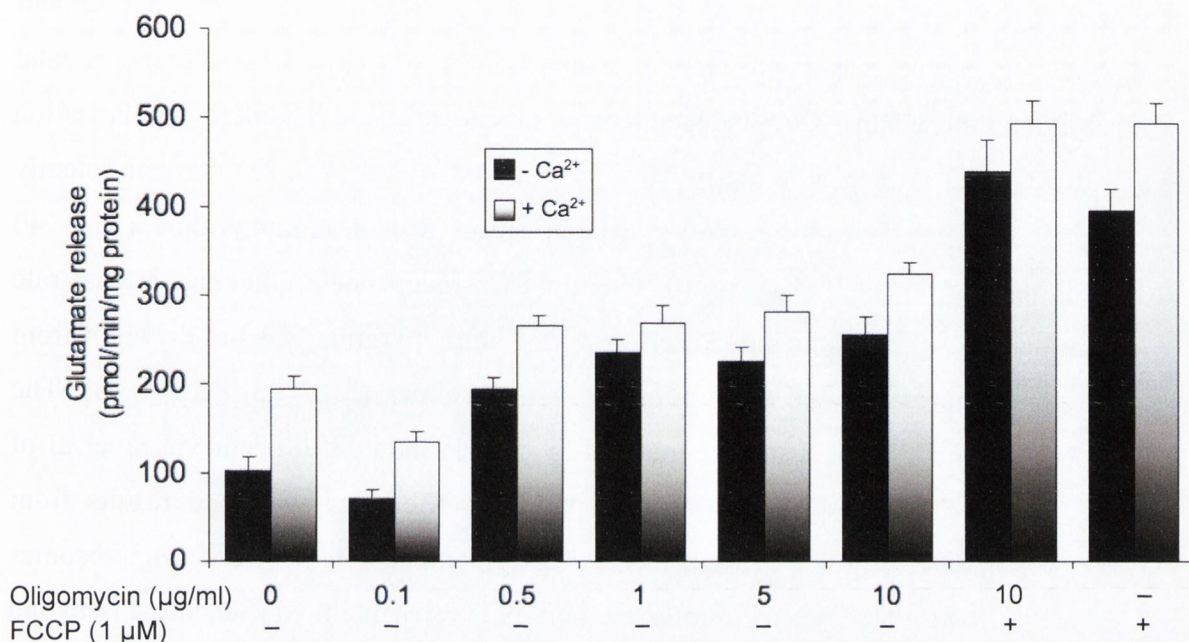


Fig. 4.23 Oligomycin and FCCP increase Ca^{2+} -independent glutamate release from synaptosomes depolarised with KCl

Oligomycin (100 ng/ml – 10 μg/ml) was added to wells containing 0.5 mg/ml synaptosomes in Kreb's buffer, 1 mM NADP^+ and 3.16 U GDH and 1 μM FCCP where shown. 1.4 mM CaCl_2 was added to one group (+ Ca^{2+}) while the other group was Ca^{2+} -free (- Ca^{2+}). Synaptosomes were incubated for 5 min and glutamate release rates were taken over 20 min. Data shown represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations.

4.4 Discussion

The results in the present chapter indicate that inhibition of the activity of complexes III and IV of *in situ* synaptosomal mitochondria increases Ca^{2+} -independent glutamate release rates from depolarised synaptosomes, as well as the Ca^{2+} -independent glutamate efflux rate from resting synaptosomes. In each of the experimental conditions the control Ca^{2+} -independent glutamate release rates after depolarisation with KCl (~ 100 pmol/min/mg protein Figs 4.2, 4.8, 4.9, 4.17, 4.23) was consistently higher than the control rates after depolarisation with 4-aminopyridine (15 – 40 pmol/min/mg, Figs 4.3, 4.10, 4.16). The low Ca^{2+} -independent glutamate release rate from control synaptosomes depolarised with 4-aminopyridine was not different from the glutamate efflux rate from polarised synaptosomes (Figs 4.3, 4.11, 4.18). The excess Ca^{2+} -independent release induced by KCl is thought to occur via reversal of the plasma membrane Na^+ -cotransport pathway (Nicholls 1989) and results from previous studies suggest a large increase in glutamate release from synaptosomes depolarised with KCl when complex IV activity is fully inhibited (Sanchez-Prieto and Gonzalez 1988; Santos *et al.* 1996). However, attention has not previously been drawn to this increase, most likely because of the nonphysiological “clamped” depolarisation brought about by KCl. The results presented here show that 4-aminopyridine, an agent that mimics the effect of action potentials on nerve terminals (Tibbs *et al.* 1989) also increased Ca^{2+} -independent glutamate release rates after inhibition of complex IV activity. Both agents also increased Ca^{2+} -independent glutamate release rates from synaptosomes after inhibition of complex III activity, a finding that has not previously been published.

Inhibition of complex III activity by 60 – 80% with 75 nM myxothiazol was found to increase Ca^{2+} -independent glutamate release rates from depolarised synaptosomes (Figs 4.1, 4.2, 4.5, 4.6). Using a similar range of concentrations of myxothiazol as was used in the present experiments, it has previously been demonstrated that inhibition of complex III activity by up to 80% did not result in major changes in oxidative phosphorylation, while inhibition by 90% completely abolished the oxygen consumption and ATP production in isolated synaptosomal mitochondria (Davey *et al.* 1998). Recent work in our lab has indicated that the threshold level of inhibition of complex III activity on oxygen consumption in *in situ* synaptosomal mitochondria

may be lower than that found in isolated synaptosomal mitochondria (Telford *et al.* unpublished). In the *in situ* synaptosomal mitochondrial model, inhibition of complex III activity by 40% reduced the rate of oxygen consumption, and ~ 50% inhibition of complex III activity brought about a reduction in the rate to ~ 65% of the control rate. Inhibition of complex III activity by ~ 70% was found to almost completely abolish the oxygen consumption rate (Telford *et al.* unpublished). At this level of inhibition mitochondrial ATP synthesis would also be expected to be minimal, and glutamate efflux from 'polarised' synaptosomes was found to at a maximum of > 100 pmol/min/mg (Fig. 4.7). The efflux was Ca^{2+} -independent (Fig. 4.3), indicating that $\Delta\psi_p$ was sufficiently depolarised (presumably by reduced ATP supply to the Na^+/K^+ ATPase) to cause reversal of the plasma membrane glutamate uptake transporters. Ca^{2+} -independent glutamate release from synaptosomes depolarised with 4-aminopyridine over same range of inhibition of complex III activity with myxothiazol (between 50% and 70%) was increased to > 200 pmol/min/mg. This suggests that the control of complex III over oxidative phosphorylation in synaptosomes is similar to the control of complex III over Ca^{2+} -independent glutamate release rates from synaptosomes. Consequently, anaerobic ATP production (via glycolysis) seems to be insufficient to support the maintenance of $\Delta\psi_p$ in resting synaptosomes when complex III is inhibited by > 50% with myxothiazol.

In each of the glutamate release experiments where complex III activity was inhibited with myxothiazol, peak levels of glutamate release occurred at concentrations of < 10 μM myxothiazol. Nonetheless, 10 μM myxothiazol was the concentration that consistently brought about the highest levels of inhibition of complex III activity, and this concentration has been shown to completely inhibit oxygen consumption and ATP production in isolated nerve terminal mitochondria (Davey *et al.* 1998). This effect on Ca^{2+} -independent glutamate release is similar to the effect on glutamate release of inhibiting complex I activity with concentrations of rotenone > 25 nM (Kilbride *et al.* 2008), and may similarly 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 hyperpolarisation response of membrane potential in different types of neurons (Jiang *et al.* 1994; Nieber *et al.* 1995; Pisani *et al.* 1999; Nicholls 2006). 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. Further studies on ATP levels in isolated nerve terminals after complex III activity inhibition with a range of myxothiazol concentrations are required to support this hypothesis.

Inhibition of complex III activity by 85 – 95% with 20 nM antimycin A increased Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with 4-aminopyridine (Figs 4.10, 4.13), KCl (Figs 4.9, 4.14) and increased glutamate efflux from ‘polarised’ synaptosomes (Figs 4.11, 4.15). The highest rates of Ca^{2+} -independent glutamate release brought about by complex III inhibition with antimycin A were similar to those with myxothiazol. For both inhibitors, > 100 pmol/min/mg glutamate was released from ‘polarised’, > 200 pmol/min/mg was released from synaptosomes depolarised with 4-aminopyridine and > 350 pmol/min/mg was released from synaptosomes depolarised with KCl. However, 80% inhibition of complex III activity with antimycin A did not increase Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with 4-aminopyridine (Fig. 4.13) while maximum 4-aminopyridine-induced glutamate release rates were recorded at < 80% inhibition of complex III activity with myxothiazol (Fig. 4.5). Similarly, 80% inhibition of complex III activity with antimycin A did not increase glutamate efflux rates from ‘polarised’ synaptosomes (Fig. 4.15) while maximum glutamate efflux rates were recorded at < 80% inhibition of complex III activity with myxothiazol (Fig. 4.7). This suggests that complex III of *in situ* nerve terminal mitochondria may have a lower energy threshold when inhibited upstream of the Q-cycle with myxothiazol than when inhibited downstream with antimycin A, which results in increased rates of glutamate release at a lower level of inhibition.

Inhibition of complex IV activity by 85 – 95% with 1 mM KCN also increased Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with 4-

aminopyridine (Figs 4.16, 4.20), KCl (Figs 4.17, 4.21) and increased glutamate efflux from 'polarised' synaptosomes (Figs 4.18, 4.22). The maximum Ca^{2+} -independent glutamate release rates were similar after depolarisation with 4-aminopyridine and KCl (~ 265 pmol/min/mg), while the maximum efflux rate of ~ 117 pmol/min/mg was 8-fold faster than the control rate. A similar increase in glutamate efflux has previously been reported in rat brain synaptosomes with when 2 mM NaCN was added, and was related to a fall in ATP/ADP ratio (Sanchez-Prieto and Gonzalez 1988). This excess level of NaCN was also shown to decrease Ca^{2+} -dependent glutamate release in a time-dependent manner, which was also related to the ATP/ADP ratio: a fall below 1.7 was found to block Ca^{2+} -dependent glutamate release from synaptosomes depolarised with KCl completely. The ATP level after incubation with a range of concentrations of KCN has not been reported on but would reveal if the increase in Ca^{2+} -independent glutamate release rates and blockage of Ca^{2+} -dependent glutamate release after depolarisation with 4-aminopyridine and KCl were similar to the previously reported threshold level. Inhibition of complex IV activity by up to 70% with KCN in isolated synaptosomal mitochondria has previously been shown to decrease ATP production by $< 10\%$, with total inhibition of ATP production at 90% inhibition of complex IV activity (Davey *et al.* 1998). A similar threshold effect in *in situ* synaptosomal mitochondria might explain the effects of complex IV inhibition on Ca^{2+} -independent and -dependent glutamate release from depolarised synaptosomes.

Inhibition of the mitochondrial ETC at sites upstream of complex V is thought to result in reversal of the ATP synthase, resulting in consumption of glycolytic ATP by complex V (Kauppinen and Nicholls 1986; Chinopoulos *et al.* 1999). Inhibition of complex V activity with oligomycin increased Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with KCl at concentrations above 0.5 $\mu\text{g/ml}$ (Fig. 4.23). This suggests that inhibition of mitochondrial ATP production increases KCl-induced Ca^{2+} -independent glutamate release rates over this time period. The rate in the presence of excess oligomycin (10 $\mu\text{g/ml}$) was ~ 256 pmol/min/mg, which was lower than the KCl-induced maximum Ca^{2+} -independent glutamate release rates after inhibition of complex III and complex IV activities respectively. Thus, glycolytic ATP production may be sufficient to partially prevent the increase in KCl-induced Ca^{2+} -independent glutamate release rates caused by inhibition of mitochondrial ATP production when ATP is not being consumed by reversed complex V. The Ca^{2+} -

dependent component of glutamate release was still evident at the highest concentrations of oligomycin, suggesting that glycolytic ATP is also sufficient to maintain exocytotic release of glutamate under these circumstances. This result is in accordance with a previously reported finding (Sanchez-Prieto *et al.* 1987).

Addition of the protonophore FCCP to synaptosomes has been shown to collapse of both $\Delta\psi_m$ (Scott and Nicholls 1980), resulting in hydrolysis of ATP by complex V (Kauppinen and Nicholls 1986) and also may reverse the vesicular proton gradient causing efflux of vesicular glutamate into the cytoplasmic pool (Sanchez-Prieto *et al.* 1987). In the present study it was shown that 1 μM FCCP increased Ca^{2+} -independent glutamate release after depolarisation with KCl to almost 400 pmol/min/mg (Fig. 4.23), a rate which was found to be higher than any brought about by inhibition any of the ETC enzymes. Preventing complex V consumption of ATP after addition of FCCP by inhibiting complex V activity did not effect the FCCP-induced increase Ca^{2+} -independent glutamate release after depolarisation with KCl. The reduced ATP after addition of FCCP has previously been shown to reduce the Ca^{2+} -dependent component of glutamate release from synaptosomes in a time-dependent manner, with complete blockage after 8 min incubation with 1 μM FCCP (Sanchez-Prieto *et al.* 1987). Depolarisation with KCl after 5 min pre-incubation with 1 μM FCCP did not affect Ca^{2+} -dependent glutamate release from synaptosomes (Fig. 4.23).

Reduced complex II/III activity has been proposed to play a role in the excitotoxic mechanism thought to occur during the pathogenesis of Huntington's disease (Mann *et al.* 1990; Browne *et al.* 1997; Tabrizi *et al.* 1999), and reduced complex IV activity has been consistently observed in post mortem Alzheimer's disease brain samples (Kish *et al.* 1992; Mutisya *et al.* 1994; Parker *et al.* 1994). Neurodegeneration has been proposed to progress from the nerve terminal in these chronic neurodegenerative disorders (Mattson *et al.* 1998). Indeed, complex IV activity in the brain has been widely shown to be reduced as part of the normal aging process (Navarro *et al.* 2002; Navarro *et al.* 2004; Navarro *et al.* 2005). However, the model of the effects of reduced activities of complex III and complex IV nerve terminal glutamate release in the present chapter indicate that these enzymes may be inhibited by at least 50% (for complex III) or 85% (for complex IV) before glutamate is excessively released. Since such large reductions in activity have not been reported in these chronic

neurodegenerative disorders, a contributing role of the nerve terminal in releasing excitotoxic amounts of glutamate in the pathogenesis of these disorders is not supported by this model.

Chapter 5

Control of $\Delta\psi_m$, $\Delta\psi_p$ and reactive oxygen species production in synaptosomes

5.1 Introduction

$\Delta\psi_m$ is the electrical charge of the proton gradient across the mitochondrial inner membrane (Nicholls and Budd 2000). As such, it represents a measure of the ability of the ETC to pump protons out of the matrix. Various techniques using either fluorescent or radiolabelled cations have been devised to estimate $\Delta\psi_m$ in *in situ* mitochondria, and the charge has been reported to be -150 mV in 'resting' synaptosomes (Scott and Nicholls 1980). Mitochondria are involved in Ca^{2+} sequestering within neurons and mitochondrial Ca^{2+} uptake is driven by $\Delta\psi_m$. Since the uptake is electrogenic, $[\text{Ca}^{2+}]_m$ overload results in decreased $\Delta\psi_m$. Among the other factors that can influence $\Delta\psi_m$ in *in situ* mitochondria are changes in ATP/ADP ratio and oxidative stress. Age-related decreases of $\Delta\psi_m$ in brain mitochondria have been reported (Joyce *et al.* 2003; Navarro and Boveris 2007; Boveris and Navarro 2008). Depolarisation of $\Delta\psi_m$ in neurons is thought to be one of the initial indicators of impending apoptosis (Nicholls and Ward 2000), and depolarisation below a threshold level induces membrane permeability via opening of the transition pore and consequent release of into the cytoplasm of proapoptotic factors including cytochrome *c* and Ca^{2+} (Nicholls and Budd 2000).

Mitochondria are known to be the primary source of reactive oxygen species (ROS) production within the cell (Chance *et al.* 1979), 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 (Shigenaga *et al.* 1994; Floyd *et al.* 2001). Among the sources of mitochondrial ROS production are the enzymes of electron transport chain that produce O_2^- , and H_2O_2 , and previous reports suggest that the levels of H_2O_2 produced by ETC enzymes increases with age (Sohal 1991; Sohal and Sohal 1991; Boveris and Navarro 2008). The enzymes are also susceptible to damage by ROS (Zhang *et al.* 1990; Navarro and Boveris 2004), which may lead to further increases in ROS production (Finkel and Holbrook 2000; Droge and Schipper 2007).

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

(Korshunov *et al.* 1997; Starkov *et al.* 2002; Starkov and Fiskum 2003). 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 (Sipos *et al.* 2003a; Tretter and Adam-Vizi 2007b). Complex I and complex III of the ETC are frequently cited as central contributors to mitochondrial H_2O_2 production (Turrens and Boveris 1980; Sugioka *et al.* 1988; Chen *et al.* 2003) and inhibition of electron transport at these sites using rotenone and antimycin A respectively invariably decreases $\Delta\psi_m$ and increase H_2O_2 production in the synaptosomal model (Sipos *et al.* 2003a, b; Tretter *et al.* 2004).

Maintenance of $\Delta\psi_p$ in the nerve terminal is essential for normal synaptic function (Scott and Nicholls 1980). Notable features of nerve terminal function that rely on $\Delta\psi_p$ include regulation of exocytotic release of neurotransmitter as well as neurotransmitter uptake from the synaptic cleft (Nicholls 1989, 1993). Neuronal $\Delta\psi_p$ is closely linked to mitochondrial activity: mitochondrial ATP drives the plasma membrane Na^+/K^+ ATPase, and mitochondrial uptake of Na^+ and Ca^{2+} contributes to the maintenance of low cytoplasmic concentrations of these cations (Erecinska *et al.* 1996). Depolarisation of $\Delta\psi_p$ by KCl has been shown to stimulate oxygen consumption in synaptosomes in a concentration dependent manner (Erecinska and Dagani 1990).

5.2 Materials and methods

5.2.1 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.* (1999). Synaptosomes were resuspended in incubation medium (4 mg/ml) and loaded with 6 μ M JC-1 for 15 min at 37°C. Following three washes in incubation medium, synaptosomes were diluted to 1 mg/ml and 100 μ l added to a pre-prepared 96-well plate containing 100 μ l incubation medium plus 2 μ l of ETC enzyme inhibitor, where appropriate, in each well. Each experimental condition was performed in triplicate on each plate. Fluorescence intensity at $\lambda = 590$ nm and at $\lambda = 535$ nm ($\lambda = 490$ nm emission) were recorded using

a SpectraMAX GeminiXS in kinetic mode (90 s interval). 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 $\lambda = 590$ nm to $\lambda = 535$ nm is a semi-quantitative measure of *in situ* mitochondrial membrane potential (Reers *et al.* 1995). In a parallel series of experiments, synaptosomes were incubated with identical ETC inhibitor concentrations and following a 15 min incubation period, samples were frozen at -80°C and stored. The activities of complexes I, III and complex IV activity were later assayed as described previously (in Sections 3.2 and 4.2) and the JC-1 ratio at 15 min plotted against the % inhibition of ETC enzyme activity.

5.2.2 Measurement of hydrogen peroxide production

H_2O_2 production was followed using the Amplex Red assay system (Mohanty *et al.* 1997). Synaptosomal pellets (1 mg) 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) and 100 μl added to 100 μl of the same medium containing amplex red and horseradish peroxidase (50 μM and 2.5 U/ml final concentrations, final volume 200 μl). Fluorescence intensity at emission $\lambda = 585$ nm ($\lambda = 550$ nm excitation) was recorded using a SpectraMAX GeminiXS in kinetic mode (32 s interval). Where appropriate, rotenone (10 μM final concentration), antimycin A (1 μM final concentration), or FCCP (10 nM – 1 μM final concentrations), were added in volumes of 2 μl .

5.2.3 LDH activity assay

Synaptosomal viability was estimated by measuring the rate oxidation of NADH to NAD^+ by lactate dehydrogenase in the presence of pyruvate. Synaptosomes (1 ml, 0.5 mg/ml) were incubated for 5 min in incubation medium in Eppendorf tubes (3 mM KCl, 140 mM NaCl, 25 mM Tris-HCl, 10 mM glucose, 2 mM MgCl_2 , pH 7.4) before being centrifuged at 3,000 g for 10 min and 100 μl of supernate extracted (blank). In another group, synaptosomes were incubated for 5 min in the same incubation medium before addition of 0.1% (v/v) of Triton X-100 before being centrifuged at 3,000 g for 10 min and 100 μl of supernate extracted. This gave a measure of the total synaptosomal LDH pool. In the remaining experiments FCCP (10 nM – 1 μM final

concentrations) was added to two groups of synaptosomes (200 μ l 0.5 mg/ml), one containing 1.4 mM CaCl_2 and the other with no Ca^{2+} in the medium and incubated for 180 min in a SpectraMAX GeminiXS well-plate reader with the plate set to shake every 30 s to prevent the synaptosomes settling at the bottom of the wells 3,000. The plate was then centrifuged at 3,000 g for 10 min and 100 μ l of supernate extracted from each well. The supernate samples were then frozen at -80°C and LDH activity was monitored by following the oxidation of NADH to NAD^+ at absorbance $\lambda = 340$ nm in an Agilent ChemStation 8453 spectrophotometer. The assay cuvettes contained 1 mM NADH, 1 mM pyruvate with a final volume of 1 ml of 10 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by the addition of 50 μ l supernate samples.

5.2.4 Measurement of $\Delta\psi_p$

$\Delta\psi_p$ was measured by modification of the method of (Nicholls 2006). An individual vial from a Molecular Devices “membrane potential assay kit, explorer format” (R-8042) containing was reconstituted in 1 ml of distilled water, dispensed into 50 μ l aliquots, and frozen. One 50 μ l was diluted to 10 ml with incubation medium (3 mM KCl, 140 mM NaCl, 25 mM Tris-HCl, 10 mM glucose, 2 mM MgCl_2 , pH 7.4), and 100 μ l added to 100 μ l of the same incubation medium with 0.1 mg of synaptosomal sample per well to give a final concentration of 0.5 mg/ml of sample. The assay mixture was allowed a 45 min pre-incubation time in the SpectraMAX GeminiXS well-plate reader with the plate set to shake every 30 s to prevent the synaptosomes settling at the bottom of the wells before experiments were initiated. Stock inhibitors (100 nM – 1 mM rotenone, 1 μ M – 1 mM myxothiazol, 500 μ M – 100 mM KCN stock concentrations) were diluted in Kreb’s to allow addition with a multi-channel pipette of a volume of 10 μ l. Fluorescence was recorded in a SpectraMAX GeminiXS well-plate reader in kinetic mode (45 s interval) at excitation $\lambda = 530$ nm and emission $\lambda = 565$ nm. The difference in fluorescence value after 15 min was divided by the initial fluorescence value and calibrated to $\Delta\psi_p$ in mV as described in Section 2.16. In a parallel series of experiments, synaptosomes were incubated with identical ETC inhibitor concentrations and following a 15 min incubation period, samples were frozen at -80°C and stored. The activities of complexes I, III and complex IV activity

were later assayed as described previously (in Sections 3.2 and 4.2) and the $\Delta\psi_p$ value at 15 min plotted against the % inhibition of ETC enzyme activity.

5.3 Results

5.3.1 Rotenone depolarises $\Delta\psi_m$ in *in situ* synaptosomal mitochondria

Synaptosomal mitochondria were partially depolarised by rotenone in a concentration dependent manner (Fig. 5.1). All concentrations of rotenone immediately reduced the JC-1 ratio upon addition to the medium, except for 1 nM, for which the ratio was significantly reduced from control after 15 min. $\Delta\psi_m$ continues to gradually depolarise at all concentrations of rotenone over the 30 min, but the highest concentration examined (10 μ M) does not completely depolarise $\Delta\psi_m$ in this time span, as addition of 1 μ M FCCP reduced the ratio from 2.13 ± 0.04 to 0.98 ± 0.03 .

When incubated with 2 mM 2-deoxyglucose (an inhibitor of glycolysis), the rotenone-induced depolarisation of $\Delta\psi_m$ was accelerated, with the drop in JC-1 ratio mostly occurring within the first 5 min (Fig. 5.2). The ratio in the presence of 25 nM rotenone dropped from 4.74 ± 0.07 to 2.70 ± 0.05 after 5 min, and was 1.61 ± 0.05 after 30 min. Addition of 1 μ M FCCP after 30 min did not affect the ratio in the presence of 1 μ M and 10 μ M rotenone, suggesting $\Delta\psi_m$ had been completely collapsed by this time by these concentrations of rotenone in combination with deoxyglucose.

Addition of the $\Delta\psi_p$ -depolarising reagent 4-aminopyridine had an immediate slight depolarising effect on $\Delta\psi_m$ at all concentrations of rotenone but not in the control (Fig. 5.3). However the effect was transient, as the rates of depolarisation by all concentrations of rotenone continued in a similar way to those in the absence of 4-aminopyridine (Fig. 5.1). A similar effect of transient depolarisation was recorded in the presence of deoxyglucose (Fig. 5.4).

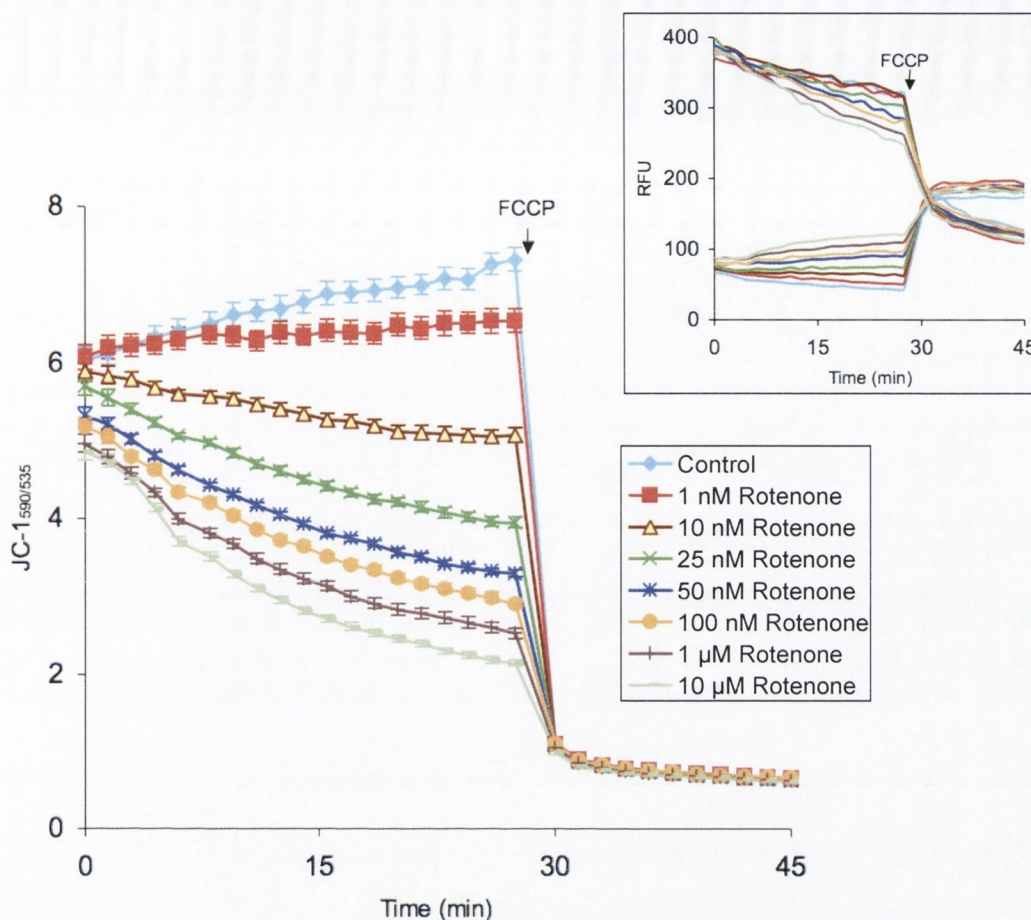


Fig. 5.1 Rotenone depolarises $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were preincubated with 6 μ M JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μ l at 0.5 mg/ml in wells containing rotenone (1 nM – 10 μ M). 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 time point. 1 μ M FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

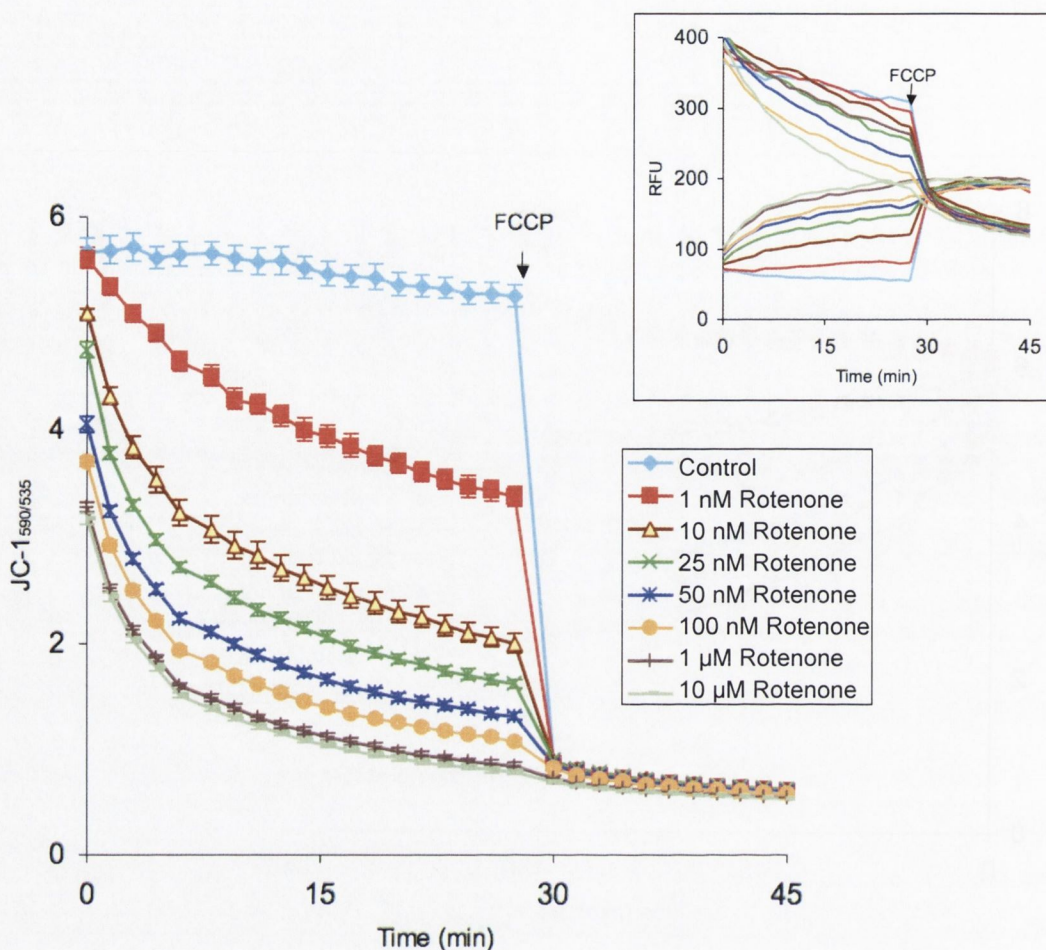


Fig. 5.2 Deoxyglucose accelerates rotenone-induced depolarisation of $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing rotenone (1 nM – 10 μM) and 2-deoxyglucose (2 mM). Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$) at each time point. 1 μM FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

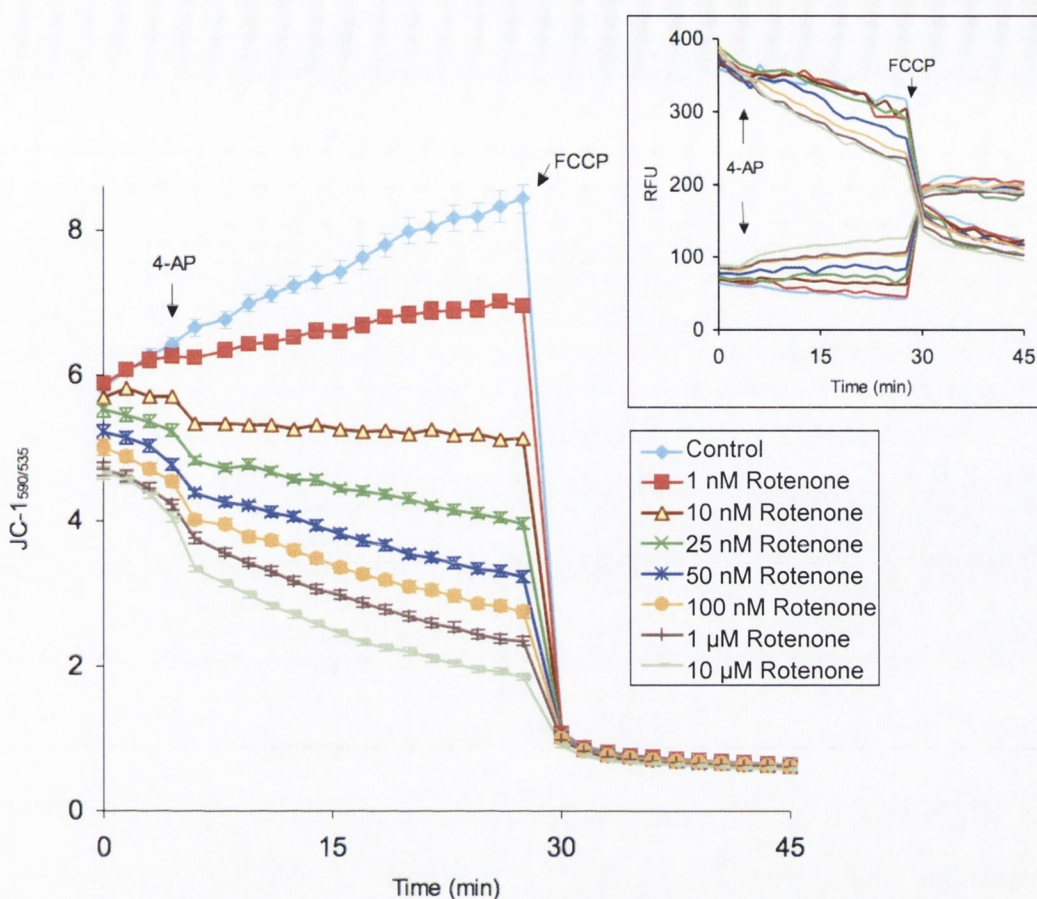


Fig. 5.3 4-aminopyridine causes a transient acceleration of rotenone-induced depolarisation of $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing rotenone (1 nM – 10 μM). 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 time point. 1 μM FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

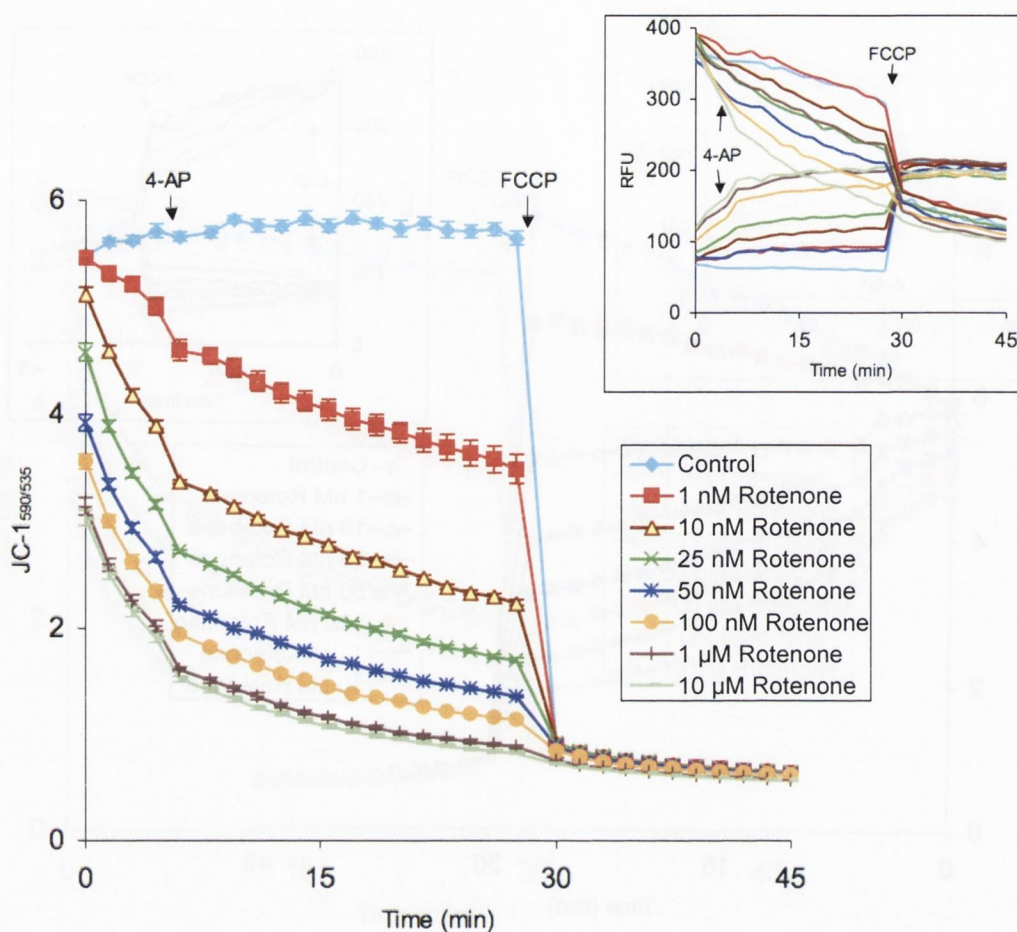


Fig. 5.4 4-aminopyridine causes a transient acceleration of rotenone- and deoxyglucose-induced depolarisation of $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing rotenone (1 nM – 10 μM) and 2-deoxyglucose (2 mM). Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$) at each time point. 1 μM FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

5.3.2 Partial inhibition of complex I activity causes partial depolarisation of $\Delta\psi_m$ in *in situ* synaptosomal mitochondria

The JC-1 ratio was found to be 6.88 ± 0.14 in synaptosomes after 15 min incubation in the presence of 10 mM glucose (Fig. 5.5, 0% inhibition). Inhibition of complex I activity by $21.5 \pm 10\%$ (using 1 nM rotenone) caused a 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 $37.3 \pm 6.3\%$ inhibition. Using 10 μ M rotenone to cause $96.7 \pm 2.6\%$ inhibition of complex I activity, the ratio was 2.70 ± 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.

The JC-1 ratio was reduced from 6.88 ± 0.14 to 5.45 ± 0.11 after 15 min in the presence of the glycolytic inhibitor 2-deoxyglucose (Fig. 5.5). $20.2 \pm 9.2\%$ (1 nM rotenone) and $25.9 \pm 8.6\%$ (10 nM rotenone) inhibition reduced the ratio to 3.93 ± 0.1 and 2.52 ± 0.11 respectively. At $44.0 \pm 7.0\%$ 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 $99.4 \pm 2.0\%$ inhibition of complex I activity (10 μ M rotenone) was not significantly different from the value after addition of FCCP, suggesting a complete collapse in $\Delta\psi_m$ under these conditions.

5.3.3 Myxothiazol partially depolarises $\Delta\psi_m$ at concentrations > 100 nM in *in situ* synaptosomal mitochondria

An initial control JC-1 ratio of 5.74 ± 0.12 was found in control synaptosomes, and this ratio was not significantly different in the presence of any concentration of myxothiazol (Fig. 5.6). However, after 5 min the ratio was reduced from 6.63 ± 0.16 in control synaptosomes to 6.10 ± 0.14 in the presence of 1 μ M myxothiazol, and 4.48 ± 0.10 in the presence of 10 μ M. After 30 min, the ratio was reduced from 7.13 ± 0.18 to 3.45 ± 0.07 by 1 μ M and 2.03 ± 0.05 by 10 μ M. The ratio fell to 0.82 ± 0.01 after addition of 1 μ M FCCP to the 10 μ M myxothiazol group, indicating $\Delta\psi_m$ was still partially maintained after 30 min at this concentration of myxothiazol.

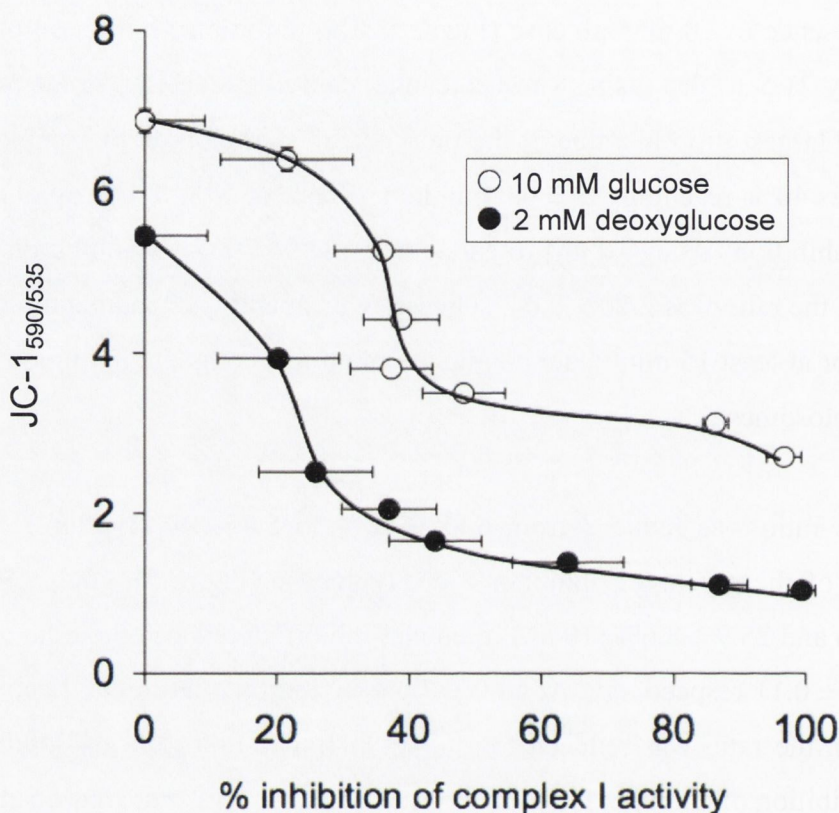


Fig. 5.5 Partial inhibition of complex I activity depolarises $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were loaded with JC-1 and resuspended (0.5 mg/ml) with rotenone (1 nM - 10 μ M) and in the presence of 10 mM glucose or 2 mM 2-deoxyglucose (in the absence of Ca^{2+}). Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$) 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 $\pm$ SEM for experiments performed in triplicate on 3 separate synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. Addition of 1 μ M FCCP, an ionophore which causes a complete collapse of $\Delta\psi_m$, results in a ratio of 1.08 ± 0.04 in control synaptosomes.

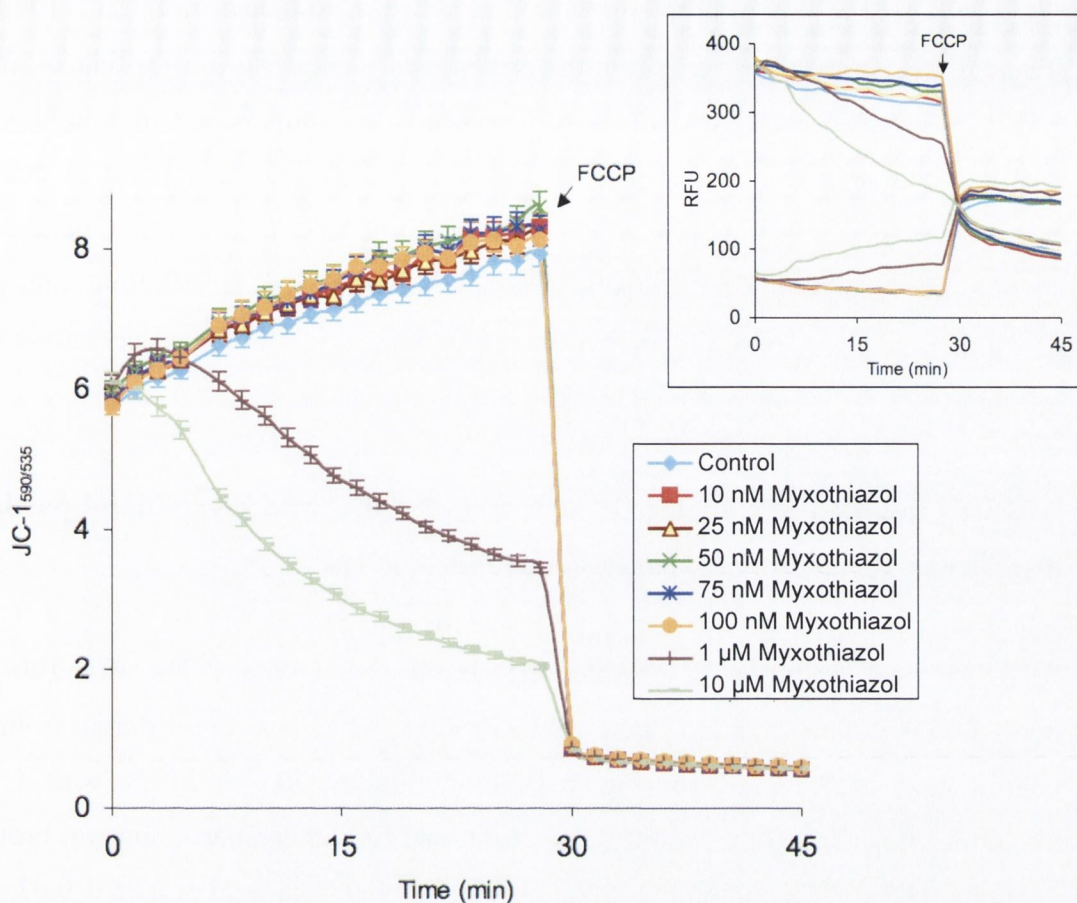


Fig. 5.6 Myxothiazol depolarises $\Delta\psi_m$ at highest concentrations in *in situ* synaptosomal mitochondria.

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing myxothiazol (10 nM – 10 μM). 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 time point. 1 μM FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

When 4-aminopyridine was added to the myxothiazol titration, there was no effect on $\Delta\psi_m$ in the control group, or at any concentration of myxothiazol from 10 – 100 nM (Fig. 5.7). The reductions in ratio in the presence of 1 and 10 μ M myxothiazol, from 6.51 ± 0.17 to 5.37 ± 0.08 and 4.01 ± 0.11 respectively, immediately after addition of 4-aminopyridine, were greater than those seen at the same time point in control experiments (Fig. 5.6). However, as with the rotenone titration, the pattern of depolarisation following the initial 4-aminopyridine-induced acceleration continued as it did in the absence of 4-aminopyridine (Fig. 5.6), suggesting the acceleration was transient.

5.3.4 Inhibition of complex III activity by > 70% with myxothiazol partially depolarises $\Delta\psi_m$ in *in situ* synaptosomal mitochondria

After 15 min, inhibition of complex III activity by $71.7 \pm 2.7\%$ with 100 nM myxothiazol resulted in a JC-1 ratio of 7.74 ± 0.18 , which was not different from the control ratio of 7.25 ± 0.16 (Fig. 5.8). Inhibition by $76.0 \pm 1.8\%$ with 1 μ M myxothiazol decreased the ratio to 4.58 ± 0.11 , and further depolarisation was brought about by $94.8 \pm 2.3\%$ inhibition of complex III activity, to a ratio of 2.86 ± 0.07 . The ratio in the presence of 1 μ M FCCP was 1.08 ± 0.04 at this time point, suggesting $\Delta\psi_m$ in *in situ* synaptosomal mitochondria can be partially maintained under conditions of almost complete inhibition of complex III activity.

After 10 min incubation with 4-aminopyridine and 5 min pre-incubation, the JC-1 ratio of 7.31 ± 0.16 was not different from control synaptosomes (Fig. 5.8). $68.1 \pm 2.4\%$ inhibition of complex III activity with 100 nM myxothiazol in the presence of 4-aminopyridine had no significant effect on $\Delta\psi_m$ (ratio 7.66 ± 0.14). $72.0 \pm 3.3\%$ inhibition by 1 μ M myxothiazol decreased the ratio to 4.02 ± 0.06 and $99.9 \pm 3.71\%$ to 2.62 ± 0.07 by 10 μ M. These data indicate that inhibition of complex III activity by up to 70% with myxothiazol does not affect $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

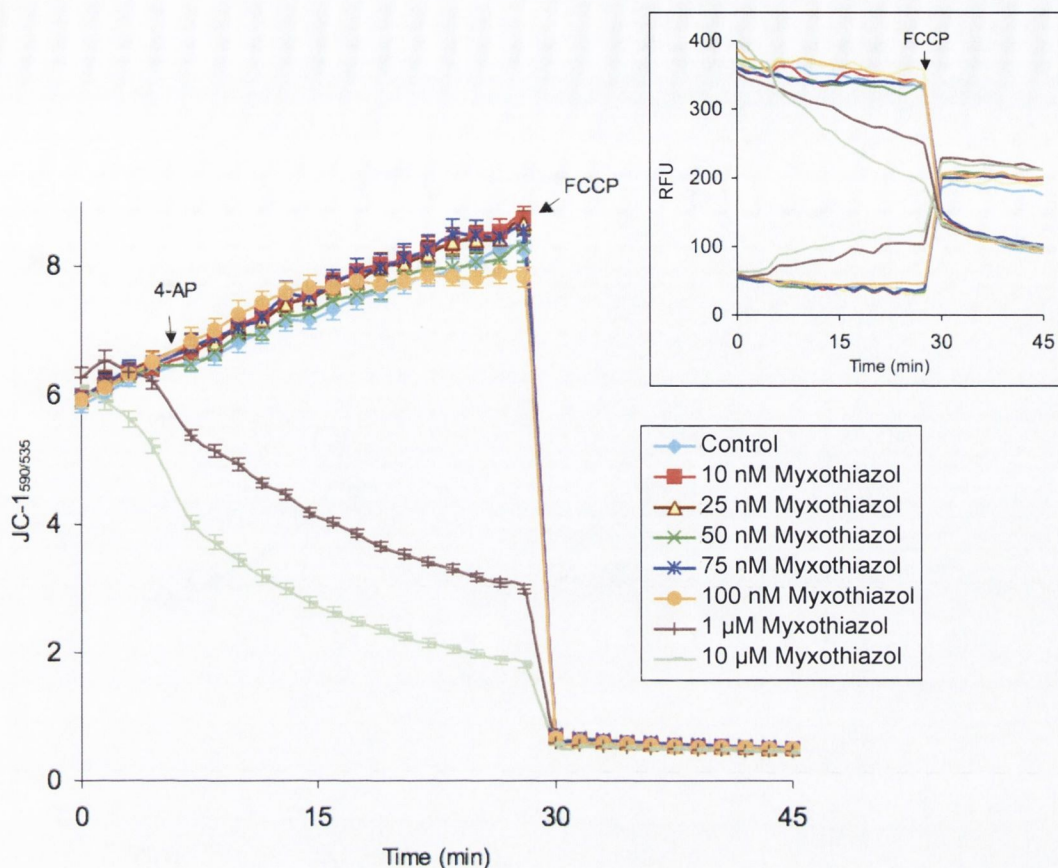


Fig. 5.7 4-aminopyridine causes a transient acceleration of myxothiazol-induced depolarisation of $\Delta\psi_m$.

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing myxothiazol (10 nM – 10 μM). 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 time point. 1 mM 4-aminopyridine was added after 5 min. 1 μM FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

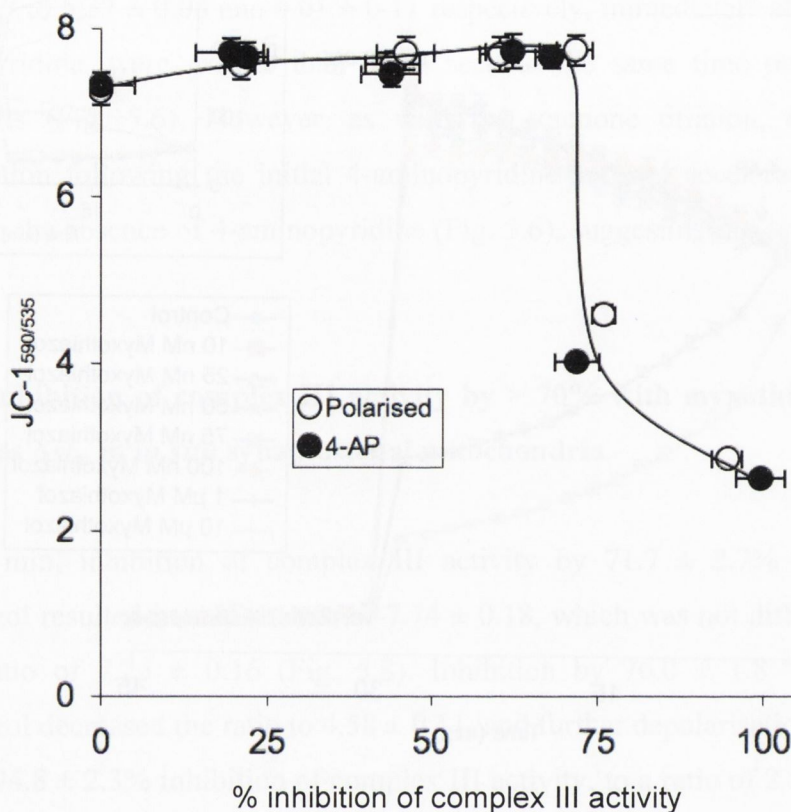


Fig. 5.8 Inhibition of complex III activity by > 70% with myxothiazol partially depolarises $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were loaded with JC-1 and resuspended (0.5 mg/ml) with myxothiazol (10 nM - 10 μ M) and in the absence of Ca^{2+} for 15 min. Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$) at each concentration of myxothiazol. The resulting ratios were plotted against percent inhibition of complex III activity brought about by that concentration of myxothiazol. Points shown represent the mean $\pm$ SEM for experiments performed in triplicate on 3 separate synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. Addition of 1 μ M FCCP, an ionophore which causes a complete collapse of $\Delta\psi_m$, results in a ratio of 1.08 ± 0.04 in control synaptosomes.

5.3.5 Antimycin A slightly depolarises $\Delta\psi_m$ in *in situ* synaptosomal mitochondria at highest concentrations

The initial control JC-1 ratio of 5.62 ± 0.07 was not immediately affected by any concentration of antimycin A (Fig. 5.9). After 15 min, $\Delta\psi_m$ began to depolarise in the presence of 40 nM and 50 nM antimycin A, and after 30 min, the ratio was reduced from 7.62 ± 0.11 to 6.45 ± 0.05 and 6.26 ± 0.07 respectively by these concentrations of inhibitor. The addition of 4-aminopyridine had an immediate depolarising effect on $\Delta\psi_m$ in groups incubated with 30, 40 and 50 nM antimycin A (Fig 5.10). The 4-aminopyridine-induced acceleration of depolarisation of continued over 30 min, and after this time the ratio was reduced from 7.83 ± 0.01 in control to 6.59 ± 0.03 in 30 nM, 6.10 ± 0.07 in 40 nM, and 5.62 ± 0.06 in 50 nM antimycin A. Addition of 1 μ M FCCP resulted in an immediate fall in ratio to 0.82 ± 0.01 in control synaptosomes, suggesting the depolarisation was slight.

5.3.6 Inhibition of complex III activity by up to 90% with antimycin A does not affect $\Delta\psi_m$ in *in situ* synaptosomal mitochondria

After 15 min, $93.9 \pm 0.4\%$ inhibition of complex III activity with 50 nM antimycin A brought about a JC-1 ratio of 6.39 ± 0.06 , which was not significantly different from the ratio in the absence of inhibitor, which was found to be 6.89 ± 0.06 (Fig. 5.11). This indicates that using antimycin A to inhibit complex III results in no effect on $\Delta\psi_m$ over this time in *in situ* synaptosomal mitochondria, even at highest levels of inhibition. In synaptosomes in which 4-aminopyridine was added after 5 min, the JC-1 ratio after 15 min was reduced from 7.08 ± 0.06 to 6.17 ± 0.03 by $93.7 \pm 0.61\%$ inhibition, brought about by 40 nM antimycin A. Inhibition by $94.2 \pm 0.62\%$ with 50 nM antimycin A resulted in a ratio of 5.77 ± 0.62 . Although these ratios indicate a significant fall in $\Delta\psi_m$ by these levels of inhibition, the ratio of 1.08 ± 0.04 brought about by 1 μ M FCCP suggest that the magnitude of depolarisation was minimal.

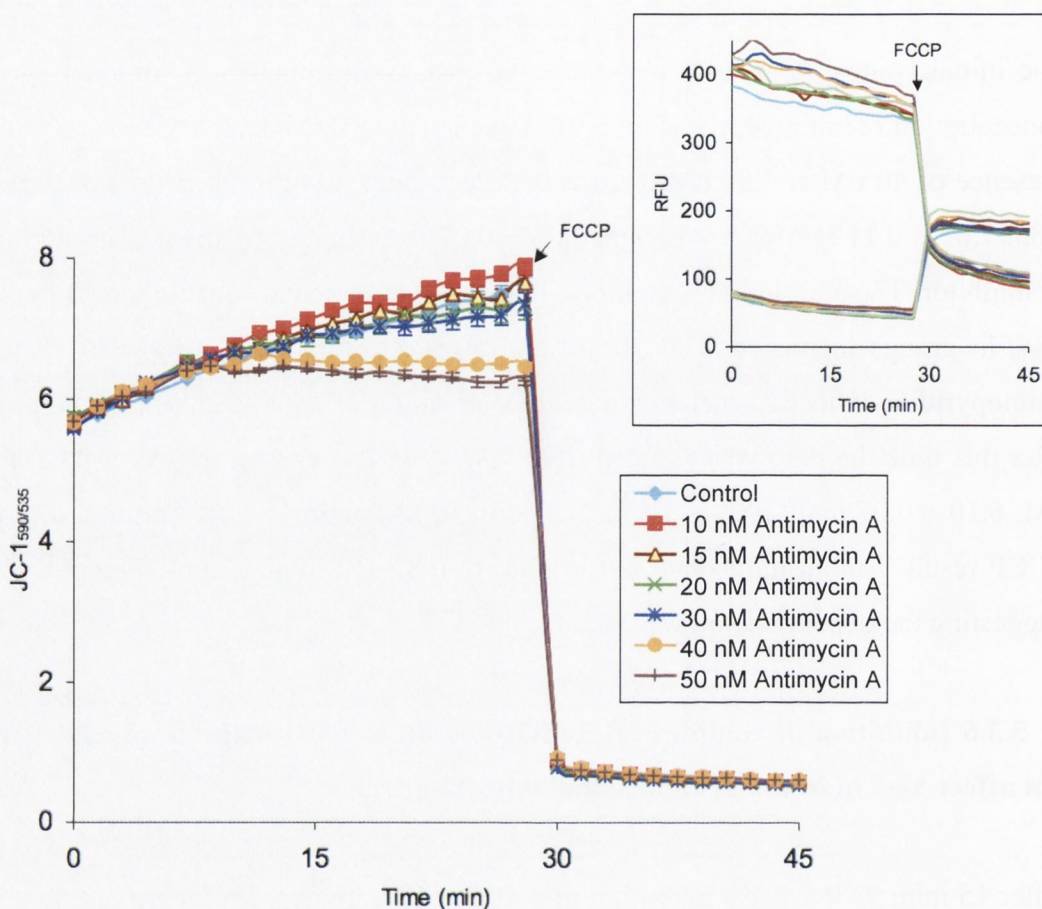


Fig. 5.9 Antimycin A partially depolarises $\Delta\psi_m$ at highest concentrations in *in situ* synaptosomal mitochondria.

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing antimycin A (10 – 50 nM). Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$) at each time point. 1 μM FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

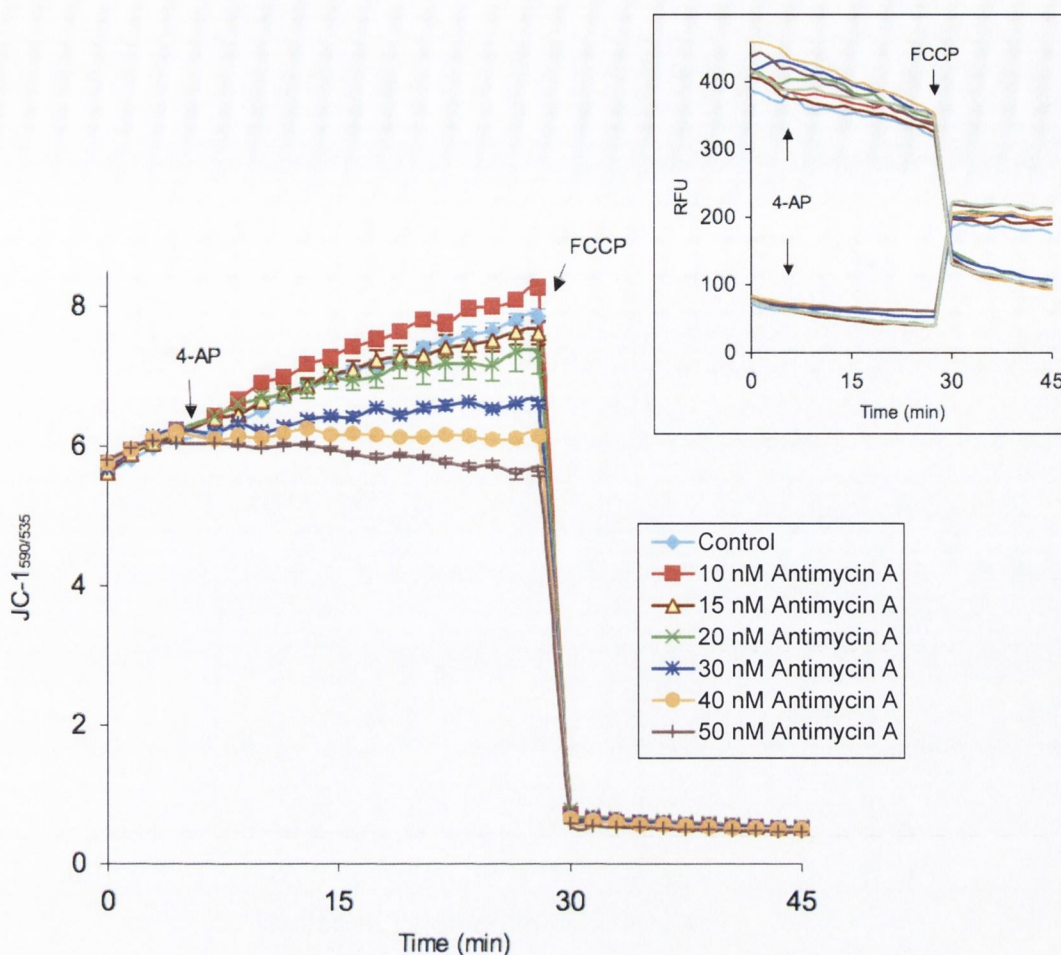


Fig. 5.10 4-aminopyridine accelerates antimycin A-induced depolarisation of $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing antimycin A (10 – 50 nM). Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$) at each time point. 1 μM FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

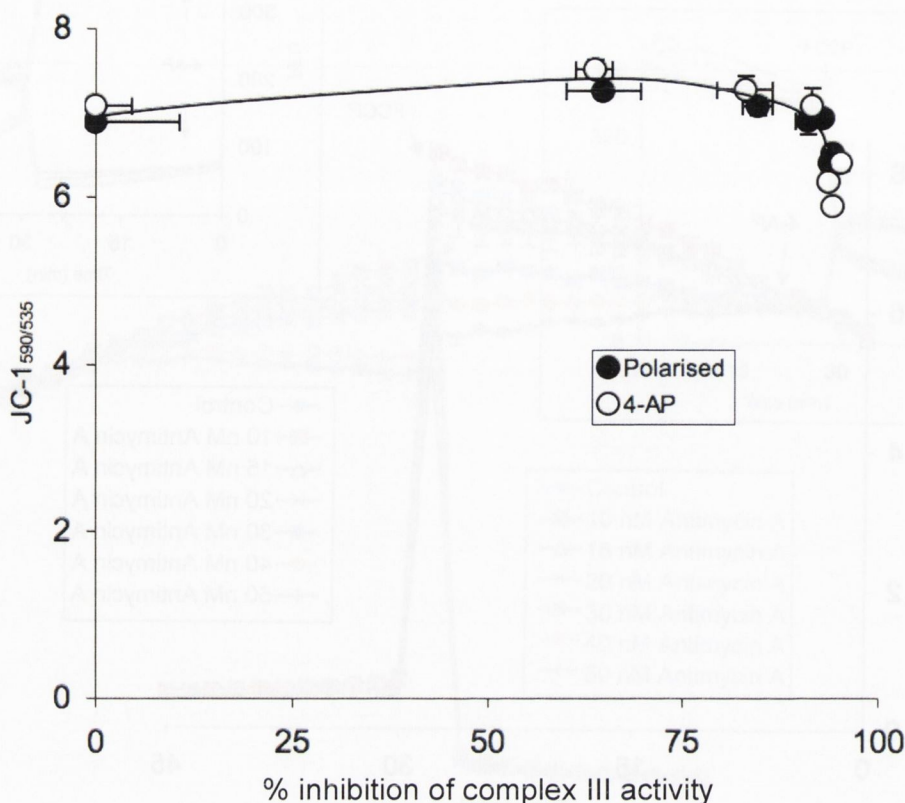


Fig. 5.11 Inhibition of complex III activity by > 90% with antimycin A partially depolarises $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were loaded with JC-1 and resuspended (0.5 mg/ml) with antimycin A (10 nM – 50 nM) and in the absence of Ca^{2+} for 15 min. Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$) at each concentration of antimycin A. The resulting ratios were plotted against percent inhibition of complex III activity brought about by that concentration of antimycin A. Points shown represent the mean $\pm$ SEM for experiments performed in triplicate on 3 separate synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. Addition of 1 μM FCCP, an ionophore which causes a complete collapse of $\Delta\psi_m$, results in a ratio of 1.08 ± 0.04 in control synaptosomes.

5.3.7 KCN causes a partial transient depolarisation of $\Delta\psi_m$ in *in situ* synaptosomal mitochondria

The initial control JC-1 ratio of 5.20 ± 0.07 was not affected by any concentration of KCN (Fig. 5.12). After 5 min the ratio reduced from 5.89 ± 0.09 to 5.00 ± 0.21 by 1 mM KCN ($p < 0.05$), but was not affected by any lower concentration of KCN. After this time point, the difference ratio in the presence of 1 mM compared to control began to decrease, and was no longer present after 20 min, when the ratio in the control group was 6.49 ± 0.12 and 6.39 ± 0.25 in the presence of 1 mM KCN. A similar pattern was observed in the presence of 4-aminopyridine, which did not affect $\Delta\psi_m$ in the presence of any concentration of KCN (Fig. 5.13).

5.3.8 Inhibition of complex IV activity has no effect on $\Delta\psi_m$ in *in situ* synaptosomal mitochondria

Inhibition of complex IV activity by $87.9 \pm 2.4\%$ with 1 mM KCN for 15 min resulted in a JC-1 ratio of 6.89 ± 0.20 (Fig. 5.14). The ratio of 6.29 ± 0.11 was not significantly different from this or any ratio brought about by any other level of inhibition of complex IV activity. In the presence of 4-aminopyridine, $88.1 \pm 3.7\%$ inhibition of complex IV activity did not affect the ratio (7.14 ± 0.17 compared to 6.43 ± 0.09 at 0% inhibition, Fig. 5.14).

5.3.9 Oligomycin partially depolarises $\Delta\psi_m$ in *in situ* synaptosomal mitochondria

The initial control JC-1 ratio of 5.54 ± 0.17 was not found to be different in the presence of 20 $\mu\text{g/ml}$ oligomycin (5.45 ± 0.16 , Fig 5.15). However, $\Delta\psi_m$ began to depolarise immediately after this point and the difference in JC-1 ratio was at a maximum after 10 min, when the ratio was reduced from 6.41 ± 0.23 to 5.02 ± 0.16 in the presence of the complex V inhibitor. Although oligomycin did not depolarise $\Delta\psi_m$ any further after this time point, however the membrane potential recovered to the level of the control group after 30 min.

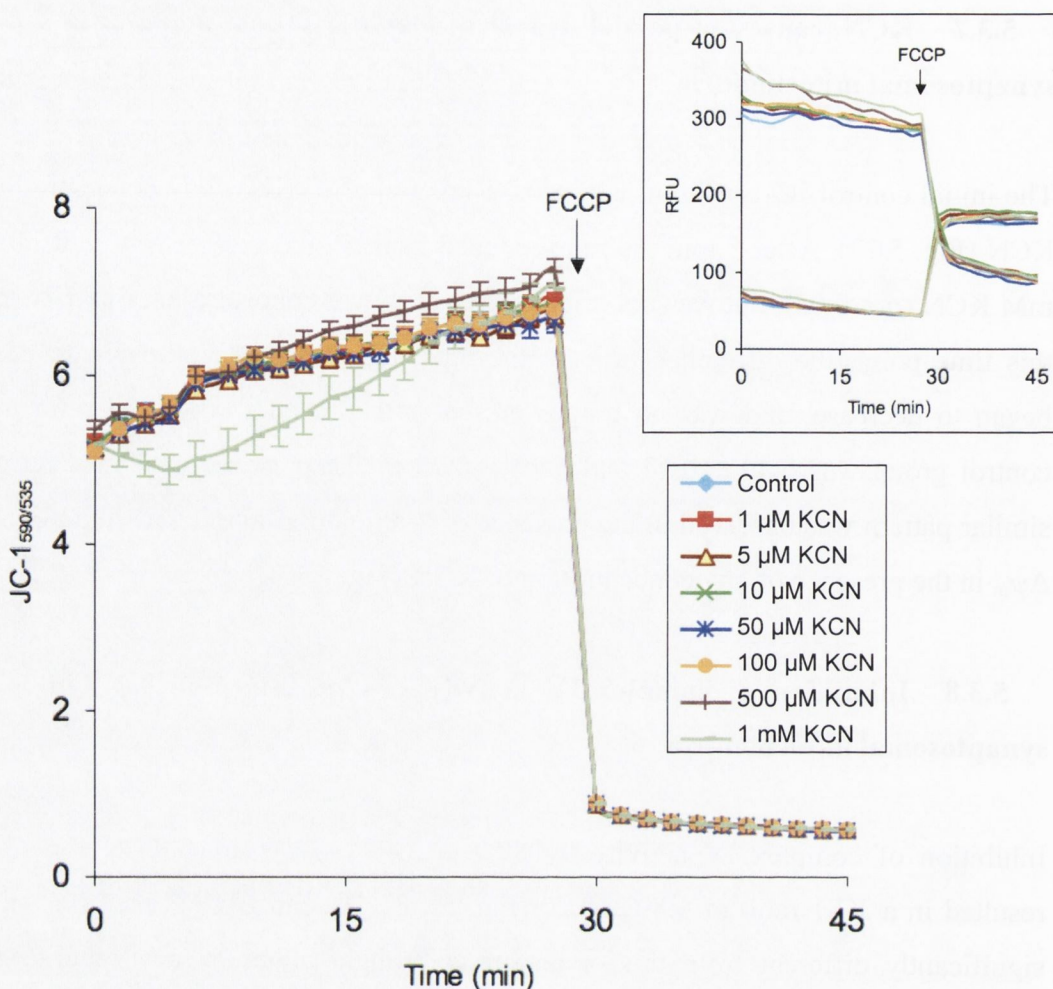


Fig. 5.12 1 mM KCN causes a partial transient depolarisation of $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing 2 μl KCN in H₂O (at the final concentrations 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) at each time point. 1 μM FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

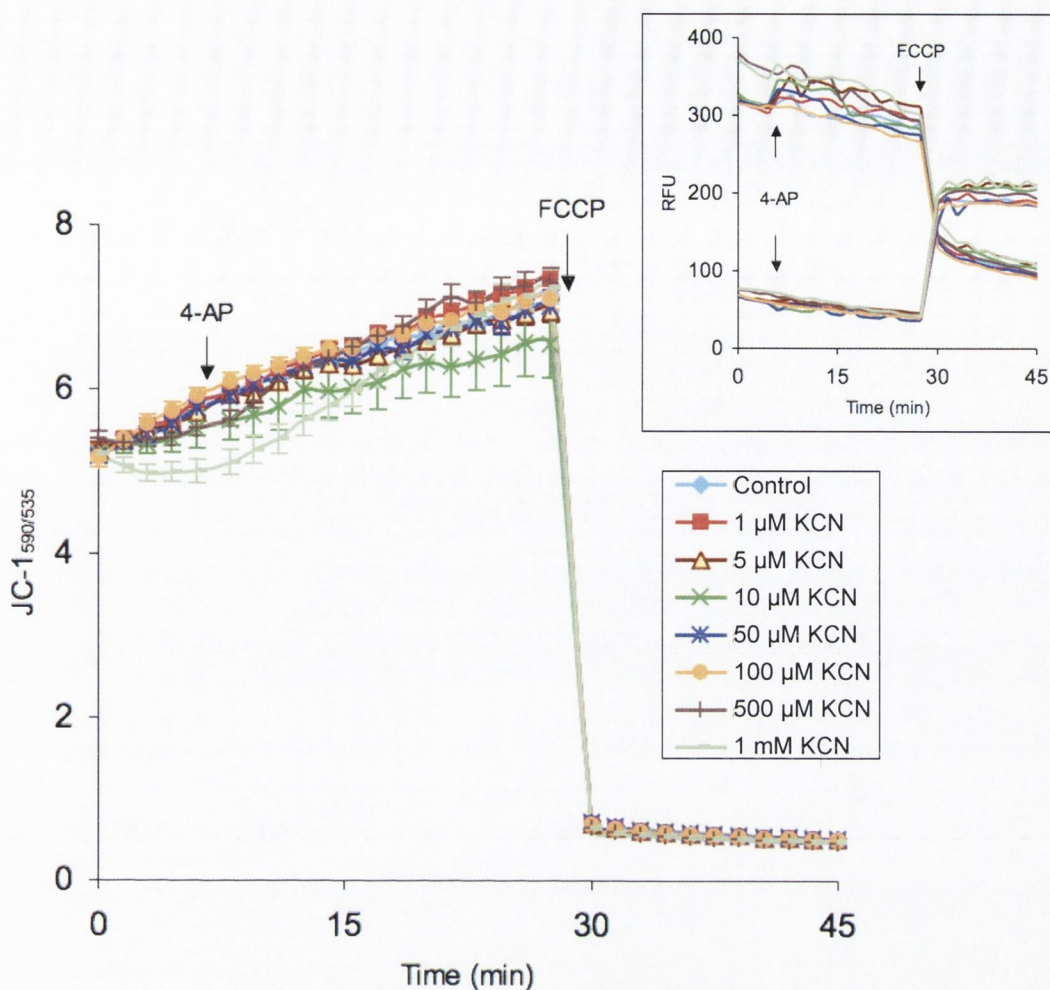


Fig. 5.13 4-aminopyridine does not affect $\Delta\psi_m$ in the presence of KCN

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing 2 μl KCN in H₂O (at the final concentrations 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) at each time point. 1 mM 4-aminopyridine was added after 5 min. 1 μM FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean ± SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

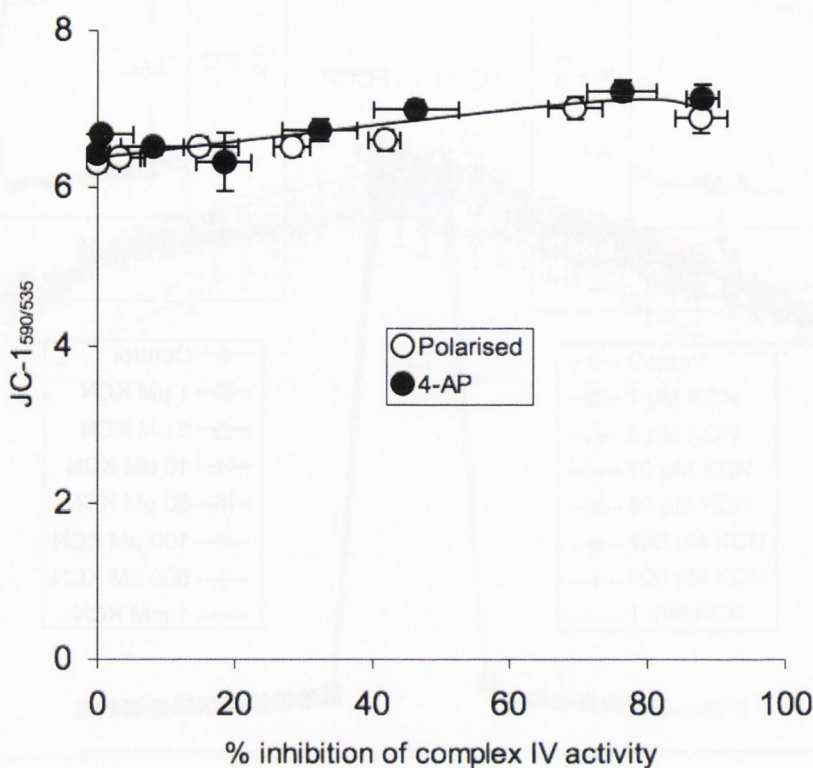


Fig. 5.14 Inhibition of complex IV activity has no effect on $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were loaded with JC-1 and resuspended (0.5 mg/ml) with KCN (1 μ M – 1 mM) and in the absence of Ca^{2+} for 15 min. Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$) at each concentration of KCN. The resulting ratios were plotted against percent inhibition of complex III activity brought about by that concentration of KCN. Points shown represent the mean $\pm$ SEM for experiments performed in triplicate on 3 separate synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. Addition of 1 μ M FCCP, an ionophore which causes a complete collapse of $\Delta\psi_m$, results in a ratio of 1.08 ± 0.04 in control synaptosomes.

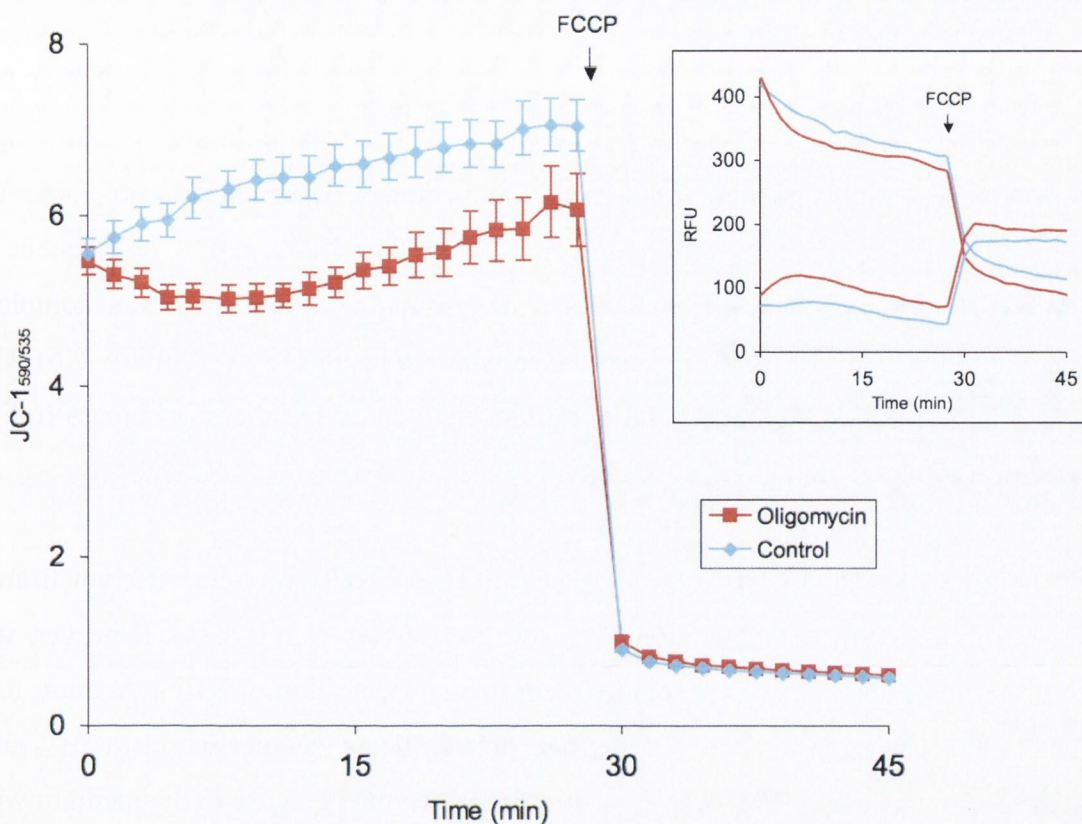


Fig. 5.15 Oligomycin partially depolarises $\Delta\psi_m$ in *in situ* synaptosomal mitochondria.

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing oligomycin (at 20 $\mu\text{g/ml}$ final concentration). Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$) at each time point. 1 μM FCCP was added where indicated to collapse $\Delta\psi_m$. Results represent the mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. Where no error bar is shown, the SEM falls within the size of the symbol. **Inset:** Typical fluorescence traces at 590 nm (upper traces) and 535 nm (lower traces).

5.3.10 Oligomycin potentiates depolarisation of $\Delta\psi_m$ brought about by metabolic inhibitors

After 15 min incubation, the JC-1 ratio of 6.59 ± 0.23 was reduced to 5.34 ± 0.22 by 20 $\mu\text{g/ml}$ oligomycin (Fig. 5.16). 10 μM rotenone reduced the ratio to 2.70 ± 0.04 , and when combined with oligomycin was 1.58 ± 0.20 . The ratio after substituting 10 mM glucose in the medium with 2 mM 2-deoxyglucose was reduced from 5.46 ± 0.12 to 4.02 ± 0.43 by addition of oligomycin. The ratio of 1.04 ± 0.03 with rotenone and deoxyglucose was not different from the group in which $\Delta\psi_m$ was completely collapsed by 1 mM FCCP, (in accordance with the result observed in Fig. 5.5) which was 0.88 ± 0.03 . Oligomycin did not affect $\Delta\psi_m$ under these circumstances (ratio of 1.26 ± 0.17).

The ratio of 6.08 ± 0.24 in the presence of 1 mM KCN was not different from the control group (in accordance with the result observed in Fig. 5.14). However, when 20 $\mu\text{g/ml}$ oligomycin was combined with this concentration of KCN, the ratio fell to 2.38 ± 0.12 , suggesting the occurrence of significant depolarisation of $\Delta\psi_m$ under these circumstances (Fig. 5.16). Substitution of 10 mM glucose in the medium with 2 mM 2-deoxyglucose did not affect this ratio, but with KCN in the absence of oligomycin, deoxyglucose caused the ratio to fall to 3.90 ± 0.17 after 15 min.

5.3.11 $\Delta\psi_m$ decreases with age in *in situ* synaptosomal mitochondria

$\Delta\psi_m$ was lower in *in situ* synaptosomal mitochondria from 18 month old rats compared to that from 6 month old rats ($p < 0.001$, Fig. 5.17). In synaptosomes isolated from 6-month old rats, the ratio was found to be 8.00 ± 0.29 , while in those from 18-month old, the ratio was found to be 7.22 ± 0.22 . 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 $\mu\text{g/ml}$ 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 synaptosomes. 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.

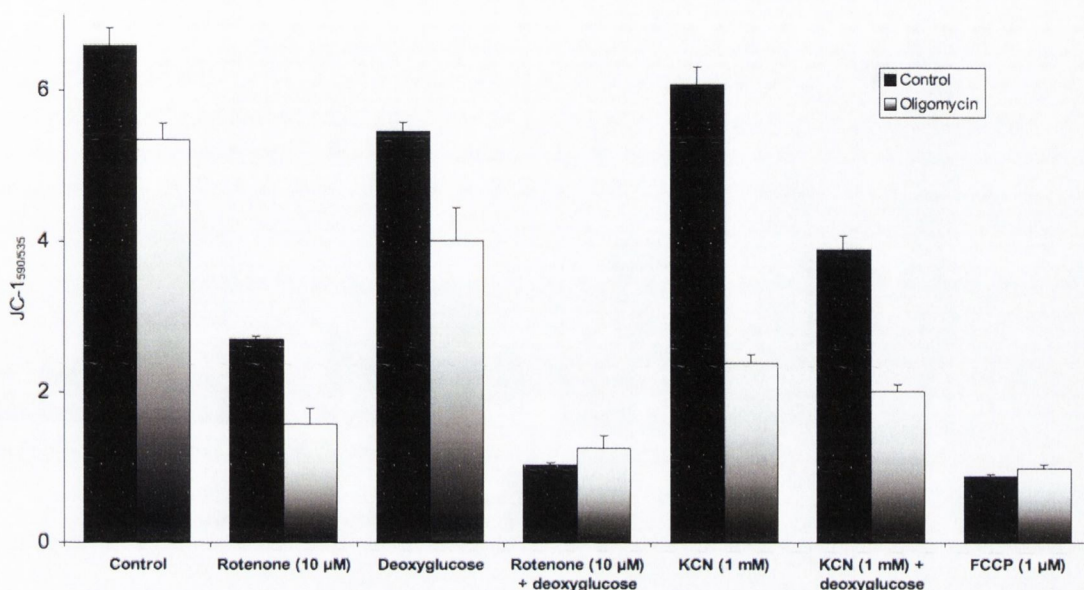


Fig. 5.16 Oligomycin potentiates depolarisation of $\Delta\psi_m$ brought about by metabolic inhibitors

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing 2 μl ETC inhibitors in ethanol/ H_2O . Oligomycin (20 $\mu\text{g}/\text{ml}$) was added where shown. Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$ nm) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$ nm) after 15 min of incubation. 1 μM FCCP collapses $\Delta\psi_m$. Results represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations.

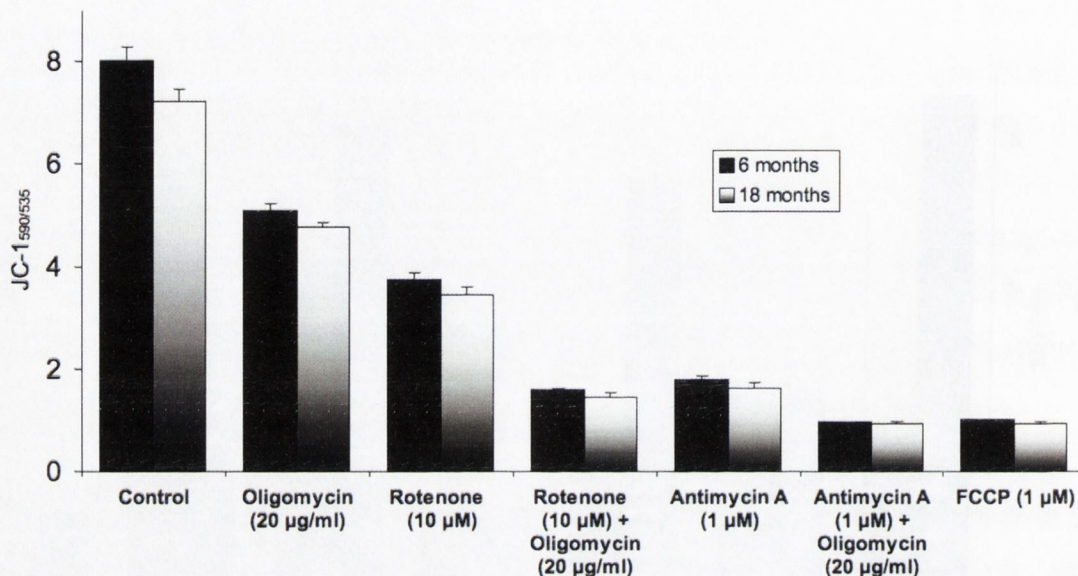


Fig. 5.17 Age-related decrease in $\Delta\psi_m$ in synaptosomes

Synaptosomes were preincubated with 6 μ M JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μ l at 0.5 mg/ml in wells containing 2 μ l ETC inhibitors in ethanol. Fluorescence intensity of the aggregate form of JC-1 (excitation λ = 490 nm, emission λ = 590 nm) was divided by that of the monomer (excitation λ = 490 nm, emission λ = 535 nm) after 15 min of incubation. 1 μ M FCCP completely collapses $\Delta\psi_m$. Results represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations.

Combined inhibition of complex III and complex V using 1 μ M antimycin A and 20 μ g/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 μ M antimycin A in 6-month (1.78 ± 0.24) and 18-month (1.63 ± 0.10) samples. It was found that incubation with oligomycin significantly reduced $\Delta\psi_m$ in synaptosomes from 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.

5.3.12 Depolarisation of $\Delta\psi_m$ reduces ROS production in synaptosomes

The initial rate of H_2O_2 production from synaptosomes was found to be 1.84 ± 0.05 pmol/min/mg protein. When 1.4 mM $CaCl_2$ was added to the medium, the rate was significantly faster, at 2.30 ± 0.06 pmol/min/mg. The presence of a range of concentrations of FCCP (10 nM – 1 μ M) did not affect the initial rate of production of H_2O_2 in either of these groups. In the absence of calcium, the rate increased spontaneously after ~ 90 min. (Fig. 5.18, left inset). The increased rate after 90 min was found to be 2.81 ± 0.02 pmol/min/mg. The extent of the increase was dependent on the concentration of FCCP (Fig. 5.18). Incubation with 200 nM resulted in a rate of 2.61 ± 0.02 pmol/min/mg, and the effect of higher concentrations was inversely proportional to the rate of production. The highest concentration, 1 μ M, caused a rate of 2.08 ± 0.07 pmol/min/mg. The rate of H_2O_2 production over this period in the presence of calcium, 1.68 ± 0.01 pmol/min/mg, was slower than the initial rate, but no significant effect was on this rate shown to occur at any concentration of FCCP.

FCCP was shown to depolarise $\Delta\psi_m$ in a concentration dependent manner using the JC-1 assay (Fig. 5.19). The control JC-1 ratio after 15 min of 7.34 ± 0.04 was reduced to 6.23 ± 0.03 by 10 nM FCCP. 1 μ M brought about the lowest ratio of 0.78 ± 0.02 .

The effect of FCCP on synaptosomal H_2O_2 production was shown to be genuine by carrying out a synaptosomal viability test using the LDH assay. This assay showed that upon addition of synaptosomes to the medium 4 – 6% have already become non-viable (Fig. 5.20). After 3 hrs incubation, 16 – 22% of the total LDH pool was released by synaptosomes. The presence of FCCP at any concentration examined does not affect the synaptosomal viability after this time (Fig. 5.20).

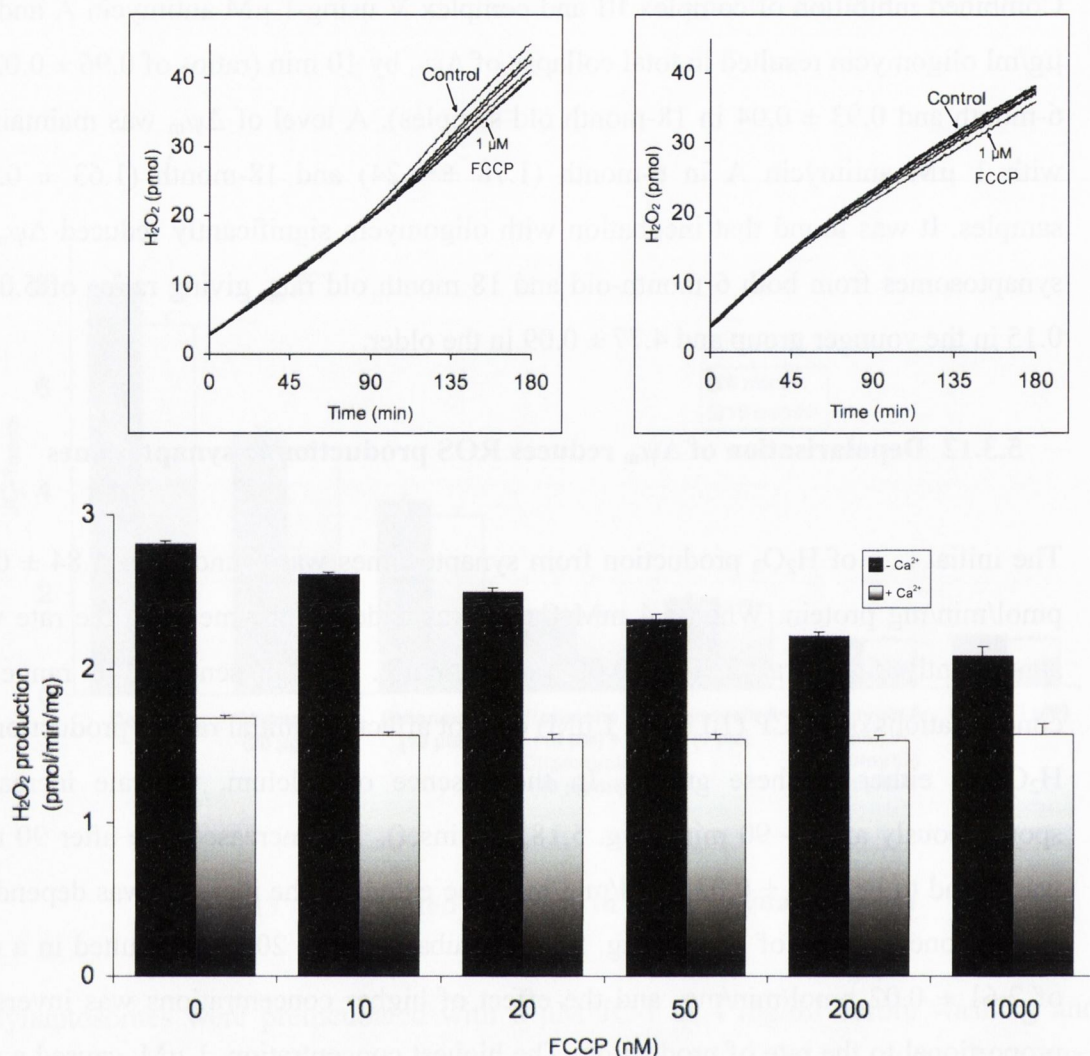


Fig. 5.18 FCCP decreases H_2O_2 production from synaptosomes after 90 min

Synaptosomes (0.5 mg/ml) were incubated with FCCP at the concentrations shown and H_2O_2 production was followed continuously using the amplex red assay system monitoring fluorescence at 585 nm (excitation 550 nm). Rates shown were calculated over the 90 – 180 min time period and represent the mean + SEM of experiments performed in triplicate on 3 synaptosomal preparations **Insets:** Typical traces of H_2O_2 production in the presence of FCCP (10 nM – 1 μM) and no Ca^{2+} (left) or 1.4 mM $CaCl_2$ added to the medium (right).

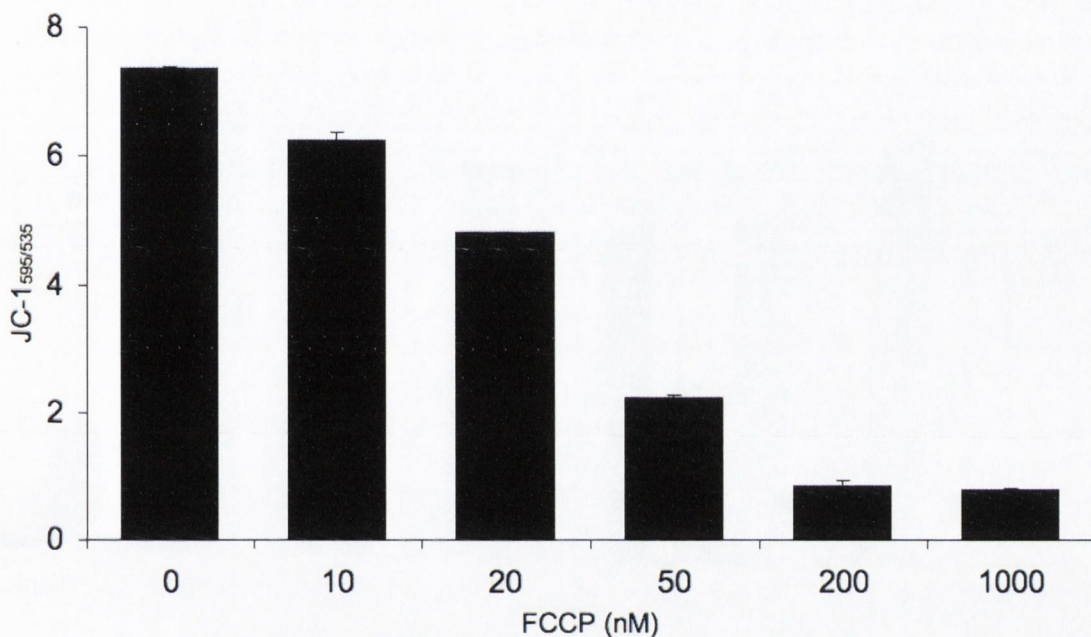


Fig. 5.19 FCCP depolarises $\Delta\psi_m$ in a concentration-dependent manner.

Synaptosomes were preincubated with 6 μM JC-1 at 4 mg/ml before washing and resuspended to a final volume of 200 μl at 0.5 mg/ml in wells containing FCCP (10 nM – 1 μM). Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$ nm) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$ nm) after 15 min of incubation. Results represent the mean + SEM of experiments done in triplicate on 3 synaptosomal preparations.

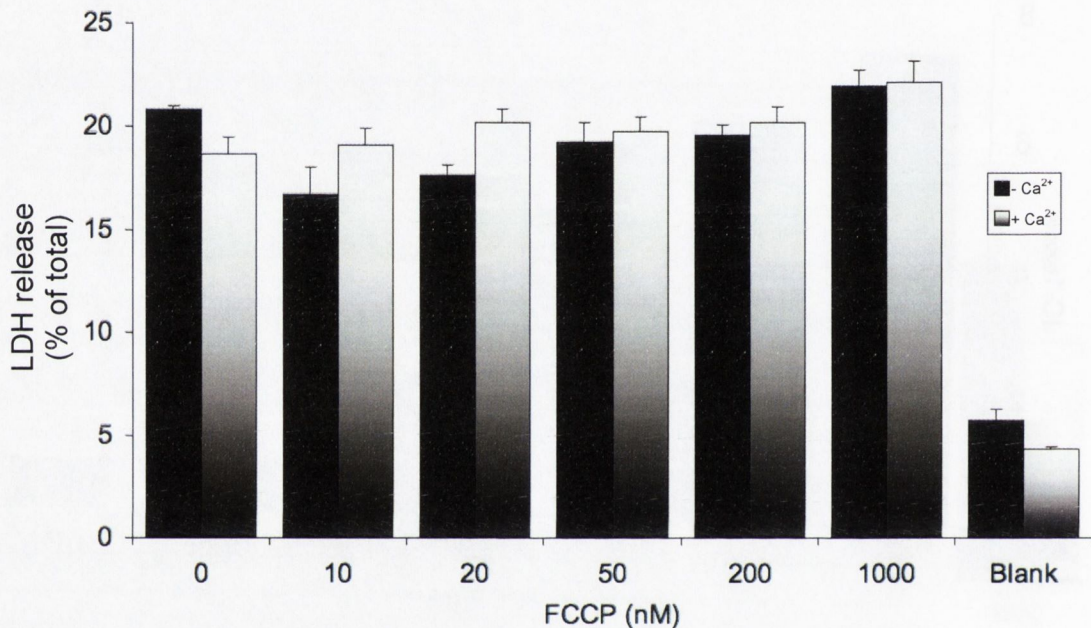


Fig. 5.20 FCCP has no effect on synaptosomal viability after 3 hours.

Synaptosomes (0.5 mg/ml) were incubated in Kreb's buffer for 3 hours in the presence of FCCP at final concentrations shown. 'Blank' synaptosomes were incubated for 5 min. Triton X-100 (0.5% v/v) was added to one group to release the total synaptosomal LDH pool. Following incubation, synaptosomes were centrifuged and supernates assayed for LDH activity. Data shown represent the mean + SEM of experiments done in triplicate on 3 separate synaptosomal preparations.

5.3.13 H₂O₂ production in synaptosomes from 6- and 18-month old rats

The rate of H₂O₂ 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. 5.21). 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 μ M rotenone, which increased the rate in both groups (Fig. 5.21, inset). 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. 5.21), which corresponds to increases of 29% and 32% compared to control. Inhibition of complex III activity with antimycin A has previously been shown to cause higher rates of H₂O₂ production in nerve terminals than inhibition of complex I activity with rotenone (Sipos *et al.* 2003a). In accordance with this work, it was found that 1 μ M antimycin A caused a 185% increase in H₂O₂ production from 6-month old and 164% increase in H₂O₂ production from 18-month old synaptosomes, giving rates of 2.81 ± 0.06 and 2.99 ± 0.05 pmol/min/mg respectively.

5.3.14 Rotenone potentiates 4-aminopyridine and KCl-induced depolarisation of $\Delta\psi_p$ in synaptosomes

The resting synaptosomal $\Delta\psi_p$ was measured to be -72.0 ± 5.9 mV (Fig. 5.22, 0 nM rotenone). Incubation with rotenone depolarised the membrane potential by a maximum of approximately 20 mV to -52.75 ± 9.7 (by 100 nM, $p < 0.05$). $\Delta\psi_p$ in the presence of 1 mM 4-aminopyridine was found to be -74.3 ± 11.4 , which was not significantly different from the resting potential. However, 1 nM rotenone in combination with 4-aminopyridine depolarised $\Delta\psi_p$ to -42.4 ± 8.9 mV (Fig. 5.22). Further depolarisation of $\Delta\psi_p$ was brought about by 100 nM rotenone (to -29.0 ± 9.1 mV) and 1 μ M (to -6.0 ± 12.1 mV) in the presence of 4-aminopyridine. The potential of -41.7 ± 10.4 mV brought about by 10 μ M rotenone and 4-aminopyridine was not different from that brought about by 1 nM rotenone in combination with 4-aminopyridine.

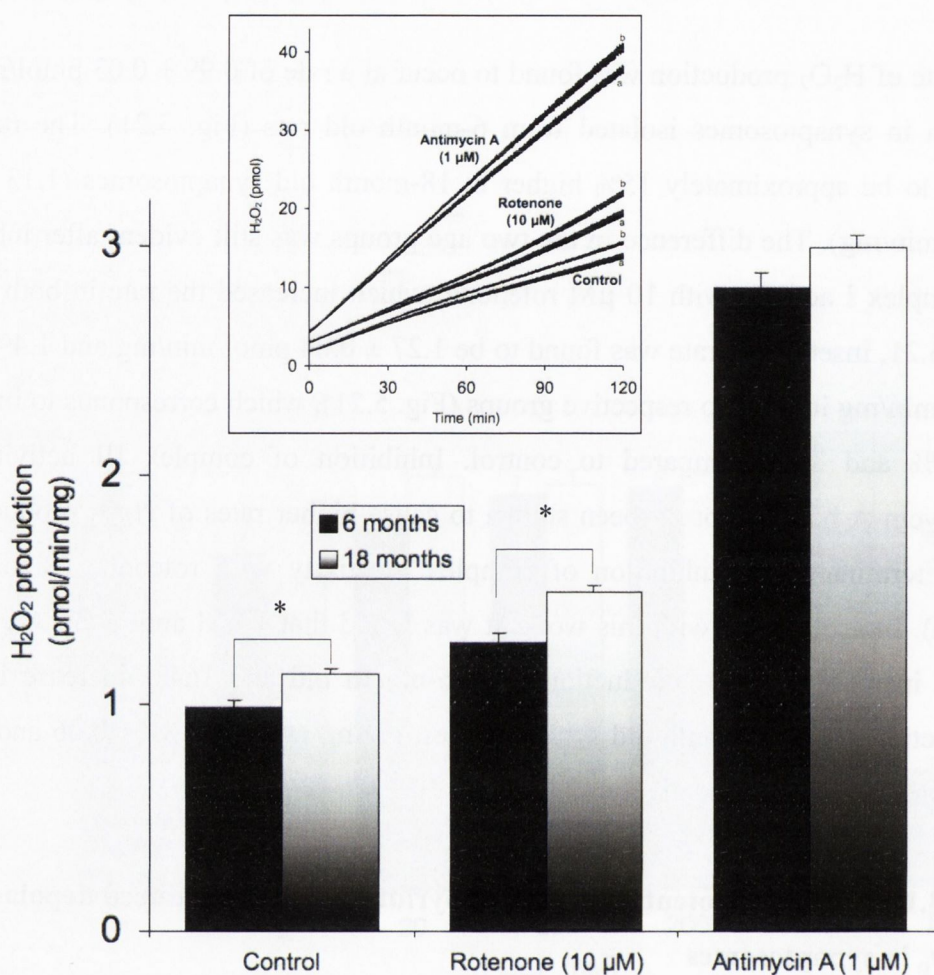


Fig. 5.21 Age-related increase in H_2O_2 production in rat brain synaptosomes.

Synaptosomes (0.5 mg/ml) were incubated with 10 μM rotenone or 1 μM antimycin A where indicated. H_2O_2 production was followed continuously using the amplex red assay system monitoring fluorescence at 585 nm (excitation 550 nm). V_{max} rates of H_2O_2 production + SEM for experiments performed in triplicate on 3 separate synaptosomal preparations are shown (* = $p < 0.05$). **Inset:** Experimental traces $\pm$ SEM from synaptosomes isolated from 6-month (**traces a**) and 18-month (**traces b**) old rats.

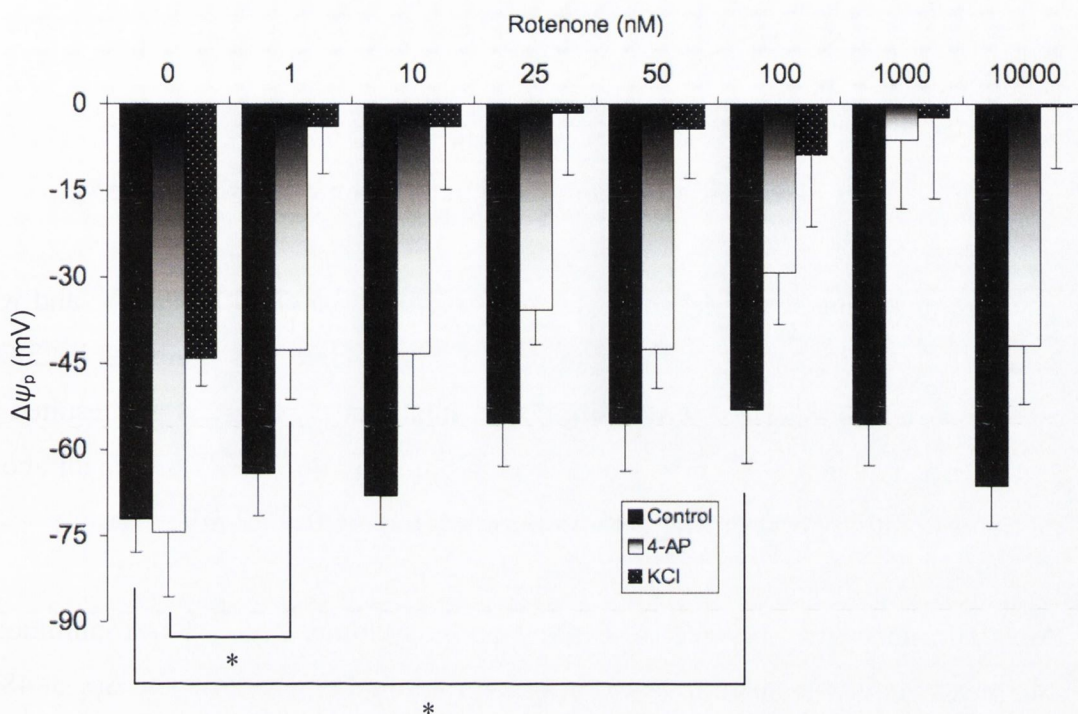


Fig. 5.22 Rotenone potentiates 4-aminopyridine- and KCl-induced depolarisation of $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of rotenone (1 nM – 10 μ M). Where shown, synaptosomes were depolarised after 5 min with 1 mM 4-aminopyridine or 40 mM KCl. The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) 10 min after depolarisation was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Results represent mean - SEM of experiments done in triplicate on 3 synaptosomal preparations. (* = p < 0.05).

In this group of experiments, 40 mM KCl depolarised $\Delta\psi_p$ by almost 30 mV to -44.1 ± 4.8 mV (Fig. 5.22). In combination with this level of KCl, $\Delta\psi_p$ was almost completely dissipated by 1 nM rotenone, which resulted in a $\Delta\psi_p$ of -3.9 ± 8.3 mV, and the levels of depolarisation by KCl at highest concentrations of rotenone were not significantly different.

5.3.15 Effects of inhibition of complex I activity on synaptosomal $\Delta\psi_p$

The resting synaptosomal $\Delta\psi_p$ after 15 min was found to be -72.0 ± 5.9 mV, and when complex I activity in *in situ* synaptosomal mitochondria was inhibited by $38.9 \pm 5.8\%$, $\Delta\psi_p$ fell to -55.1 ± 7.9 mV (Fig. 5.23). Inhibition by $86.4 \pm 1.9\%$ resulted in a similar $\Delta\psi_p$ of -55.3 ± 7.4 mV. However, inhibition by $96.7 \pm 2.6\%$ brought about a partial restoration of $\Delta\psi_p$ towards the control level, to 66.0 ± 7.2 mV.

After 10 min in the presence of 1 mM 4-aminopyridine, $21.9 \pm 8.0\%$ inhibition of complex I activity resulted in a $\Delta\psi_p$ of -42.4 ± 8.9 mV (Fig. 5.24). The $\Delta\psi_p$ at $48.4 \pm 6.0\%$ inhibition of complex I activity was -42.2 ± 7.0 mV. Higher levels of inhibition depolarised $\Delta\psi_p$ further, and $91.3 \pm 2.3\%$ inhibition almost brought about almost complete depolarisation to 6.0 ± 12.1 mV. However, the result of $92.9 \pm 2.2\%$ inhibition was a $\Delta\psi_p$ of -41.7 ± 10.4 mV.

After depolarisation with KCl for 10 min, $\Delta\psi_p$ was reduced to -44.1 ± 4.8 mV, and inhibition of complex I activity by $20.5 \pm 10.0\%$ depolarised $\Delta\psi_p$ to -3.9 ± 8.3 mV (Fig. 5.25). $\Delta\psi_p$ remained at this level of almost complete depolarisation at all points up to $96.0 \pm 2.6\%$ inhibition of complex I activity when a value of -0.4 ± 10.7 mV was measured.

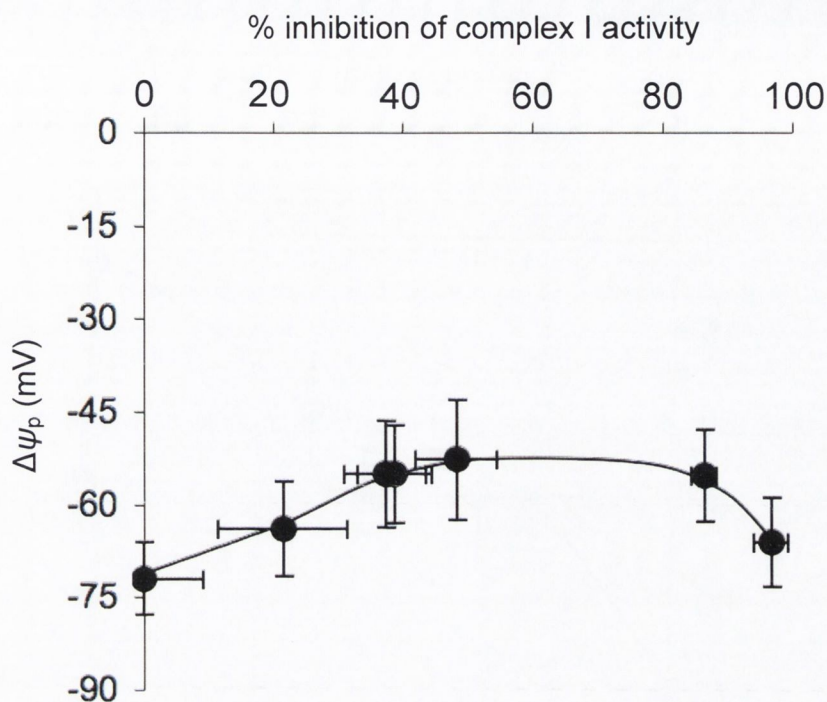


Fig. 5.23 Inhibition of complex I activity partially depolarises $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of rotenone (1 nM – 10 μ M). The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) after 15 min was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Levels of depolarisation 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. Results represent mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations.

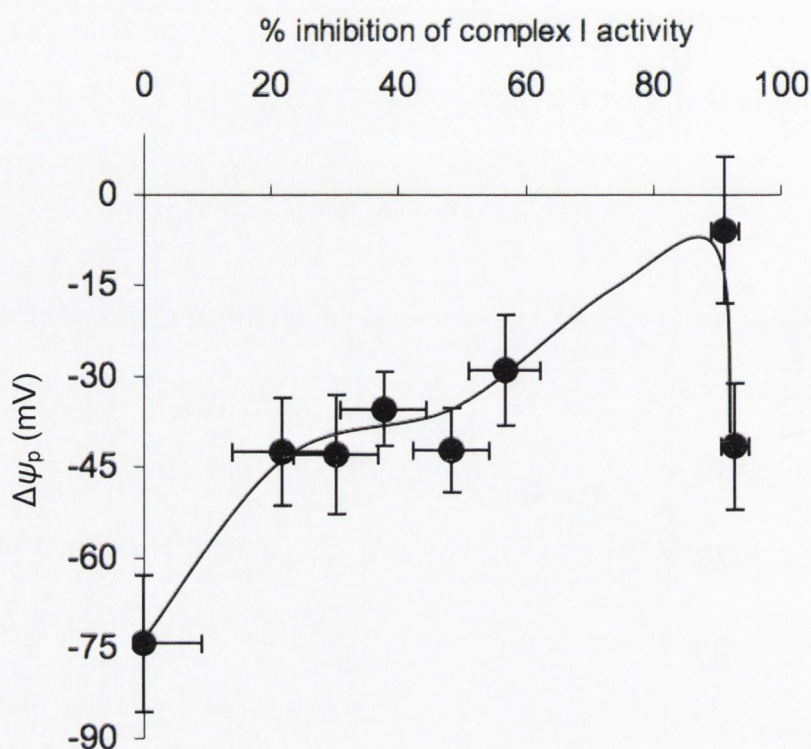


Fig. 5.24 Inhibition of complex I activity potentiates 4-aminopyridine-induced depolarisation of $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of rotenone (1 nM – 10 μ M). Synaptosomes were depolarised after 5 min with 1 mM 4-aminopyridine. The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) 10 min after depolarisation was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Levels of depolarisation 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. Results represent mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations.

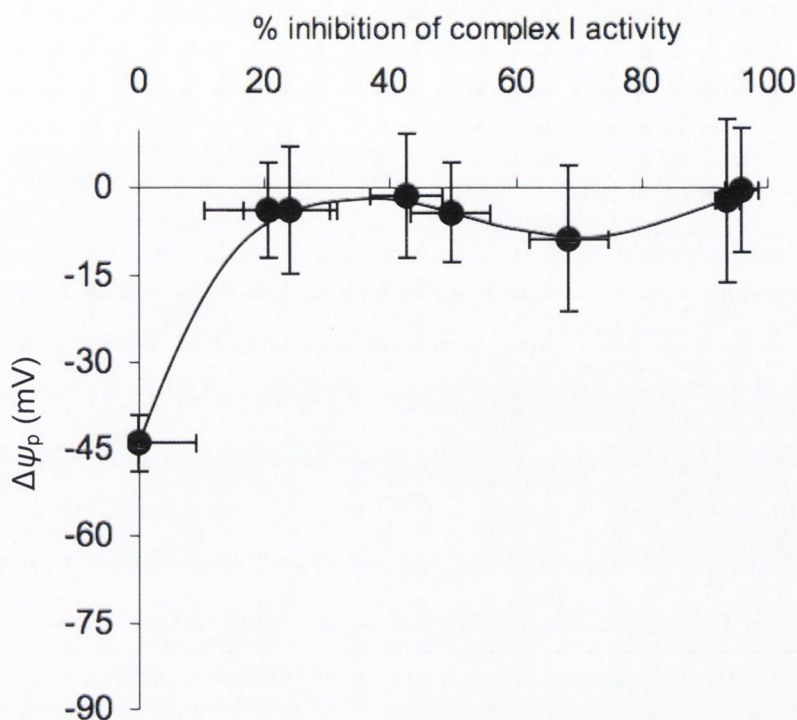


Fig. 5.25 Inhibition of complex I activity potentiates KCl-induced depolarisation of $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of rotenone (1 nM – 10 μ M). Synaptosomes were depolarised after 5 min with 40 mM KCl. The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) 10 min after depolarisation was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Levels of depolarisation 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. Results represent mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations.

5.3.16 Myxothiazol depolarises $\Delta\psi_p$ and potentiates 4-aminopyridine-induced depolarisation in synaptosomes

The resting $\Delta\psi_p$ of -70.6 ± 7.1 mV was reduced to -58.1 ± 5.6 mV by 10 nM myxothiazol, and higher concentrations reduced $\Delta\psi_p$ in a concentration dependent manner (Fig. 5.26). The highest concentration of myxothiazol examined, 10 μ M, resulted in a $\Delta\psi_p$ of -40.1 ± 6.4 mV ($p < 0.05$). The $\Delta\psi_p$ after 10 min in the presence of 1 mM 4-aminopyridine was found to be 82.6 ± 5.6 mV, and 10 nM reduced $\Delta\psi_p$ to 52.4 ± 9.5 mV under these conditions. 100 nM myxothiazol reduced $\Delta\psi_p$ to -35.0 ± 8.4 mV, and this was the highest level of depolarisation brought about by any concentration of the inhibitor in the presence of 4-aminopyridine. Depolarisation with KCl resulted in a $\Delta\psi_p$ of -34.4 ± 1.1 mV, which was reduced to -14.3 ± 10.2 mV by 25 nM myxothiazol. Higher concentrations of myxothiazol did not bring about further depolarisation of $\Delta\psi_p$ however, and the $\Delta\psi_p$ of 27.5 ± 8.8 mV in the presence of 1 μ M was not different from the control. Contrary to this trend, 10 μ M myxothiazol almost completely depolarised $\Delta\psi_p$ to -5.8 ± 7.5 mV in the presence of KCl.

5.3.17 Effects of inhibition of complex III activity on synaptosomal $\Delta\psi_p$

Inhibition of complex III activity with myxothiazol caused partial depolarisation of synaptosomal $\Delta\psi_p$ in a relationship that was almost linear (Fig. 5.27). A $60.2 \pm 3.0\%$ inhibition of complex III activity depolarised $\Delta\psi_p$ to 48.9 ± 6.5 mV, and a $\Delta\psi_p$ of -40.1 ± 6.4 mV was recorded at $94.7 \pm 2.3\%$ inhibition.

Inhibition of complex III activity by $19.4 \pm 5.1\%$ depolarised $\Delta\psi_p$ to 52.4 ± 9.5 mV in the presence of 1 mM 4-aminopyridine (Fig. 5.28). Further inhibition of complex III continued to depolarise $\Delta\psi_p$ to -35.0 ± 8.4 mV at $68.1 \pm 2.4\%$ inhibition of complex III activity. At the slightly higher level of $72.0 \pm 5.8\%$ inhibition, $\Delta\psi_p$ was found to return to -60.0 ± 7.5 mV, and the charge of -59.6 ± 8.6 mV after $99.9 \pm 3.7\%$ inhibition was not different.

The relationship between the inhibition complex III activity and $\Delta\psi_p$ after depolarisation with KCl was also almost linear (Fig. 5.29). The charge ranged from -34.4 ± 1.12 mV at $0. \pm 11.7\%$ inhibition to -5.8 ± 7.5 mV at $98.5 \pm 1.1\%$ inhibition.

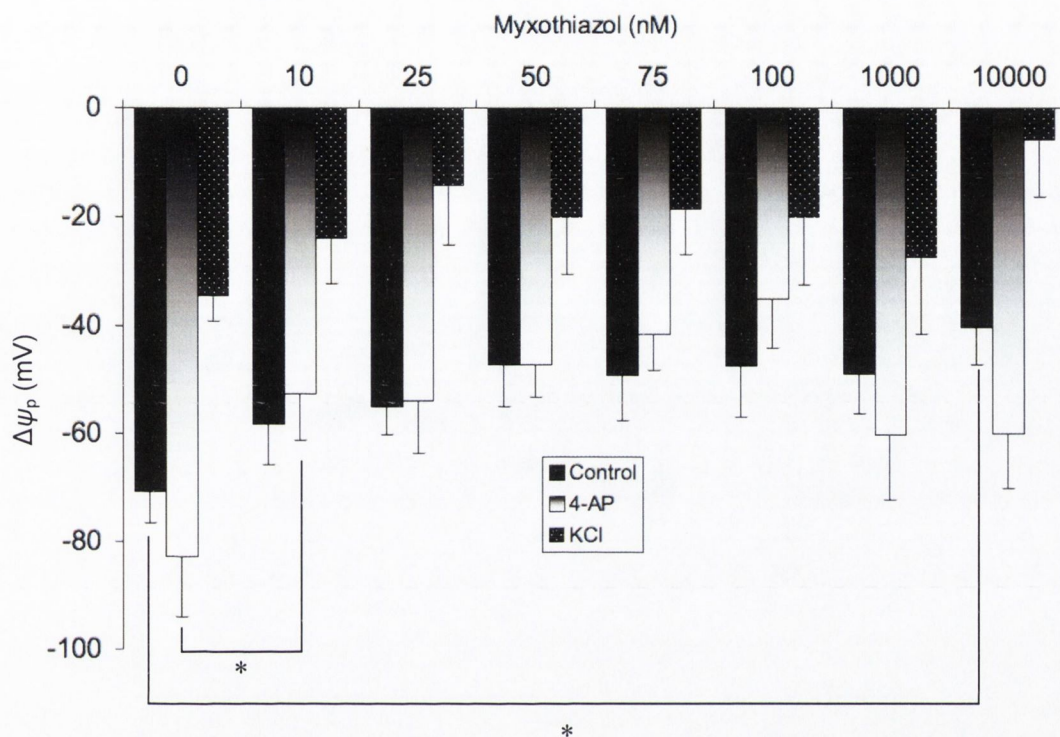


Fig. 5.26 Myxothiazol depolarises $\Delta\psi_p$ and potentiates 4-aminopyridine- and KCl-induced depolarisation in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of myxothiazol (10 nM – 10 μ M). Where shown, synaptosomes were depolarised after 5 min with 1 mM 4-aminopyridine or 40 mM KCl. The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) 10 min after depolarisation was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Results represent mean - SEM of experiments done in triplicate on 3 synaptosomal preparations. (* = p < 0.05).

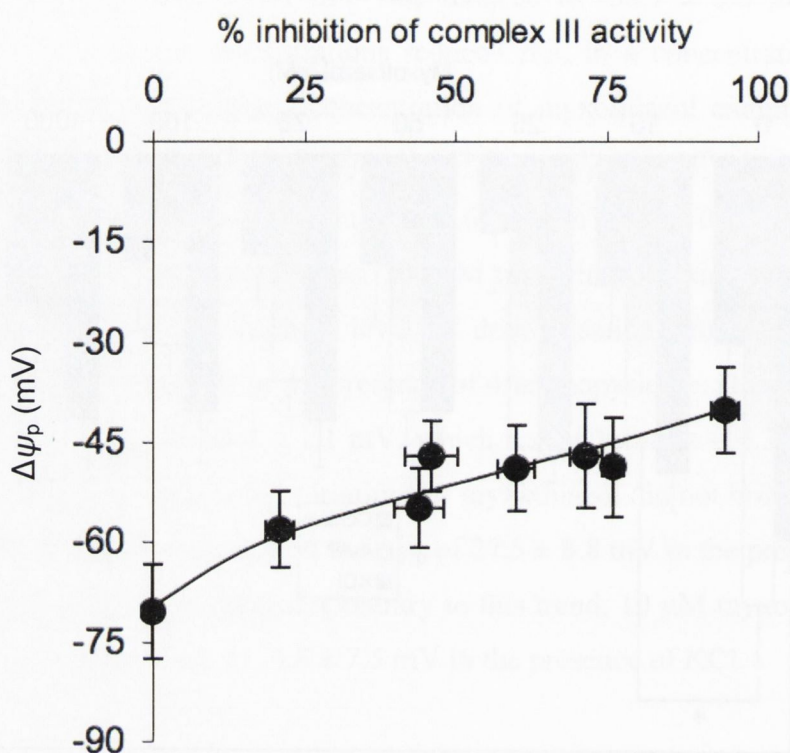


Fig. 5.27 Inhibition of complex III activity partially depolarises $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of myxothiazol (10 nM – 10 μ M). The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) after 15 min was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Levels of depolarisation at each concentration of myxothiazol were plotted against percent inhibition of complex III activity brought about by that concentration of myxothiazol. Freehand curves were drawn through the results. Results represent mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations.

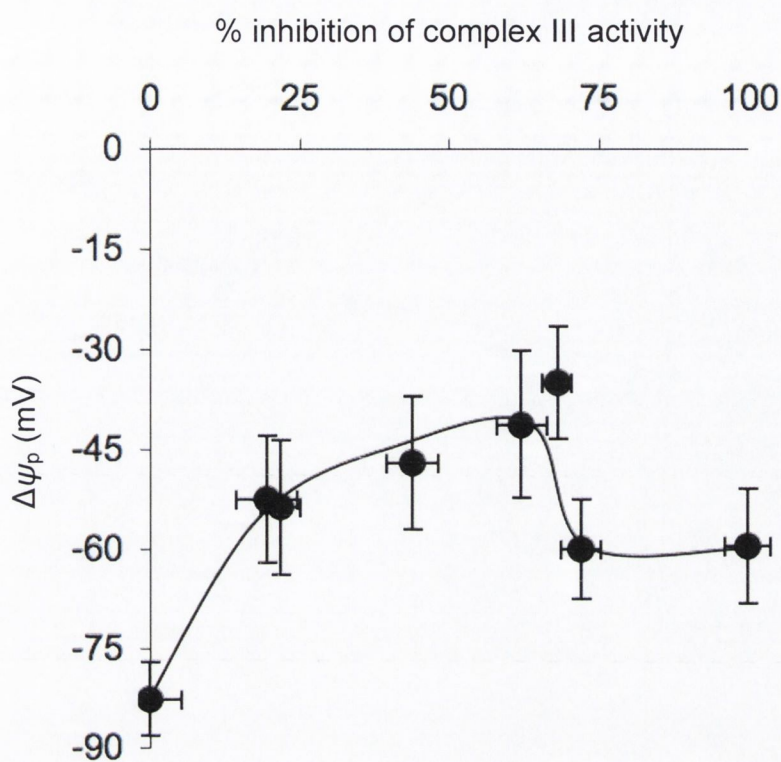


Fig. 5.28 Inhibition of complex III activity accentuates 4-aminopyridine-induced depolarisation of $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of myxothiazol (10 nM – 10 μ M). Synaptosomes were depolarised after 5 min with 1 mM 4-aminopyridine. The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) 10 min after depolarisation was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Levels of depolarisation at each concentration of myxothiazol were plotted against percent inhibition of complex III activity brought about by that concentration of myxothiazol. Freehand curves were drawn through the results. Results represent mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations.

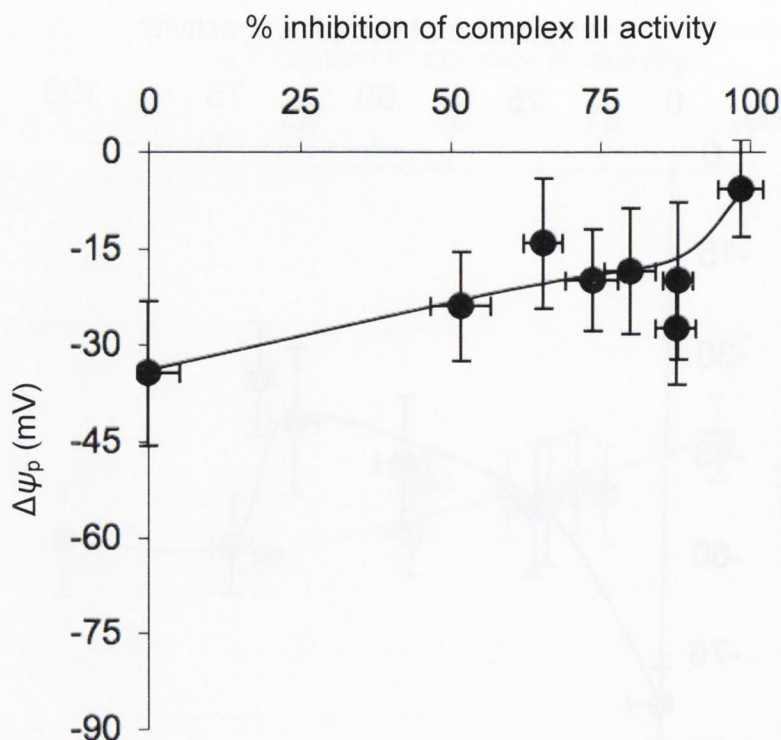


Fig. 5.29 Inhibition of complex III activity accentuates KCl-induced depolarisation of $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of myxothiazol (10 nM – 10 μ M). Synaptosomes were depolarised after 5 min with 40 mM KCl. The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) 10 min after depolarisation was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Levels of depolarisation at each concentration of myxothiazol were plotted against percent inhibition of complex III activity brought about by that concentration of myxothiazol. Freehand curves were drawn through the results. Results represent mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations.

5.3.18 KCN depolarises the resting synaptosomal $\Delta\psi_p$ but does not affect 4-aminopyridine and KCl-induced depolarisation of $\Delta\psi_p$

The resting membrane potential of -78.0 ± 5.7 mV was depolarised to 60.0 ± 7.7 mV by 5 μ M KCN (Fig. 5.30). 100 μ M brought about further depolarisation to -38.3 ± 11.0 mV, which was found to be the maximum level in the absence of any depolarising agent. Depolarisation with 4-aminopyridine resulted in a $\Delta\psi_p$ of -63.5 ± 9.0 mV, and $\Delta\psi_p$ after incubation with 500 μ M KCN was 37.0 ± 15.8 . This level of was not significantly different from control, nor was $\Delta\psi_p$ found to be significantly different at any other concentration of KCN when depolarised with 4-aminopyridine.

5.3.19 Effects of inhibition of complex IV activity on synaptosomal $\Delta\psi_p$

$\Delta\psi_p$ was partially depolarised in synaptosomes by inhibition of complex IV activity by $3.2 \pm 4.6\%$ (Fig. 5.31). Highest levels of inhibition did not depolarise $\Delta\psi_p$ any further. Inhibition of complex IV activity did not significantly affect the 4-aminopyridine- (Fig. 5.32) or KCl-induced depolarisation of $\Delta\psi_p$ (Fig. 5.33) at any level of inhibition.

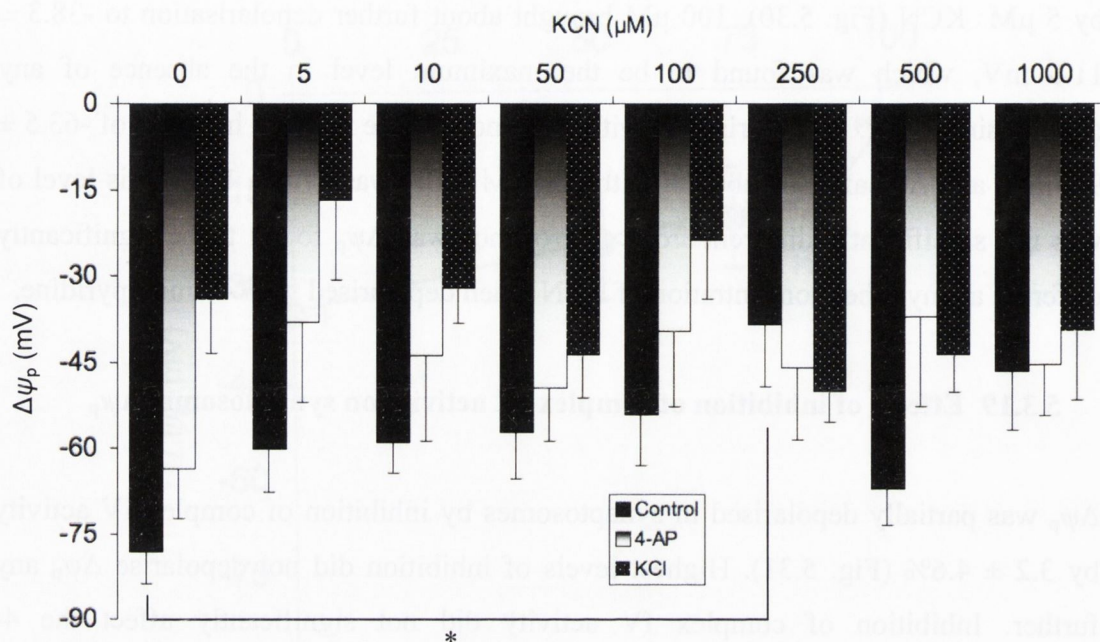


Fig. 5.30 KCN does not affect 4-aminopyridine and KCl-induced depolarisation of $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of KCN (5 μ M – 1 mM). Where shown, synaptosomes were depolarised after 5 min with 1 mM 4-aminopyridine or 40 mM KCl. The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) 10 min after depolarisation was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Results represent mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations. (* = p < 0.05).

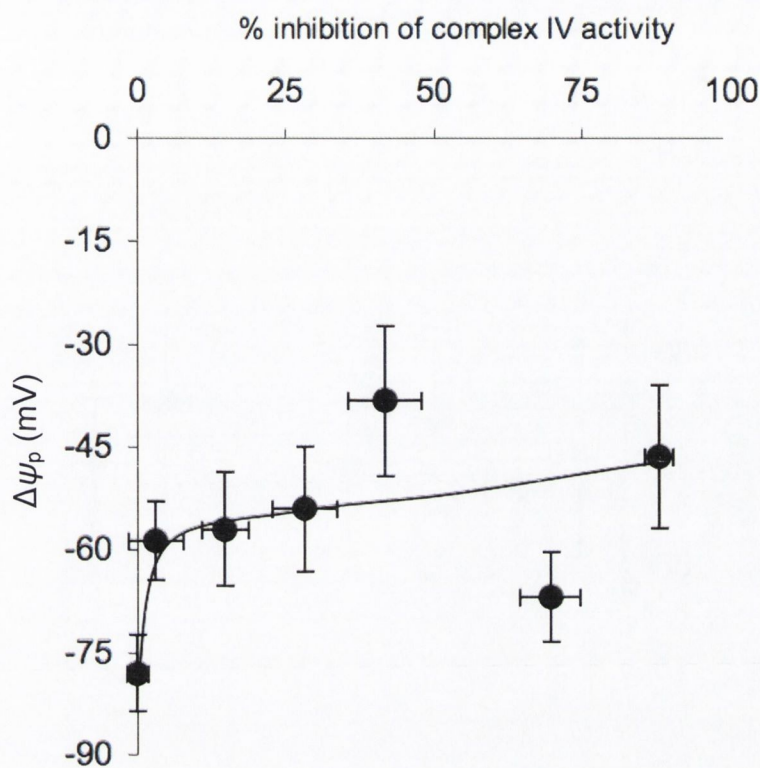


Fig. 5.31 Inhibition of complex IV activity partially depolarises $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of KCN (5 μ M – 1 mM). The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) after 15 min was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Levels of depolarisation at each concentration of KCN were plotted against percent inhibition of complex IV activity brought about by that concentration of KCN. Freehand curves were drawn through the results. Results represent mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations.

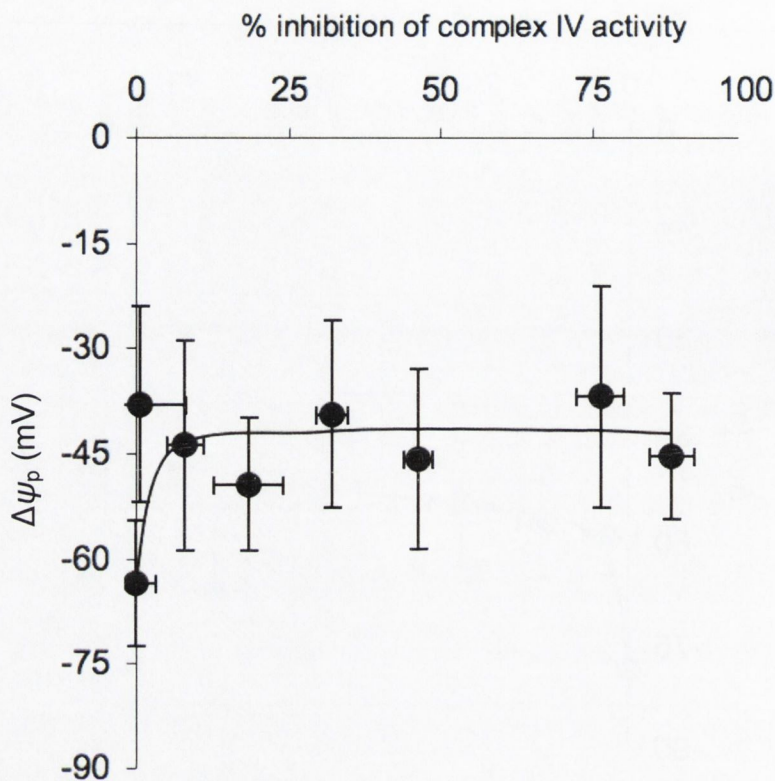


Fig. 5.32 Inhibition of complex IV does not affect 4-aminopyridine depolarisation of $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of 10 μ l KCN in buffer at final concentrations of 10 nM – 10 mM. Synaptosomes were depolarised after 5 min with 1 mM 4-aminopyridine. The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) 10 min after depolarisation was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Levels of depolarisation at each concentration of KCN were plotted against percent inhibition of complex III activity brought about by that concentration of KCN. Freehand curves were drawn through the results. Results represent mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations.

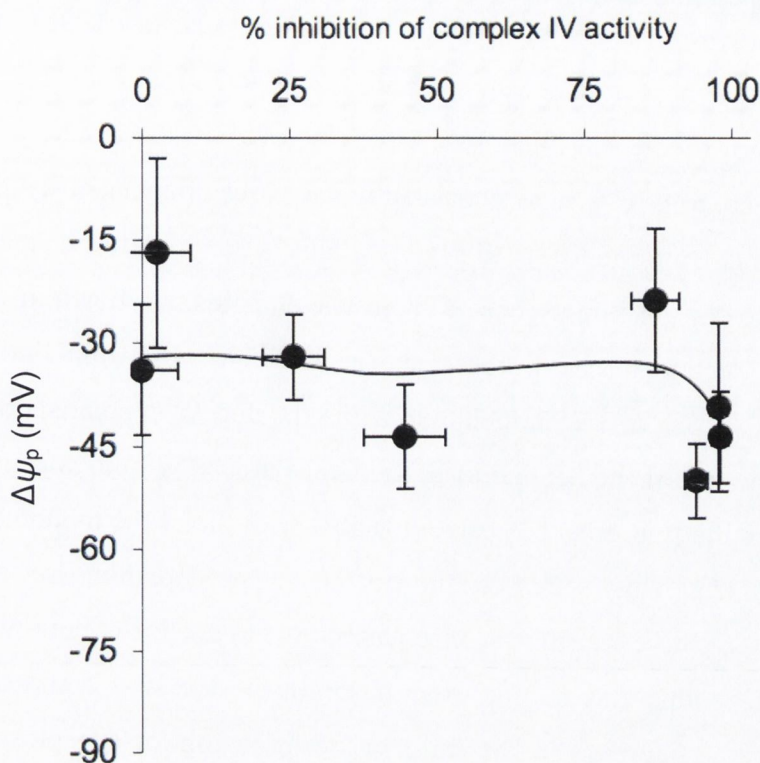


Fig. 5.33 Inhibition of complex IV activity does not affect KCl-induced depolarisation of $\Delta\psi_p$ in synaptosomes.

Synaptosomes (0.5 mg/ml) were pre-incubated with PMPI for 45 min at final volume of 200 μ l with PMPI before addition of KCN (5 μ M – 1 mM). Synaptosomes were depolarised after 5 min with 40 mM KCl. The difference in fluorescence signal (excitation λ = 550 nm, emission λ = 585 nm) 10 min after depolarisation was calibrated to the differences in signal brought about by known amounts of $\Delta\psi_p$ depolarisation by KCl. Levels of depolarisation at each concentration of KCN were plotted against percent inhibition of complex IV activity brought about by that concentration of KCN. Freehand curves were drawn through the results. Results represent mean $\pm$ SEM of experiments done in triplicate on 3 synaptosomal preparations.

5.4 Discussion

The ratio of JC-1 emission at $\lambda = 590$ nm to $\lambda = 535$ nm (excitation $\lambda = 490$ nm) is a semi-quantitative measure of *in situ* $\Delta\psi_m$ (Reers *et al.* 1995). Under resting conditions, the synaptosomal $\Delta\psi_m$ has been shown to be maintained at ~ -150 mV for at least 30 min (Scott and Nicholls 1980). However, in the results presented in this chapter, the control JC-1 ratio consistently increases spontaneously from the initial time point (e.g. from ~ 6 to ~ 7 in Fig 5.1). This is due to a discrepancy in the rate of decline in the control fluorescence at both wavelengths, as shown in the inset traces. Although a decline in the fluorescence at $\lambda = 590$ nm would indicate efflux of aggregate JC-1 out of the mitochondrial matrix due to depolarisation of $\Delta\psi_m$, this could only be true if the decline in fluorescence at $\lambda = 590$ nm was matched with an increase with the fluorescence peak associated with the monomeric form of the dye ($\lambda = 535$). This was not shown to be the situation in 'resting' control synaptosomes, where the fluorescence was shown to decline at both wavelengths (e.g. Fig 5.1, inset – this may be due to an instability in the JC-1 compound). Therefore, the spontaneous increase in the ratio in 'resting' control synaptosomes cannot be attributed to a hyperpolarisation of the *in situ* synaptosomal $\Delta\psi_m$. This indicates the importance of examining both traces as well as the ratio when drawing conclusions from the results given by the JC-1 probe. Hence, all of the effects of metabolic inhibitors on the fluorescence ratio are described relative to the control ratio at that time point.

The results in this chapter indicate that $\Delta\psi_m$ is susceptible to depolarisation by partial inhibition of complex I activity with low concentrations of rotenone (Figs 5.1, 5.5). This depolarisation is accelerated by inhibition of glycolysis with 2-deoxyglucose (Fig. 5.2). The $\Delta\psi_m$ after 15 min was lowered by $< 40\%$ inhibition of complex I activity compared to the control and by $< 20\%$ inhibition of complex I activity when glycolysis was also inhibited (Fig. 5.5). In contrast, inhibition of complex III activity by up to 70% with 100 nM myxothiazol did not affect $\Delta\psi_m$ (Figs 5.6, 5.8), and inhibition of complex III activity by up to 85% with 30 nM antimycin A did not affect $\Delta\psi_m$ (Figs 5.9, 5.11). Inhibition of complex IV activity with KCN did not affect $\Delta\psi_m$ at any concentration (Figs 5.12, 5.14).

It has previously been demonstrated that inhibition of complex III with myxothiazol activity by up to 80% did not result in major changes in oxidative phosphorylation, while inhibition by 90% completely abolished the oxygen consumption and ATP production in isolated synaptosomal mitochondria (Davey *et al.* 1998). Recent work in our lab has indicated that the threshold level of inhibition of complex III activity on oxygen consumption in *in situ* synaptosomal mitochondria may be lower than that reported in isolated synaptosomal mitochondria (Telford *et al.* unpublished). In the *in situ* synaptosomal mitochondrial model, inhibition of complex III activity by ~ 40% reduced the rate of oxygen consumption, and ~ 50% inhibition of complex III activity brought about a reduction in the rate to ~ 65% of the control rate. Inhibition of complex III activity by ~ 70% was found to almost completely abolish the oxygen consumption rate (Telford *et al.* unpublished). At ~ 70% inhibition of complex III activity $\Delta\psi_m$ was found to be partially depolarised in the present chapter (Fig. 5.8), but inhibition of complex III activity between 50 and 70% did not affect $\Delta\psi_m$ (Fig. 5.8). Similarly, inhibition of complex IV activity with KCN did not depolarise $\Delta\psi_m$ at any level of inhibition, although inhibition of complex IV activity by up to 70% with KCN in isolated synaptosomal mitochondria has previously been shown to decrease ATP production by < 10%, with total inhibition of ATP production at 90% inhibition of complex IV activity (Davey *et al.* 1998).

Total inhibition of complex I activity 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), and 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 was reduced in synaptosomes, complex V 'compensated' by operating in reverse, thus acting as an alternative proton pump, but resulting in hydrolysis of glycolytic ATP (Kauppinen and Nicholls 1986; Nicholls 2006). The level of $\Delta\psi_m$ maintained in this situation is sub-optimal (Figs 5.5, 5.16; (Scott and Nicholls 1980; Chinopoulos *et al.* 1999). Removal of the supply of glycolytic ATP by substituting 2-deoxyglucose for glucose causes a complete depolarisation of $\Delta\psi_m$ at highest levels of inhibition of complex I activity (Fig. 5.5). Indeed, the threshold level of inhibition

under these conditions is reduced to < 20% decrease in complex I activity when $\Delta\psi_m$ is affected, suggesting the ATP synthase may begin compensating at low levels of inhibition of complex I activity.

The results in this chapter suggest that inhibition of complex III activity or inhibition of complex IV activity may have a similar reversing effect on the ATP synthase. The clearest evidence for this comes from Figs 5.16 and 5.17. In Fig. 5.16, 1 mM KCN was shown to have no effect on $\Delta\psi_m$. However, when combined with inhibition of complex V activity with oligomycin (20 $\mu\text{g/ml}$), $\Delta\psi_m$ was shown to be significantly depolarised (Fig. 5.16). Similarly, 1 μM antimycin A partially depolarised $\Delta\psi_m$ in 6- and 18-month old rats, while combination of this concentration of complex III inhibitor with inhibition of complex V activity with oligomycin (20 $\mu\text{g/ml}$) resulted in depolarisation of $\Delta\psi_m$ to the same level that the protonophore FCCP did in excess (1 μM , Fig. 5.17). These results have implications for the interpretation of the results of inhibiting complex III activity with a range of concentrations of antimycin A (Fig. 5.11) and complex IV activity with a range of concentrations of KCN (Fig. 5.14) on $\Delta\psi_m$. The finding that inhibition of complex IV activity by $\sim 90\%$ with KCN in isolated synaptosomal mitochondria results in total inhibition of ATP production (Davey *et al.* 1998) also suggests that complex V activity would be working in reverse under these conditions, and therefore may be contributing to the maintenance of $\Delta\psi_m$.

Nonetheless, it may be stated that $\Delta\psi_m$ in *in situ* synaptosomal mitochondria is more susceptible to depolarisation by low-level inhibition of complex I activity than similar low-level inhibition of the activities of the other ETC complexes. This finding is in accordance with previous reports, which find lower energy thresholds in complex I of brain mitochondria than the other ETC complexes (Davey *et al.* 1997; Davey *et al.* 1998; Rossignol *et al.* 2003). Specifically, it has been shown that after inhibition of complex I activity with rotenone by up to 25% does not cause major changes in oxidative phosphorylation, inhibition by more than this level decreased oxygen consumption and ATP production linearly in relation to complex I activity in isolated synaptosomal mitochondria (Davey *et al.* 1998). Recent work in our lab has indicated that the threshold level of inhibition of complex I activity on oxygen consumption in *in situ* synaptosomal mitochondria may be lower than that reported in isolated

synaptosomal mitochondria (Telford *et al.* unpublished). Fig. 5.5 shows that inhibition of complex I activity by $< 40\%$ caused a depolarisation of $\Delta\psi_m$ of *in situ* synaptosomal mitochondria. This low 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; Schapira *et al.* 1990). 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 inhibition of complex I activity (Fig. 5.5).

Depolarisation of $\Delta\psi_p$ with 4-aminopyridine caused a slight acceleration of the rotenone-induced depolarisation of $\Delta\psi_m$ immediately after addition, but no effect was seen in synaptosomes where rotenone was absent (Fig. 5.3). The initial acceleration upon addition was not sustained for more than one or two time points. A similar effect was shown to occur in the presence of rotenone and deoxyglucose (Fig. 5.4) and 1 μM myxothiazol (Fig. 5.7, 5th time point compared to Fig. 5.6, 5th time point). A more sustained acceleration of antimycin A-induced depolarisation of $\Delta\psi_m$ was observed (Fig. 5.10 compared to Fig. 5.9). However, the difference in $\Delta\psi_m$ between resting synaptosomes and those in which $\Delta\psi_p$ was depolarised with 4-aminopyridine was negligible after 15 min after inhibition of complex III activity with myxothiazol (Fig. 5.8), antimycin A (Fig. 5.11) and inhibition of complex IV activity with KCN (Fig. 5.14).

The relationship between $\Delta\psi_m$ and oxidative stress in the brain has been the subject of much recent research. H_2O_2 has been found to depolarise $\Delta\psi_m$ in *in situ* synaptosomal mitochondria in a concentration-dependent manner (Chinopoulos *et al.* 1999, 2000), and partial depolarisation of $\Delta\psi_m$ by 'mild uncoupling' of proton pumping from ATP production has been found to reduce H_2O_2 production in isolated brain mitochondria (Starkov and Fiskum 2003; Fiskum *et al.* 2004; Tretter and Adam-Vizi 2007a). This led to the hypothesis that administration of pharmacological agents that partially depolarise $\Delta\psi_m$ may be neuroprotective by lowering mitochondrial free radical production (Brand *et al.* 2004; Andrews *et al.* 2005). However, uncoupling with a range of concentrations of FCCP has recently been shown to have no effect on H_2O_2 production in *in situ* nerve terminal mitochondria (Tretter and Adam-Vizi 2007b).

However, the latter study reported on the effects of FCCP over a limited time period (< 5 min) and used a low concentration of Amplex Red ($1 \mu\text{M}$).

Using $50 \mu\text{M}$ Amplex Red, it was shown that FCCP did not affect synaptosomal H_2O_2 production over 90 min after initial resuspension (Fig. 5.18, insets). The initial rate of H_2O_2 production was elevated in synaptosomes that were resuspended in medium that included 1.4 mM CaCl_2 compared to those resuspended in the absence of Ca^{2+} for ~ 60 min. After this time point, the rate of H_2O_2 production from synaptosomes in the presence of Ca^{2+} began to slow down (Fig. 5.18, right inset). In contrast, the rate in the absence of Ca^{2+} remained constant for ~ 90 min, after which it increased spontaneously (Fig. 5.18, left inset). However, the increase in rate was attenuated by FCCP in a concentration-dependent manner for the following 90 min (Fig. 5.18). FCCP also depolarised $\Delta\psi_{\text{m}}$ in a concentration-dependent manner (Fig. 5.19), but did not affect synaptosomal viability over this time period (Fig. 5.20). The rate of H_2O_2 production from synaptosomes resuspended in the presence of Ca^{2+} was also lowered by FCCP in a concentration-dependent manner, although to a lesser extent than those resuspended in the absence of Ca^{2+} (Fig. 5.18). This suggests that depolarisation of $\Delta\psi_{\text{m}}$ reduced H_2O_2 production from synaptosomes, with most reduction by total uncoupling of proton pumping from ATP production in *in situ* mitochondria. The mechanism of reduction is not clear, and further experiments on Ca^{2+} -induced H_2O_2 production in synaptosomes are required.

The aging process in the brain involves changes to mitochondria including increased levels of ROS production and reduction in bioenergetic capacity. Elevated rates of H_2O_2 production in synaptosomes from 18-month old rats compared to those from 6-month old were found (Fig. 5.21). Simultaneously, $\Delta\psi_{\text{m}}$ was found to be lower in the older group (Fig. 5.17), and this correlated to reduced oxygen consumption in the older group (Kilbride *et al.* 2008). These data indicate that an age-related decline in the 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 (Finkel and Holbrook 2000; Droge and Schipper 2007), 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 (Cardoso *et al.* 1998).

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 enzyme assay results (Kilbride *et al.* 2008). Age-related reductions in complex II and complex III activities have previously been demonstrated in mouse brain mitochondria (Kwong and Sohal 2000), and decreased complex IV activity with age has been widely reported in rat brain (Navarro and Boveris 2004, 2007). Under conditions of reduced electron flow through the electron transport chain, a reduction in proton pumping through 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 (Fig. 5.21; Sipos *et al.* 2003a, b). The complex I iron-sulphur centres (Herrero and Barja 1997; Genova *et al.* 2001) and the flavin moiety (Liu *et al.* 2002) 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 inhibition of complex I activity with rotenone suggesting that the rotenone ROS production at the rotenone-binding site complex I is not responsible for the elevated H_2O_2 production in the older group (Ramsay and Singer 1992). Furthermore, complex I activity was not found to be reduced in the older group, suggesting no oxidative damage at this site in the 18-month old nerve terminals. Inhibition of complex III using antimycin A produced higher levels of H_2O_2 production. However, at least some of the H_2O_2 induced by this inhibitor in synaptosomes is due to reverse electron transport through complex I, and can be partially inhibited by rotenone (Sipos *et al.* 2003b). Complex III has been associated with increased mitochondrial H_2O_2 production (Chen *et al.* 2003) 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 (Adam-Vizi 2005; Tretter and Adam-Vizi 2005), and

this enzyme may also be among the sources of the excess H_2O_2 produced in the synaptosomes isolated from older animals.

In the present chapter rats of two age groups were compared, 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' (Coleman *et al.* 2004). 'Old age' is the highest risk factor for many common chronic neurodegenerative disorders in which deterioration of mitochondrial function is implicated in their aetiology (Lin and Beal 2006). The results show elevated levels of nerve terminal H_2O_2 production and reduced level of $\Delta\psi_m$ 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 (Finkel and Holbrook 2000; Droge and Schipper 2007). This increase in ROS production and associated decline in mitochondrial function may play a role in the pathogenesis of chronic neurodegenerative disorders in which aging is considered the highest risk factor such as Alzheimer's disease and Parkinson's disease (Beal 1995, 1998; Lin and Beal 2006).

The resting synaptosomal $\Delta\psi_p$ was found to be within the range of -70 – -80 mV, and was shown to be not significantly changed in the presence of 1 mM 4-aminopridine for 10 min (Figs 5.22, 5.26, 5.30). This finding is similar to previous reports, which used TPP^+ (Tibbs *et al.* 1989) and ^{86}Rb (Tibbs *et al.* 1996) to measure $\Delta\psi_p$ after addition of 1 mM 4-aminopridine. The intermittent depolarising of synaptosomes after addition of 1 mM 4-aminopridine is not reflected in a time-averaged fluorescence signal over the entire synaptosomal population (Nicholls 2003). Nonetheless, inhibition of complex I activity by ~ 40% resulted in a depolarisation of $\Delta\psi_p$ to -35 – -45 mV in the presence of 4-aminopridine (Fig. 5.24) but ~ $\Delta\psi_p$ at this level of inhibition of complex I activity in resting synaptosomes was ~ -55 mV (Fig. 5.23). Inhibition of complex I activity by ~ 90% depolarised $\Delta\psi_p$ to < -10 mV (Fig 5.24). No level of inhibition the activities of complex III (Fig. 5.28) or complex IV (Fig. 5.32) depolarised $\Delta\psi_p$ to this extent. KCl depolarised synaptosomes to -35 – -45 mV in the absence of any inhibitor (Figs 5.22, 5.26, 5.30). Inhibition of complex I activity by between ~ 20% and ~ 100% depolarised $\Delta\psi_p$ to < -10 mV after

depolarisation with KCl. $\Delta\psi_p$ was only depolarised to this level by KCl under conditions of almost complete inhibition of complex III activity (Fig. 5.29) and not by any level of inhibition of complex IV activity (Fig. 5.33). This suggests $\Delta\psi_p$ is more susceptible to inhibition of complex I activity than to inhibition of complex III or complex IV activity in depolarised synaptosomes.

When complex I and complex III activities were inhibited by $> 90\%$ or $> 70\%$ with $10\ \mu\text{M}$ rotenone and $1 - 10\ \mu\text{M}$ myxothiazol respectively, $\Delta\psi_p$ in synaptosomes depolarised with 4-aminopyridine was closer to the control level than at lower levels of inhibition (Figs 5.24, 5.28). This effect 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 hyperpolarisation 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 hyperpolarisation effect which lasted over 20 min after application of $10\ \mu\text{M}$ rotenone, but show shorter hyperpolarisation phases at $3\ \mu\text{M}$ and $1\ \mu\text{M}$ rotenone (Bonsi *et al.* 2004). The duration of hyperpolarisation 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. Further studies on synaptosomal ATP levels after addition of 4-aminopyridine and inhibition of complex I and complex III activities (and indeed inhibition of complex IV activity, where the effect was not apparent) with a range of inhibitor concentrations are required to investigate this hypothesis.

Ion circuitry across the mitochondrial and plasma membranes are closely interlinked and depolarisation of $\Delta\psi_m$ using FCCP has previously been shown to partially depolarise $\Delta\psi_p$ in synaptosomes in a concentration dependent manner (Tretter *et al.* 1998). Partial depolarisation of $\Delta\psi_m$ by $\sim 40\% - \sim 90\%$ inhibition of complex I activity after 15 min (Fig. 5.5) correlated to a partial depolarisation of $\Delta\psi_p$ at the same

time point (Fig. 5.23). Inhibition of complex I activity by $> \sim 90\%$ resulted in further depolarising of $\Delta\psi_m$ (Fig. 5.5) but had a repolarising effect on $\Delta\psi_p$ in resting synaptosomes compared to lower levels of inhibition of complex I activity (Fig. 5.23). Partial inhibition of complex III activity by $> 40\%$ with myxothiazol decreased $\Delta\psi_p$ in resting synaptosomes (Fig. 5.27) but did not affect $\Delta\psi_m$ until $\sim 70\%$ inhibition of complex III activity was achieved (Fig. 5.8). This level of inhibition of complex III activity correlated to the maximum level of depolarisation of $\Delta\psi_p$ in the presence of 4-aminopyridine (Fig. 5.28), but inhibition by $> \sim 90\%$ had a repolarising effect on $\Delta\psi_p$ (towards the control level), whereas $> \sim 90\%$ inhibition of complex III activity resulted in a lower $\Delta\psi_m$ than any lower level of inhibition of complex III activity (Fig. 5.8). Partial inhibition of complex IV activity in resting synaptosomes resulted in depolarisation of $\Delta\psi_p$ (Fig. 5.31) but no depolarisation of $\Delta\psi_m$ was found under these conditions (Fig. 5.14). Thus, no general relationship between the pattern of depolarisation of $\Delta\psi_m$ and that of $\Delta\psi_p$ after inhibition of ETC enzymes was observed in polarised synaptosomes or those depolarised with 4-aminopyridine.

Nonetheless, both $\Delta\psi_m$ and $\Delta\psi_p$ were shown to be more susceptible to depolarisation after reduced complex I activity than reductions in the activities of complex III and complex IV. This may be of consequence in the pathogenesis of Parkinson's disease, in which complex I activity has been shown to be reduced in post mortem studies on substantia nigra (Schapira *et al.* 1989; Schapira *et al.* 1990) and frontal cortex (Parker *et al.* 2008) homogenates. Increasing evidence suggests that neuronal cell death commences in the nerve terminal, and reduced $\Delta\psi_m$ in isolated nerve terminals has been shown to correlate to induction of pro-apoptotic stimuli including cytochrome c release, increased $[Ca^{2+}]_i$ and caspase activation (Mattson *et al.* 1998). Reduced $\Delta\psi_p$ in isolated nerve terminals has also been shown to result in increased $[Na^+]_i$ (Deri and Adam-Vizi 1993), which has been shown to be an early trigger of apoptosis (Bortner *et al.* 2001). The nerve terminal rotenone model of the $\sim 40\%$ reduction complex I activity in Parkinson's disease brain shows reduced $\Delta\psi_m$ (Fig. 5.5) and reduced $\Delta\psi_p$ in resting synaptosomes (Fig. 5.23) and those depolarised with 4-aminopyridine (Fig. 5.24) and the effects of such reductions may contribute neuronal apoptotic cell death and the chronic neurodegeneration associated with this disorder.

Chapter 6

General Discussion

6.1 Discussion

The results in this thesis address the effects of inhibition of the enzymes of the ETC in *in situ* synaptosomal mitochondria on nerve terminal physiology, including glutamate release rates from depolarised and resting synaptosomes, $\Delta\psi_m$, $\Delta\psi_p$, ATP level, and H_2O_2 production. Inhibition of complex I activity with a range of concentrations of rotenone showed that Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with 4-aminopyridine were increased by 35 – 40% inhibition of complex I activity (Fig. 3.12, Table 6.1). $\Delta\psi_m$ was reduced by inhibition of complex I activity by 35 – 40% (Fig. 5.5). In contrast, inhibition of complex III activity (using a range of concentrations of myxothiazol) showed that Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with 4-aminopyridine was not increased until 50 – 60% inhibition of complex III activity was reached (Fig. 4.5), and ~ 70% inhibition of complex III activity with myxothiazol was necessary to bring about a reduction in $\Delta\psi_m$ (Fig. 5.8). Inhibition of complex III activity at a site after the Q-cycle with antimycin A was shown to have a very high threshold before Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with 4-aminopyridine or $\Delta\psi_m$ were affected (85 – 90% inhibition of complex III activity, Fig. 4.13). A similar level of inhibition of complex IV activity with KCN was required to increase Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with 4-aminopyridine (Fig. 4.20, Table 6.1). Therefore, mitochondrial control over Ca^{2+} -independent glutamate release rates from depolarised synaptosomes was found to be most susceptible to inhibition at complex I, and susceptibility to inhibition was shown to decrease continuously at later points of intervention along the ETC.

The increased Ca^{2+} -independent glutamate release rates from depolarised synaptosomes after inhibition of ETC enzymes is most likely via reversal of the glutamatergic uptake transporters. EAAT1 and EAAT2 transporters each cotransport three Na^+ ions and one H^+ and countertransport one K^+ ion for each glutamate molecule they transport (Nicholls and Attwell 1990). Ion circuitry across the mitochondrial and plasma membranes are closely interlinked (Fig. 1.3) and therefore depolarisation of $\Delta\psi_m$ might be expected to alter the $\Delta\psi_p$, as the ability of $\Delta\psi_m$ to sequester cations is reduced under these conditions (Tretter *et al.* 1998). However no clear correlation between $\Delta\psi_m$ and $\Delta\psi_p$ was found in synaptosomes (Chapter 5).

Therefore, the reversal of the Na^+ cotransport pathway is more likely to be due to reduced Na^+/K^+ ATPase activity after impeded ATP production.

In Chapter 3 it was shown that a reduction of synaptosomal ATP level by 25 – 30% over 15 min by partial inhibition of complex I activity with 25 nM rotenone (Fig. 3.13) correlated to increased Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with both 4-aminopyridine and KCl. However, a similar reduction in synaptosomal ATP level after 15 min in resting synaptosomes failed to illicit a significant increase in glutamate efflux. Inhibition of complex III activity with a range of concentrations of myxothiazol showed that glutamate efflux rates from resting synaptosomes was increased by 50 – 60% inhibition of complex III activity was reached (Fig. 4.7, Table 6.2). Inhibition of complex III activity after the Q-cycle with antimycin A was shown to also increase Ca^{2+} -independent glutamate efflux rates from polarised synaptosomes, although the threshold of inhibition was higher using this inhibitor (85 – 90% inhibition of complex III activity, Fig. 4.15). A similar level of inhibition of complex IV activity with KCN increased the Ca^{2+} -independent glutamate efflux rates from polarised synaptosomes (Fig. 4.22, Table 6.1). Further studies are required to examine the ATP level that correlates to increased glutamate efflux from polarised synaptosomes after inhibition of complex III and complex IV activity. Indeed, continuous monitoring of ATP level, or at least measurement of the ATP level at frequent time points would be very informative as regards the relationship between ATP level and Ca^{2+} -independent glutamate release rates from depolarised synaptosomes.

Although continuous monitoring of ATP level in synaptosomes has not previously been performed, information on ETC activity can be deduced by synaptosomal oxygen consumption, and experiments on ATP production after inhibition of the ETC enzymes have previously been performed on isolated rat brain synaptosomal mitochondria (Davey *et al.* 1997; Davey *et al.* 1998). In particular, complex I was shown to have tightest control of any of the ETC enzymes over flux through the ETC, resulting in a low threshold of inhibition before ATP production and oxygen consumption were reduced (~ 25% in isolated whole brain synaptosomal mitochondria (Davey *et al.* 1998)). The tighter control over ATP production and oxygen consumption in isolated synaptosomal mitochondria has been shown to be

Complex	Ca ²⁺ -independent glutamate release			$\Delta\psi_m$
	4-AP	KCl	Efflux	
I	35 - 40	40 - 45	NA	35 - 40
III (Upstream of Q-cycle)	50 - 60	~ 80	50 - 60	~ 70
III (Downstream of Q-cycle)	85 - 90	90 - 95	85 - 90	~ 90
IV	85 - 90	~ 95	85 - 90	NA
I + deoxyglucose	20 - 30	40 - 50	20 - 30	20 - 30

Table 6.1 ETC inhibition thresholds in synaptosomes before significant effects on glutamate release and $\Delta\psi_m$ were observed

Results are given as a % of control activity of samples containing no inhibitor. Complex I activity was inhibited with rotenone. Complex III activity was inhibited upstream of the Q-cycle by myxothiazol and downstream of the Q-cycle by antimycin A. Complex IV activity was inhibited with KCN. 2 mM deoxyglucose was used as inhibitor of glycolysis. Glutamate release with the absence of any added Ca²⁺ was recorded continuously over 20 min following 5 min pre-incubation and addition of 1 mM 4-aminopyridine (4-AP) or 40 mM KCl (KCl) or no addition (Efflux). $\Delta\psi_m$ after 15 min pre-incubation with inhibitor was measured semi-quantitatively using the fluorescent probe JC-1. Glutamate efflux from polarised synaptosomes after inhibition of complex I activity did not exhibit a threshold effect, nor did depolarisation of $\Delta\psi_m$ after inhibition of complex IV activity. (NA = Not Applicable – A threshold effect was not demonstrated under these conditions).

evident in ETC enzyme control of oxygen consumption in *in situ* synaptosomal mitochondria (Telford *et al.* Unpublished). Indeed, the rate of oxygen consumption in *in situ* synaptosomal mitochondria was found to be decreased at a lower level of inhibition of complex I activity with rotenone than in isolated synaptosomal mitochondria (~ 15%). This level of inhibition matches the level that induced an increase in Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with 4-aminopyridine and when deoxyglucose was also inhibited (Table 6.1).

The energy threshold for inhibition of complex I activity before effects on ATP production and oxygen consumption has consistently shown to be lower than the other ETC enzymes. Inhibition of complex III with myxothiazol activity by up to 80% did not result in major changes in oxidative phosphorylation, while inhibition by 90% completely abolished the oxygen consumption and ATP production in isolated synaptosomal mitochondria (Davey *et al.* 1998). Recent work in our lab has indicated that the threshold level of inhibition of complex III activity on oxygen consumption in *in situ* synaptosomal mitochondria may be lower than that found in isolated synaptosomal mitochondria (Telford *et al.* unpublished). In the *in situ* synaptosomal mitochondrial model, inhibition of complex III activity by ~ 40% reduced the rate of oxygen consumption, and ~ 50% inhibition of complex III activity brought about a reduction in the rate to ~ 65% of the control rate. Inhibition of complex III activity by ~ 70% was found to almost completely abolish the oxygen consumption rate (Telford *et al.* unpublished). At ~ 70% inhibition of complex III activity glutamate efflux from resting synaptosomes was at a maximum (Fig. 4.7) Ca^{2+} -independent glutamate release rates after depolarisation with 4-aminopyridine were also at a maximum (Fig. 4.8) $\Delta\psi_p$ after depolarisation with 4-aminopyridine was depolarised by more than at any other level of complex III activity (Fig. 5.28) and $\Delta\psi_m$ was found to be partially depolarised (Fig. 5.8).

Inhibition of complex IV activity with KCN has previously been demonstrated that a decrease of complex IV activity by up to ~ 70% in isolated synaptosomal mitochondria decreased ATP production by < 10%, with total inhibition of ATP production at 90% inhibition of complex IV activity (Davey *et al.* 1998).

4-aminopyridine was consistently found to increase Ca^{2+} -independent glutamate release rates at lower levels of inhibition of each of the ETC enzymes than KCl. There are two possible explanations for this effect. In each of the experimental conditions the control Ca^{2+} -independent glutamate release rates after depolarisation with KCl was ~ 100 pmol/min/mg protein. This rate was consistently higher than the control rates after depolarisation with 4-aminopyridine, which was 15 – 40 pmol/min/mg. The excess Ca^{2+} -independent glutamate release induced by KCl is thought to occur via reversal of the plasma membrane Na^+ -cotransport pathway (Nicholls 1989). Thus, subtle increases in Ca^{2+} -independent glutamate release rates after depolarisation may be masked by the higher control Ca^{2+} -independent glutamate release rates brought about by depolarisation by KCl. Depolarisation of synaptosomes with 4-aminopyridine is thought not to induce reversal of the glutamate Na^+ -cotransport pathway in the absence of any metabolic inhibitor (Tibbs *et al.* 1996a), and glutamate release after depolarisation with 4-aminopyridine in the absence of Na^{2+} was not different from efflux rates from resting synaptosomes.

Alternatively, the difference in threshold levels may be related to the difference in ATP demand after depolarisation with 4-aminopyridine compared with KCl. In Chapter 3 it was shown that combination of inhibition of complex I activity with 10 μM rotenone decreased the ATP level by $\sim 7\%$ more in synaptosomes depolarised with 4-aminopyridine than those depolarised with KCl (Fig. 3.19). Perhaps partial inhibition of the activities of complexes III and IV combined with depolarisation of synaptosomes with 4-aminopyridine results in a higher demand on ATP than the same level of inhibition combined with depolarisation with KCl. Further experiments to measure the ATP level after partial inhibition of the activities of complexes III and IV are required to investigate the merits of such a hypothesis. Nonetheless, the effect demonstrates the importance of using 4-aminopyridine, an agent that mimics the effect of action potentials on nerve terminals (Tibbs *et al.* 1989) in such synaptosomal experiments, as depolarisation of $\Delta\psi_p$ with KCl is “clamped” and nonphysiological.

Glutamate efflux from 'polarised' synaptosomes was found to occur at a maximum of ~ 66 pmol/min/mg after inhibition of complex I activity (Fig. 3.14, Table 6.2). This rate was not significantly faster than the control efflux rate found in the absence of inhibitor, which was consistently found to be between 5 and 30 pmol/min/mg. The corresponding $\Delta\psi_p$ after 15 min was found to be depolarized by ~ 20 mV to ~ -53 mV (Fig. 5.23, Table 6.2). However, inhibition of complex III activity by $\sim 72\%$ with myxothiazol increased the rate of glutamate efflux to ~ 117 pmol/min/mg (Fig. 4.7) and inhibition of complex IV activity by $\sim 87\%$ with KCN increased the efflux rate to ~ 175 pmol/min/mg (Fig. 4.22). The corresponding $\Delta\psi_p$ at these maxima release conditions for complex III and complex IV were ~ -47 mV and ~ -46 mV respectively. The glutamate release under these conditions was found to be Ca^{2+} -independent, suggesting non-vesicular release mechanism. Increased glutamate release from resting synaptosomes after metabolic inhibition has been proposed to occur via reversal of uptake transporters, which rely on normal functioning of the Na^+/K^+ ATPase to maintain the Na^+ and K^+ gradients across the plasma membrane (Nicholls 1989, 1993). The results presented here indicate that partial inhibition of complex III activity and complete inhibition of complex IV activity caused reversal of this transport system, resulting in significant increase in glutamate efflux as $\Delta\psi_p$ is depolarised past ~ -50 mV (Fig. 6.1A). Also, the maximum efflux rates were shown to increase continuously at later points of intervention along the ETC (Table 6.2).

The corresponding Ca^{2+} -independent glutamate release rates after depolarisation with 4-aminopyridine were also shown to increase continuously at later points of intervention along the ETC (Table 6.2). Inhibition of complex IV activity by $> 85\%$ brought about the maximum Ca^{2+} -independent release rate after depolarisation with 4-aminopyridine (~ 265 pmol/min/mg protein) and inhibition of complex I activity by 60 – 90% caused release of ~ 200 pmol/min/mg protein. A maximum rate of ~ 230 pmol/min/mg was recorded after 70 – 90% inhibition of complex III activity with myxothiazol. Paradoxically, the corresponding values of $\Delta\psi_p$ at these levels of inhibition were shown to decrease continuously at later points of intervention along the ETC (Table 6.2). Nonetheless, when Ca^{2+} -independent glutamate release rates after depolarisation with 4-aminopyridine were plotted against the corresponding $\Delta\psi_p$ for the entire complex I, III and IV titrations, a clear trend was evident: depolarisation beyond a threshold $\Delta\psi_p$ of $-45 - -35$ mV increased the release rates (Fig. 6.1B).

Complex	Inhibition of activity (% of control)	Ca ²⁺ -independent glutamate release (pmol/min/mg)			$\Delta\psi_p$ (mV)		
		4-AP	KCl	Efflux	4-AP	KCl	Polarised
I	60 - 90	~ 200	~ 300	~ 66	~ -6	~ -10	~ -53
III	70 - 90	~ 230	~ 400	~ 117	~ -35	~ -20	~ -47
IV	> 85	~ 265	~ 265	~ 175	~ -45	~ -39	~ -46

Table 6.2 Maximum glutamate release rates from synaptosomes after inhibition of ETC enzyme activity and relationship to $\Delta\psi_p$

The activities of complexes I, III and IV were inhibited with a range of concentrations of rotenone, myxothiazol and KCN respectively. Glutamate release in the absence of any added Ca²⁺ was recorded continuously over 20 min following 5 min pre-incubation and addition of 1 mM 4-aminopyridine (4-AP) or 40 mM KCl (KCl) or no addition (Efflux) and maximum rates after inhibition of each ETC enzyme were found to occur at the levels of inhibition shown. $\Delta\psi_p$ was measured using the fluorescent probe PMPI, and values shown are the $\Delta\psi_p$ found that were corresponding to the level of inhibition of ETC enzymes leading to the maximum Ca²⁺-independent glutamate release rates.

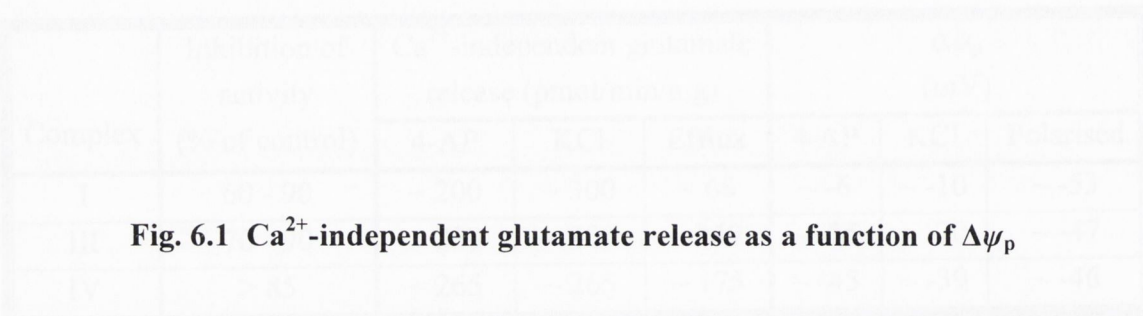
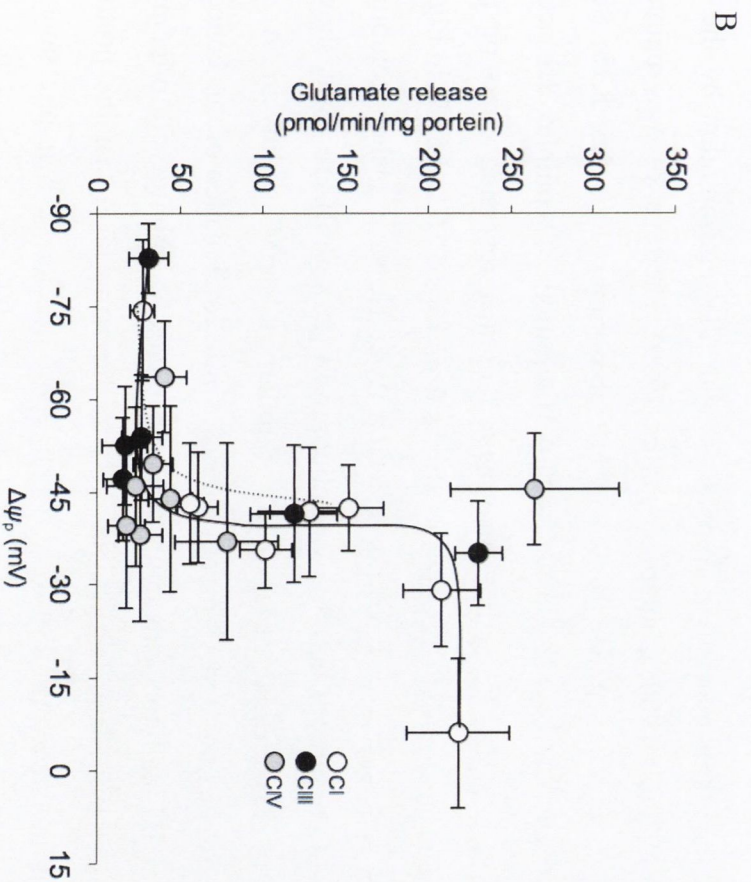
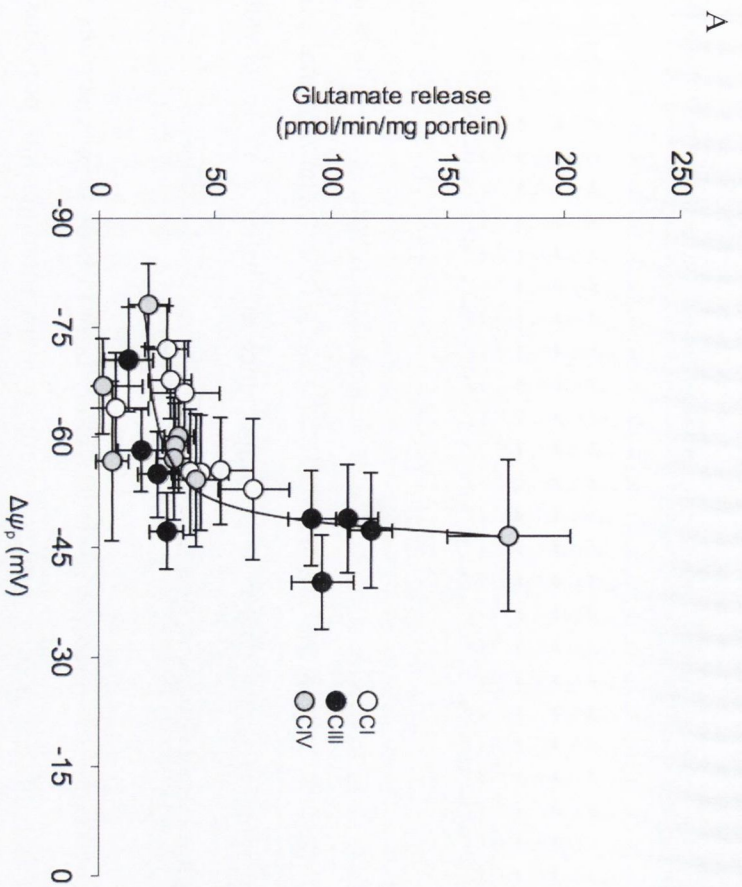


Fig. 6.1 Ca^{2+} -independent glutamate release as a function of $\Delta\psi_p$

Complex I activity was inhibited with rotenone (1 nM – 10 μM), complex III activity with myxothiazol (10 nM – 10 μM) and complex IV activity with KCN (5 μM – 1 mM) and $\Delta\psi_p$ measured fluorimetrically with PMPI 15 min after addition of inhibitor. **A** Glutamate efflux from resting synaptosomes was measured over 20 min following 5 min pre-incubation with inhibitor. **B** Glutamate release from synaptosomes depolarised with 1 mM 4-aminopyridine was measured over 20 min following 5 min pre-incubation with inhibitor. Freehand curves were drawn through the points (dotted line in B represents glutamate efflux curve shown in A).



Inhibition of ETC enzyme activity and depolarisation with KCl resulted no clear trend with regards to point of inhibition along the ETC: inhibition of complex I activity by 60 – 90% increased Ca^{2+} -independent glutamate release rates to a maximum of ~ 300 pmol/min/mg, inhibition of complex III activity with myxothiazol by $\sim 90\%$ increased Ca^{2+} -independent glutamate release rates to ~ 400 pmol/min/mg, but inhibition of complex IV activity by $\sim 90\%$ increased Ca^{2+} -independent glutamate release rates to only ~ 265 pmol/min/mg (Table 6.2). The corresponding $\Delta\psi_p$ values were ~ -10 mV, ~ -20 mV and ~ -39 mV for each of the respective enzymes. Thus, was found to be not directly related to the level of $\Delta\psi_p$ after depolarisation with KCl. This is likely to be at least partially due to the effects of KCl on reversal of synaptosomal glutamate uptake transporters, and reiterates the importance of using a more physiological means of depolarisation.

The role of complex V in ATP depletion after inhibition of ETC enzymes remains unclear. Total inhibition of complex I activity with rotenone has previously been found to reverse complex V activity in synaptosomes, resulting in proton pumping out of the mitochondria matrix via complex V and ATP consumption by the enzyme (Scott and Nicholls 1980; Chinopoulos *et al.* 1999). The level of $\Delta\psi_m$ maintained in this situation was previously shown to be sub-optimal (Figs 5.5, 5.16; Scott and Nicholls 1980; Chinopoulos *et al.* 1999). Inhibition of complex III activity or inhibition of complex IV activity may have a similar reversing effect on the ATP synthase. The clearest evidence for this comes from Figs 5.16 and 5.17. In Fig. 5.16, 1 mM KCN was shown to have no effect on $\Delta\psi_m$. However, when combined with inhibition of complex V activity with oligomycin (20 $\mu\text{g/ml}$), $\Delta\psi_m$ was shown to be significantly depolarised (Fig. 5.16). Similarly, 1 μM antimycin A partially depolarised $\Delta\psi_m$ in 6- and 18-month old rats, while combination of this concentration of complex III inhibitor with inhibition of complex V activity with oligomycin (20 $\mu\text{g/ml}$) resulted in depolarisation of $\Delta\psi_m$ to the same level that the protonophore FCCP did in excess (1 μM , Fig. 5.17). These results have implications for the interpretation of the results of titrating out the complex IV activity with KCN (Fig. 5.14) on $\Delta\psi_m$. Under these circumstances, $\Delta\psi_m$ was maintained at the control charge at all levels of complex IV inhibition, but proton pumping by reversed complex V may contribute to the maintenance of $\Delta\psi_m$, at least at high levels of inhibition.

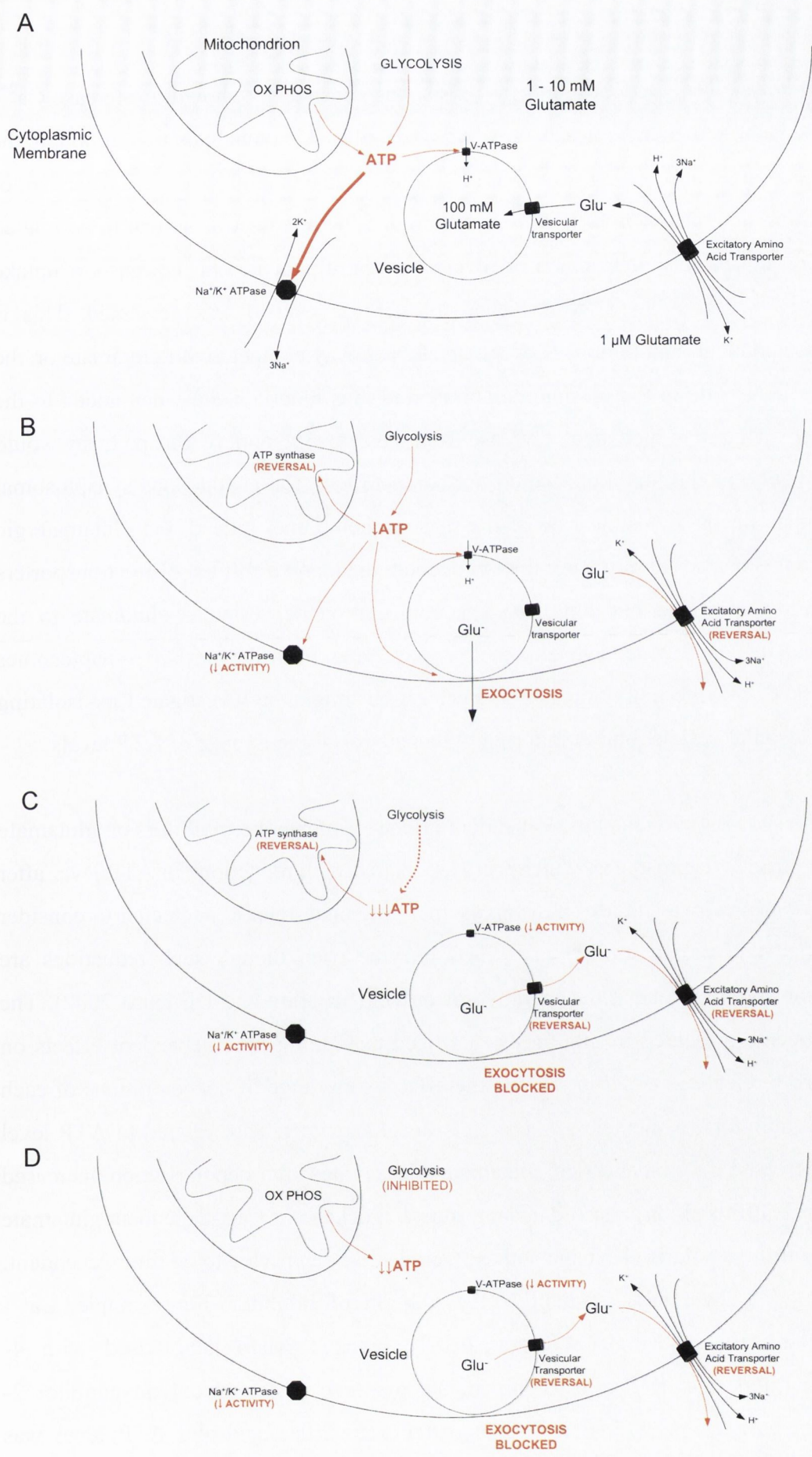
The contribution of complex V to maintenance of $\Delta\psi_m$ after partial inhibition of ETC enzyme activity remains to be investigated. However, the effects of inhibition of ATP consumption by reversed complex V after inhibition of complex I activity with rotenone on Ca^{2+} -independent glutamate release rates from depolarised synaptosomes was examined (Fig. 3.10). In this experiment, oligomycin was added in excess (20 $\mu\text{g/ml}$) to totally inhibit complex V activity, and rotenone was added (1 nM – 10 μM) to inhibit complex I activity by different extents. Although corresponding complex I activity assays were not performed, pattern of increase in Ca^{2+} -independent glutamate release rates after titration with rotenone in the absence of oligomycin was similar to that shown by inhibition of complex I activity with this range of concentrations of rotenone (Fig. 3.13). However, the increase in release brought about by rotenone was not prevented by inhibition of complex V activity oligomycin. This indicates that ATP consumption by complex V may not contribute to the ATP depletion that brings about increased in Ca^{2+} -independent glutamate release rates from depolarised synaptosomes.

Indeed, the increase in Ca^{2+} -independent glutamate release rates to 300 – 400 pmol/min/mg by oligomycin alone indicates that mitochondrial ATP is important in the causal mechanism of this increase. Subsequent investigation of the effects of lower concentrations of oligomycin on glutamate release resulted in increased Ca^{2+} -independent glutamate release rates from synaptosomes depolarised with KCl, although the increase was not as great as in the presence of 20 $\mu\text{g/ml}$. The effects of total complex V inhibition combined with partial inhibition of other ETC enzyme activity on Ca^{2+} -independent glutamate release rates from depolarised synaptosomes remains to be investigated.

Inhibition of glycolysis increased glutamate efflux from resting synaptosomes to ~ 250 pmol/min/mg between 60 – 90% inhibition of complex I activity (Fig. 3.14) and Ca^{2+} -independent glutamate release rates after depolarisation with 4-aminopyridine were similar at this level of inhibition of complex I activity when glycolysis was inhibited. The effects of inhibition of ETC enzymes and glycolysis and on glutamate release from synaptosomes are summarised and the possible mechanisms for such effects are proposed in Fig. 6.2.

Fig. 6.2 Proposed mechanisms of interaction between bioenergetic and glutamatergic systems in nerve terminals

A: In resting synaptosomes, up to 70% of ATP is used by Na^+/K^+ ATPase to maintain $\Delta\psi_p$ at $-70 - -80$ mV, and also by the vesicular ATPase (v-ATPase), which pumps H^+ across the vesicular membrane, creating an electrochemical gradient across the vesicular membrane of $\sim +100$ mV. Glutamate uptake into vesicles is driven by this charge, and transport across the plasma membrane relies on cotransport of 3 Na^+ and one proton, and the counter-transport of one K^+ . **B:** Inhibition of complex I activity by $> 40\%$ lowers the ATP level, causing reduced Na^+/K^+ ATPase activity, a fall in $\Delta\psi_p$ to ~ 55 mV and reversal of the plasma membrane glutamate transport system, as well as reversal of the ATP synthase. However, glycolysis is capable of sustaining the ATP level above that required for Ca^{2+} -dependent vesicular (exocytotic) release of glutamate. Depolarisation with KCl results in Ca^{2+} -independent release of glutamate via reversal of the plasma membrane glutamate transport system, but the ATP level is not reduced any more than in polarised synaptosomes. **C:** Inhibition of complex III or complex IV activity or inhibition of complex I activity combined with inhibition of glycolysis can lower the ATP level to an extent that reduces Na^+/K^+ ATPase activity, causing a fall in $\Delta\psi_p$ to < -50 mV, reversal of the glutamate transport system, reversal of the ATP synthase and blockage of exocytosis from depolarised synaptosomes. The activity of v-ATPase is also likely to be reduced in this scenario, resulting in reversal of the vesicular glutamate transporter and leakage of the vesicular glutamate pool into the cytoplasmic pool. **D:** Inhibition of glycolysis reduces Na^+/K^+ ATPase activity, reversal of the plasma membrane glutamate transport system, and blocks Ca^{2+} -dependent exocytotic release of glutamate from depolarised synaptosomes. The activity of v-ATPase is also likely to be reduced in this scenario, resulting in reversal of the vesicular glutamate transporter and leakage of the vesicular pool into the cytoplasmic pool. However, mitochondria maintain a level of ATP (30 – 35% of control) for at least 15 min after inhibition of glycolysis.



6.2 Future work

Inhibition of ETC enzymes and glycolysis was consistently shown to increase Ca^{2+} -independent glutamate release rates from depolarised synaptosomes, although the increase varied according to which ETC enzyme was being inhibited and the extent of inhibition of each enzyme. However, although the release was shown to be related to $\Delta\psi_p$ and a likely mechanism of reversal of the glutamate Na^+ cotransport uptake pathway was postulated, no evidence for such a reversal was presented. This is because conventional inhibitors of the uptake pathway compete with glutamate on the extracellular side of the plasma membrane and thus block uptake when added to the medium. Therefore blockage of release from the cytosolic pool by this pathway would be unlikely, as the inhibitors would not compete with the endogenous synaptosomal glutamate on the cytosolic side of the plasma membrane. Indeed, the glutamatergic uptake mechanism in nerve terminal is understudied, and a full list of the transporters involved has yet to be compiled. The contribution of vesicular glutamate to the increase in Ca^{2+} -independent glutamate release rates from depolarised synaptosomes after ETC enzyme inhibition is also unclear, but might be investigated by isolating synaptosomal vesicles and monitoring glutamate release at a range of ATP levels.

Despite showing obvious time-dependent effects of metabolic inhibitors on glutamate release from synaptosomes, which were associated with reductions in ATP level after inhibition, previous studies on synaptosomal glutamate release neglected to consider the effects of partial inhibition of ETC enzyme even though such reductions are common in chronic neurodegenerative disorders (Schapira and DiMauro 2002). The experiments presented in this thesis sacrificed examining time-dependent effects on glutamate release in order to include the effects of a range of concentrations of each of the inhibitors. Nonetheless, the effects on glutamate release related to ATP level (i.e. increased Ca^{2+} -independent glutamate release rates after depolarisation, increased glutamate efflux from polarised synaptosomes, blockage of Ca^{2+} -dependent glutamate release from depolarised synaptosomes) would still be expected to be time-dependent, as the ATP level falls gradually after addition of inhibitor. For example, Ca^{2+} -dependent glutamate release was partially blocked when depolarised with 4-aminopyridine and KCl after 5 min in the presence of the glycolytic inhibitor 2-deoxyglucose (Figs 3.15, 3.16). Thus, after 5 min the cytosolic ATP level was

sufficiently reduced so that exocytosis could no longer proceed as normal. Inhibition of glycolysis also increased glutamate efflux from resting synaptosomes (Fig. 3.5) with the efflux rate taken after 5 min pre-incubation with deoxyglucose. Further experiments are required to establish the point at which deoxyglucose depletes the ATP level sufficiently to depolarise $\Delta\psi_p$ and reverse the glutamate uptake pathway. This would involve parallel continuous monitoring of ATP level, $\Delta\psi_p$ and glutamate release. Such experiments on glutamate release would have to be performed in a more sensitive (both temporally and fluorimetrically) well-plate reader than the SpectraMAX Gemini used in these experiments, lacked the speed and accuracy required to record changes in glutamate release over short time periods (< 20 min).

Indeed, inhibition of glycolysis would eventually be expected to result in depletion of the endogenous synaptosomal NADH and FADH₂ pools. Under these circumstances, electron transfer to complex I and complex II of the ETC would cease, resulting in inhibition of ETC proton pumping and oxidative phosphorylation. The time span of this depletion was not examined, and could be performed fluorimetrically (at least for the NAD(P)H pool, at excitation $\lambda = 340$ nm and emission $\lambda = 360$ nm, if the sensitivity of the fluorimeter is sufficient). Synaptosomes have been shown to express a plasma membrane pyruvate uptake transporter, and inhibition of glycolysis combined with addition of excess pyruvate to the medium would allow ETC activity to continue as normal while the effects of inhibition of glycolysis alone on glutamate release/ $\Delta\psi_p$ were monitored.

Previous studies on glutamate release have disregarded increases in Ca²⁺-independent release glutamate release rates from metabolically impaired synaptosomes after depolarisation, presumably because of the non-physiological Ca²⁺-independent release brought about by KCl. 1 mM 4-aminopyridine was here shown to not stimulate any excess Ca²⁺-independent release glutamate release in control samples, but the rates increased after metabolic inhibition. However, this concentration high (Tibbs *et al.* 1996a), and results in rapid repeated action potential-like depolarisations, similar to those thought to occur in glutamatergic nerve terminals in the substantia nigra during Parkinson's Disease (Bezard *et al.* 1997; Obeso *et al.* 2004). A recent study found that complex I activity was reduced in Parkinson's disease frontal cortex (Parker *et al.* 2008) and a genetic predisposition to susceptibility (due to a mutation in the complex

I ND5 subunit; Parker and Parks 2005) was postulated, suggesting complex I activity may be widespread in the idiopathic form of the disease. Similarly, systemic infusion of rotenone to rats at a low concentration results in selective degeneration of dopaminergic substantia nigra neurons similar to that seen in Parkinson's disease (Betarbet *et al.* 2000). This opens up the question of why this region is selectively affected if complex I activity is also reduced in other areas of the brain. Future work might examine the effects of a range of concentrations of 4-aminopyridine on glutamate release from synaptosomes with reduced complex I activity, with the aim of modelling release rates during normal glutamatergic neurotransmission in brain areas that do not degenerate during Parkinson's disease. Also, differences in effects of partial inhibition of complex I activity on oxygen consumption and ATP production between mitochondria isolated from different brain regions have previously been demonstrated (Davey *et al.* 1997). Such differences may contribute to different effects on glutamate release after partial inhibition of complex I activity depending on brain region. Thus comparison of the effects of inhibition complex I activity in isolated substantia nigra synaptosomes with those isolated from other brain regions might contribute to the explanation of the apparent susceptibility of this area to degeneration during Parkinson's Disease.

Susceptibility of Ca^{2+} -independent release glutamate release rates from depolarised synaptosomes to low level inhibition of complex I activity was shown to be increased in nerve terminals from 18-month old rats compared to those from 6-month old rats. Simultaneously, $\Delta\psi_m$ was shown to be lower and H_2O_2 production was found to be higher in the older rats. In other work done in our lab on samples from the synaptosomal mitochondria, ETC enzyme activity was shown to be reduced (Kilbride *et al.* 2008). These age-related changes were observed in rats considered to be of 'late-middle age' (Coleman *et al.* 2004). Further investigation of these parameters in rats of 'old age' is warranted to examine whether such deterioration continues in brain nerve terminal mitochondria, and may generate information on why old age is considered a high risk factor in many chronic neurodegenerative disorders.

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Publications

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 depo-

larized 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 homogenates 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), according 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 fluorimetric 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 $\lambda = 460$ 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'-tetraethylbenzimidazoly carbocyanine 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-1, 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 $\lambda = 590$ nm and at $\lambda = 535$ nm ($\lambda = 490$ 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 $\pm$ 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 $37.8 \pm 6.8\%$ (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, whereas 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.

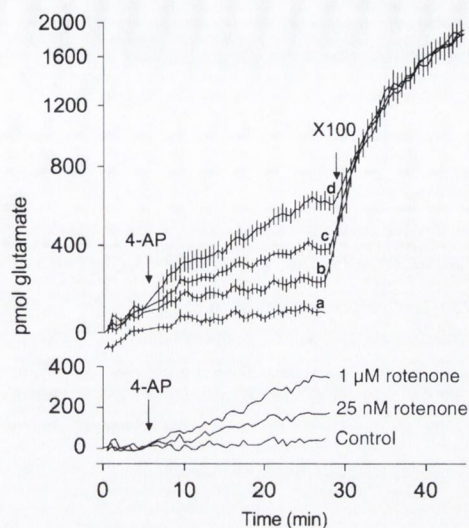


Fig. 1 Representative traces showing the effect of rotenone on Ca^{2+} -independent release of glutamate from synaptosomes depolarized with 4-aminopyridine. Synaptosomes were pre-incubated at 1 mg/mL for 5 min before addition to assay mixture and readings were taken over a 32-s period. Trace **a** shows 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 $\pm$ 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 $42.7 \pm 5.6\%$ inhibition of complex I activity. When depolarized with KCl, 100 nM rotenone, which caused $68.4 \pm 6.3\%$ 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

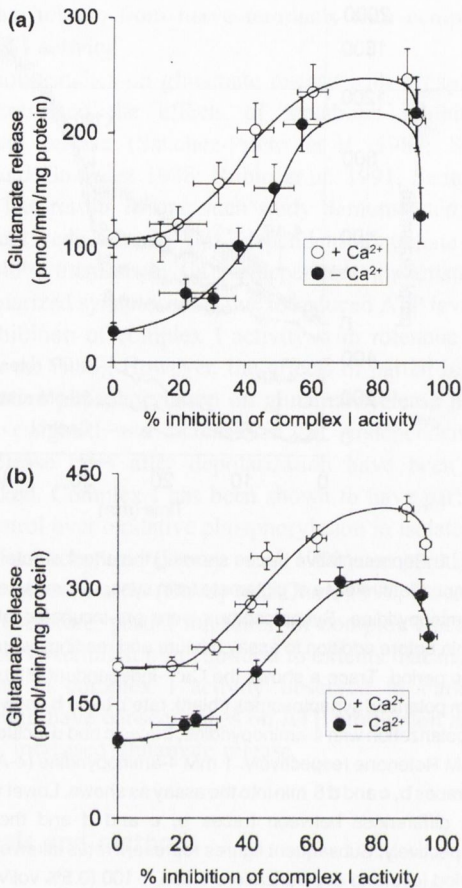


Fig. 2 Partial inhibition of complex I activity increases release of non-vesicular glutamate stores from depolarized synaptosomes. Synaptosomes (0.5 mg/mL) were pre-incubated at 37°C for 5 min in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarized with (a) 1 mM 4-aminopyridine or (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 Ca²⁺-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.

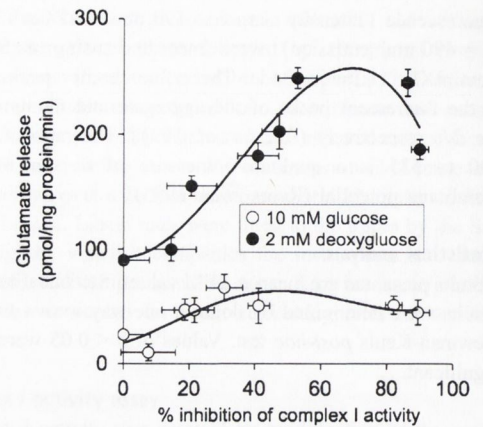


Fig. 3 Partial inhibition of complex I activity combined with inhibition of glycolysis increases glutamate efflux from synaptosomes. Synaptosomes (0.5 mg/mL) were incubated with 10 mM glucose (○) or 2 mM deoxyglucose (●) and rotenone (1 nM – 10 μM) and in the presence of 1.4 mM CaCl₂. Rates of glutamate 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 CaCl₂ at any concentration of rotenone (results not shown), indicating that the increase in glutamate efflux is Ca²⁺-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 Ca²⁺-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 CaCl₂ from 107.8 ± 16.1 to 84 ± 8.2 pmol/min/mg, when depolarized with 4-aminopyridine (Figs 2a and 4a). This suggests that Ca²⁺-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 CaCl₂ was reduced when

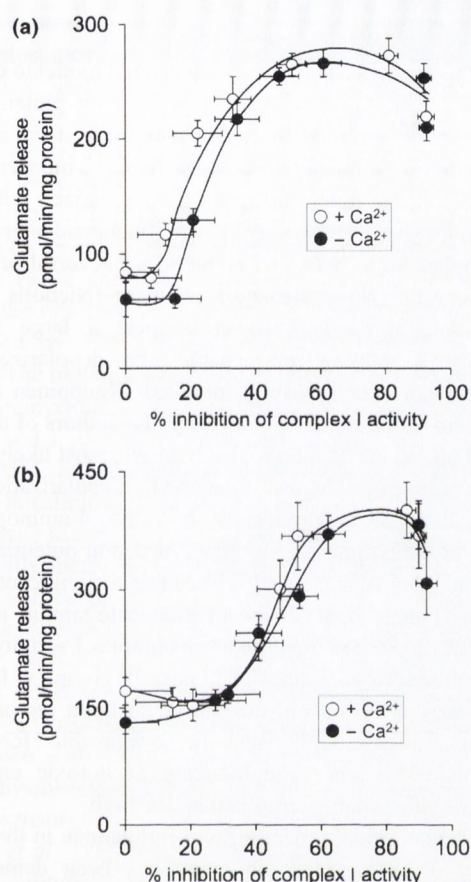


Fig. 4 Inhibition of complex I activity and glycolysis blocks the Ca²⁺-dependent component of glutamate release from depolarized synaptosomes. Synaptosomes (0.5 mg/mL) were pre-incubated for 5 min with 2 mM deoxyglucose in the presence (○) or absence (●) of 1.4 mM CaCl₂ before being depolarized with (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.

glycolysis is inhibited (comparing Figs 2 and 4, 0% inhibition) and increased in the absence of CaCl₂.

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).

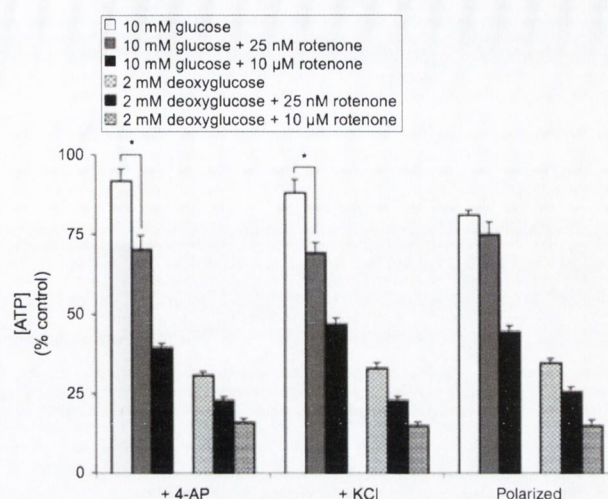


Fig. 5 Partial inhibition of complex I activity controls ATP levels in synaptosomes. Synaptosomes (0.5 mg/mL) were divided into groups and incubated at 37°C with 10 mM glucose or 2 mM 2-deoxyglucose and rotenone as indicated. ATP from synaptosomes was PCA extracted immediately upon resuspension and again 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.

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 depolarising 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 ΔΨ_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 ΔΨ_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

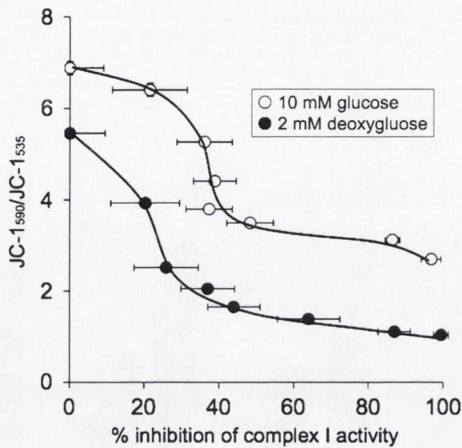


Fig. 6 Partial inhibition of complex I activity causes a fall in $\Delta\psi_m$ in synaptosomes. Synaptosomes were loaded with JC-1 and resuspended (0.5 mg/mL) with 10 mM glucose (○) or 2 mM deoxyglucose (●) and rotenone (1 nM – 10 μ M) and in the absence of Ca^{2+} . Fluorescence intensity of the aggregate form of JC-1 (excitation $\lambda = 490$ nm, emission $\lambda = 590$) was divided by that of the monomer (excitation $\lambda = 490$ nm, emission $\lambda = 535$) 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 $\pm$ 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 $37.3 \pm 6.3\%$ inhibition. When 1 μ M carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) was added to completely collapse $\Delta\psi_m$, the ratio was 1.08 ± 0.04 . Using 10 μ M rotenone to cause $96.7 \pm 2.6\%$ 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 $20.2 \pm 9.2\%$ (1 nM rotenone) and $25.9 \pm 8.6\%$ (10 nM rotenone) inhibition reduced the ratio to 3.93 ± 0.1 and 2.52 ± 0.11 respectively. At $44 \pm 7\%$ 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 $99.4 \pm 2\%$ inhibition of complex I activity (10 μ M 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 μ M 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 μ M

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 μ M rotenone are added, and 10 μ M 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 μ M rotenone. Nonetheless, 10 μ M 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 μ M rotenone, but show shorter hyperpolarization phases at 3 μ M and 1 μ M 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 depolariza-

tion 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 synaptosomes (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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