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CRITICAL AGE-RELATED LOSS OF COFACTORS OF NEURON CYTOCHROME C OXIDASE REVERSED BY ESTROGEN

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Abstract

The mechanistic basis for the correlation between mitochondrial dysfunction and neurodegenerative disease is unclear, but evidence supports involvement of cytochrome C oxidase (CCO) deficits with age. Neurons isolated from the brains of 24 month and 9 month rats and cultured in common conditions provide a model of intrinsic neuronal aging. *In situ* CCO activity was decreased in 24 month neurons relative to 9 month neurons. Possible CCO-related deficits include holoenzyme activity, cofactor, and substrate. No difference was found between neurons from 24 month and 9 month rats in mitochondrial counts per neuron, CCO activity in submitochondrial particles, or basal respiration. Immunostaining for cytochrome C in individual mitochondria revealed an age-related deficit of this electron donor. 24 month neurons did not have adequate respiratory capacity to upregulate respiration after a glutamate stimulus, in spite of a two-fold upregulation of respiration seen in 9 month neurons. Respiration in 24 month neurons was inhibited by lower concentrations of potassium cyanide, suggesting a 50% deficit in functional enzyme in 24 month compared to 9 month neurons. In addition to cytochrome C, CCO requires cardiolipin to function. Staining with nonylacridine orange revealed an age-related deficit in cardiolipin. Treatment of 24 month neurons with 17- β -estradiol restored cardiolipin levels (10 ng/mL) and upregulated respiration under glutamate stress (1 pg/mL). Attempts to induce mitochondrial turnover by neuronal multiplication also rejuvenated CCO activity in 24 month neurons. These data suggest cytochrome C and cardiolipin levels are deficient in 24 month neurons, preventing normal upregulation of respiration needed for oxidative phosphorylation in response to stress. Furthermore, the data suggest this deficit can be corrected with estrogen treatment.

Keywords

aging; neuron culture; cytochrome C oxidase; mitochondria; estrogen; brain

INTRODUCTION

Changes in cytochrome C oxidase (CCO) function and quantity are widely documented characteristics of the aging mammalian brain (Bertoni-Freddari *et al.*, 2004; Bertoni-Freddari *et al.*, 2004; Curti *et al.*, 1990; Davey & Clark, 1996; Fattoretti *et al.*, 2006; Sen *et al.*, 2007; Weinreb *et al.*, 2007). The importance of brain dependence on oxygen for activity is highlighted by the fact that the brain only comprises about 2% of the human body's mass, but it consumes approximately 20% of the body's oxygen (Grande 1979). CCO converts oxygen to water in the final step of the electron transport chain, driving aerobic ATP

production and therefore supplying most of the phosphorylative energy to the brain. In spite of an age-related increase in total CCO protein levels, CCO activity decreases in homogenates of 24 month rat hippocampal and cerebellar brain regions (Fattoretti *et al.*, 2006). Other studies show a significant decrease in CCO protein content in the aging hippocampus (Weinreb *et al.*, 2007). CCO activity also declines with age in isolated synaptic mitochondria (Sen *et al.*, 2007; Bertoni-Freddari *et al.*, 2006). However, all of the samples extracted from 24 month rat brains are subject to degradation during homogenization and are inextricably linked to the aging environment of the brain including the vasculature, hormone levels, and immune system. These interfering factors compromise the interpretation of whether activity differences are due to aging neurons or are caused by the aging brain environment, alternatives that greatly impact the design of anti-aging therapy. By isolating viable cortical and hippocampal neurons from 9 month and 24 month rat brains under common culture conditions, we have removed these variables to study CCO changes with age that are intrinsic to neurons.

Although brain CCO activity has been studied for over 40 years (Moraczewski & Anderson, 1966), advancements in techniques have been restrained by methods of sample preparation. Age-related differences in CCO activity observed in brain homogenates (Fattoretti *et al.*, 2006) and synaptic mitochondria (Sen *et al.*, 2007; Bertoni-Freddari *et al.*, 2006) have, in the past, failed to differentiate between the aging neurons and the brain's vasculature, hormones, and immune system. Our method of isolating neurons from the brains of adult rats and growing them in common culture conditions has allowed us to apply well-established techniques to the study of intrinsic differences of CCO, *in situ*, in live 9 month and 24 month neurons separated from the aging environment of the brain. In this study, we found that an age-related deficit in CCO is maintained in viable neurons even when the cells are isolated to culture conditions that are common among the ages, and performed additional studies in isolated neurons to determine the cause of such deficiencies. We also evaluated whether mitochondrial turnover could be induced by neuronal multiplication (Brewer, 1999) to rejuvenate CCO activity in 24 month neurons.

For CCO to reduce oxygen, both the electron source, reduced cytochrome C, as well as an anionic lipid environment containing cardiolipin are necessary (Robinson *et al.*, 1990). We evaluated whether deficits in cytochrome C or cardiolipin could account for the decrease in CCO activity with age. To test the effects of these deficits on mitochondria *in situ* in neurons, we measured cellular respiration of cells isolated from E18, 9 month, and 24 month rats under basal and glutamate-challenged conditions to increase calcium and consume ATP to maximize activity of CCO. We chose glutamate as a stressor because of the efficacy of the Alzheimer's Disease drug memantine, an NMDA-receptor antagonist (Wilcock *et al.*, 2002; Reisberg *et al.*, 2003), implicates glutamate as an effector of age-related neurodegeneration. Also, the demand for ATP during glutamate stimulation of NMDA receptors uses 80% of the spare respiratory capacity in cerebellar granule neurons isolated from rat pups (Yadava & Nicholls, 2007). With age, a depletion of spare respiratory capacity could reduce viability in response to stress. While 9 month and 24 month neurons showed adequate basal respiration, the high energy demand imposed by glutamate adequately upregulated respiration only in the 9 month neurons. As an independent measure of functional CCO, we titrated increasing concentrations of potassium cyanide (Barber *et al.*, 1978; Padmaja & Panikkar, 1989) onto viable neurons to compare capacities of 24 month relative to 9 month neurons. Since Brewer *et al.* (2006) showed that low concentrations of estrogen (as low as 0.1 pg/mL) dose-dependently restored calcium homeostasis in aging neurons, we tested the ability of estrogen treatment of neurons to reverse age-related structural deficits in cardiolipin as well as functional deficits in CCO activity after glutamate stress. We report that estrogen reverses cardiolipin deficits and respiratory response to glutamate in 24 month neurons.

MATERIALS AND METHODS

Cell Culture

Adult rat neurons were cultured according to the method of Brewer (Brewer, 1997; Brewer & Torricelli 2007). Male fisher 344 rats, which have a median life span of 24 months, were used for all experiments (Solleveld *et al.*, 1984). The rats were fed rat chow *ad libitum* and weighed 350–500 g at the time of sacrifice. Cortical and hippocampal neurons were extracted from brains of embryonic day 18 (E18), 9 month (9 month), and 24–25 month (24 month) rats. Once dissected, the hippocampi or cortices were sliced to 0.5 mm with a McIlwain chopper (Campden Instruments, Lafayette, IN), digested in 2 mg/mL papain (Worthington, Lakewood, NJ), and triturated in Hibernate A/B27 minus calcium (BrainBits LLC.com). Cells were separated from debris on an Optiprep (Sigma-Aldrich, St. Louis, MO) gradient and resuspended in Neurobasal A/B27, 0.5 mM glutamine, 5 ng/mL basic human recombinant FGF2 (Invitrogen, Carlsbad, CA). Cells were then plated on poly-D-lysine (Sigma-Aldrich, St. Louis, MO)-coated glass cover slips (Assistent brand, Carolina Biological, Burlington, NC) at densities of 320 cells/mm² – 500 cells/mm², and cultured at 37°C, 5% CO₂, 9% O₂ for 5 – 12 days

In situ Cytochrome C Oxidase Activity

Multiplication of 24 month neurons (Reisberg *et al.*, 2003) was achieved by plating neurons at the low density of 40 cells/mm² and addition of human recombinant FGF2 (5 ng/mL) every 4 days. Viable neurons were released from culture slips using 0.25% trypsin. Cells were transferred to 24-well plates, rinsed once with 37°C 0.1 M Na-phosphate buffer, pH 7.0, and stained with 250 µL solution/well. Staining solution contained 0.1 M Na-phosphate, 0.13% cytochrome C, 0.14% diaminobenzidine (Sigma-Aldrich) and was kept from light until the time of the experiment (24 month & Johnson, 1989). A negative control solution was made without cytochrome C. After 60 minutes incubation at 37°C, staining solution was removed and slips were rinsed three times with PBS. Slips were mounted using Aquamount (Fisher Scientific, Hampton, NH). The density of the diaminobenzidine (DAB) reaction product was measured using phase optics and a Spot camera (Diagnostic Instruments, Sterling Heights, MI) initially and after 60 minutes. Substrate consumption was compared among the ages and treatments using ImagePro software (Media Cybernetics, Bethesda, MD) (Frasch *et al.*, 1978).

Mitochondrial counts

Mitochondria were stained *in situ* in 24 month, 9 month, and E18 hippocampal neurons cultured 5–7 days with 250 nM MitoTracker Red (Molecular Probes, Carlsbad, CA). They were imaged using 60x oil immersion fluorescent imaging (Olympus IX-71), and a Retiga Exi cooled CCD camera (QImaging, Surrey, BC, Canada). Based on constant brightness thresh24 months with area limits, mitochondria were counted using Image Pro software.

Cytochrome C Immunoreactivity

Mitochondria were immunostained *in situ* with anti-cytochrome c primary antibodies (PharMingen, San Jose, CA) and AlexaFluor 488 goat-anti-mouse secondary antibodies (Invitrogen, Carlsbad, CA). The mitochondrial area of this stain was measured using ImagePro Software, as was the density of the stain within the area.

Respiration

Cells plated at 500/mm² were cultured for 7 – 12 days on 15 mm diameter poly-D-lysine coated glass cover slips. Prior to respiration measurements, six fields of cells were photographed with 20x phase optics (Olympus, Center Valley, PA) and counted for

normalization purposes. Slips were also photographed after respiration measurements to confirm the presence of cells. We monitored basal, CCO-inhibited, and glutamate-challenged neuronal respiration using the Oxygraph-2K (Oroboros, Innsbruck, Austria). Two polarographic oxygen sensors connected to temperature-controlled, continuously stirred, sealed chambers measure oxygen concentration within the chamber over time. Early experiments optimized 500 RPM as the best stirring speed for full cell adherence with minimum electrode noise. DatLab4 software (Oroboros) calibrated the instrument using atmospheric oxygen concentration (21%) and 0% oxygen concentration (obtained by addition of sodium dithionite to water) as reference points. We derived oxygen consumption rates from DatLab4-generated graphs of oxygen concentration and flux in real time. Typical measurements in the Oxygraph are taken from cell suspensions, but neuronal bioenergetics rely on attachment to a substrate. One prior study, Jekabsons and Nicholls (2004), successfully measured oxygen consumption in attached cerebellar granule neurons from very young rats. Our system measures oxygen consumption in a closed chamber in cortical neurons of varying ages of rats. We developed 14.5 mm diameter silicone (Kwik-cast, World Precision Instruments, Sarasota, FL) disc-topped stirbars with Teflon star bases (Nalgene, Rochester, NY) to accommodate 500 cells/mm² neuronal cultures adherent to 15 mm glass cover slips. The silicone was chosen as an optimal material as it did not absorb a significant amount of oxygen when added to the chamber alone. The cells adhered to the cover slip, balanced atop the specialized stirbar, during the titration of drugs, and had an open interface with the medium within the sealed 1-mL chamber and direct interaction with the titrants. Oxygen consumption was measured as a change in oxygen concentration in the chamber as oxygen was converted to water, detected by the connected polarographic oxygen sensors, over time. The rate of respiration was derived from this measurement using DatLab4 software. For inhibition of CCO, freshly prepared potassium cyanide (Sigma-Aldrich, 20781-0) was titrated in concentrations from 10 – 100 μ M from 100x stocks in 2–5 minute increments using a 25 μ L syringe with a 75 mm needle (Hamilton, Reno, NV) until a steady rate was established. Stimulation of respiration with glutamate (U.S. Biological, Swampscott, MA; G7115) was also delivered in 2–5 minute increments at concentrations from 25 – 800 μ M from 100x stock in Neurobasal/B27 or Neurobasal A/B27. Means and S.E.M. were calculated using Plot-It software (Scientific Programming Enterprises).

Mitochondrial Cytochrome C Oxidase Activity

Submitochondrial particles were isolated from 9 month and 24 month rat cortical neurons cultured for 5 – 7 days on 50×24 mm glass cover slips according to the method of Almeida and Medina (1998). Cytochrome C oxidase activity was then measured as oxidation of cytochrome C using the MS427 microplate assay kit for rat CCO activity from Mitosciences (Eugene, OR) at 550 nm in the power wave microplate spectrophotometer (Bio-Tek, Winooski, VT) every 5 minutes over a 180-minute time course or until substrate supply was exhausted.

Estrogen treatments and cardiolipin measures

Following 7–12 days in culture, some cultures were treated with the pharmacologic dose of 10 ng/mL 17- β -estradiol or vehicle. Cells were treated with 1 μ M nonylacridine orange (Invitrogen, Carlsbad, CA) from a 5 mM stock in 100% ethanol to stain cardiolipin (Mileykovskaya *et al.*, 2001). After a 10 minute incubation period, the cells were rinsed four times with warm PBS (Invitrogen, Carlsbad, CA) and fixed with 4% paraformaldehyde (electron microscopy sciences, Hatfield, MA) in PBS for 10 minutes at room temperature. They were again rinsed four times with PBS and mounted using Aquamount (Lerner Laboratories, Pittsburgh, PA). The slips were analyzed at 60× magnification under oil immersion with a B-1A fluorescent filter (Nikon, Huntley, IL). Basal respiration and glutamate-stimulated respiration of 9 month and 24 month neurons were measured in

cultures treated 2–5 days with 1 pg/mL 17- β -estradiol, a very low dose testing the limits of treatment efficacy (Mannella and Brinton, 2006).

RESULTS

In order to dissect the age-related deficiency in brain CCO activity, we used multiple approaches a) with excess cytochrome C *in situ* in fixed neurons, b) in the presence of endogenous substrates and cofactors in live, intact neurons, and c) with excess cytochrome C in mitochondrial extracts. Previous studies have shown, based on staining for neurofilament, GFAP, and Ox-42, that cultures of 9 month and 24 month neurons are ~80% neuronal, ~5% astroglia, ~10% oligodendroglia, and ~5% microglia (Patel & Brewer, 2003). Here, from each 9 month rat cortex of 760 $\pm$ 20 mg (mean $\pm$ S.E. n= 7 preparations) we isolated an average of 11 $\pm$ 1 million live cells of which 80% are assumed to be neurons. From 24 month cortices of 720 $\pm$ 50 mg, 10 $\pm$ 1 million live cells were isolated. There are no age-related differences in these averages. Compared to an average of 83,000 neurons/mm³ of rat cortex *in vivo* (Korbo et al., 1990), we isolated 14–15,000 live cells/mm³ or 14% of the neurons originally present in the rat brain cortex, independent of age. After 10 days in culture, 30% of the neurons survived from what was plated, independent of age.

Age-related deficit in cytochrome C oxidase activity *in situ*

To determine whether the observed age-related brain deficits in respiration were intrinsic to neurons, we measured CCO activity *in situ*. Figure 1 shows an *in situ* 29% decrease in CCO activity measured as electron transfer from reduced cytochrome C to diaminobenzidine in 24 month compared to 9 month neurons. Neurons in culture for 7–10 days show a significant decline in CCO activity in the presence of excess cytochrome C with age of the animal from which they were isolated. We hypothesized that growth-factor-stimulated mitosis in neurons would be accompanied by mitochondrial turnover or mitogenesis that would replace damaged mitochondria. We previously observed that hippocampal neurons isolated from a 381 g Sprague Dawley rat and plated at 320 cells/mm² with FGF2 supplementation increased in density from 55 live cells/mm² to 450 neurofilament positive cells/mm² after 5 days in culture with 70% of these staining positive for added BrdU, a clear sign of neuron multiplication (Brewer 1999). Here, 24 month neurons plated at a lower density of 40 neurons/mm² in the presence of FGF2 increased in density from 27 $\pm$ 2 neurons/mm² at day 2 to 84 $\pm$ 11 neurons/mm² at day 7 (n=12, p<0.001). Figure 1 shows that such neuron multiplication restores cytochrome C oxidase activity in 24 month neurons to that of 9 month neurons.

Mitochondrial counts and area are the same in 24 month and 9 month neurons

To rule out the possibility that decreased CCO activity with age is due to a decreased number of total functional mitochondria or more depolarized mitochondria, neurons in culture were stained with Mito-Tracker RedTM and counted using the count function of ImagePro software (Media Cybernetics, Bethesda, MD) as individual mitochondria/neuron. The high-sensitivity software is able to detect boundaries of individual mitochondria and isolate and count individual objects that cannot be detected by the human eye. Mito-Tracker Red density decreases when fewer mitochondria are present or when mitochondria are more depolarized. Figure 2 shows that there is the same number of mitochondria per cell in 24 month neurons as there is in 9 month neurons. Therefore, the 29% loss of activity in 24 month neurons in Figure 1 is not explained by fewer mitochondria/neuron.

Cytochrome C Immunoreactivity

Activity loss in whole neurons is also not explainable by mitochondrial swelling or shrinking, because Figure 3 reveals that mitochondrial area per cell is the same in 9 month

and 24 month neurons as measured by cytochrome c immunostaining. However, an age-related decline in substrate content within the mitochondrial area is revealed by the density measurement of this stain. This substrate deficit reduces the neuron's capability to utilize oxygen to generate a proton gradient and power the production of ATP.

Basal respiration is similar in E18, 9 month, and 24 month neurons

Age-related deficiencies in oxidative phosphorylation have been noted in heart and liver mitochondrial activity (Meng *et al.*, 2007) as well as in brain mitochondria from organ homogenates supplied with excess substrates and without stimulation by calcium (Sen *et al.*, 2007). Based on these reports and the data in Figure 1, here, we expected that the decline in CCO activity with age would similarly be measured as a decrease in basal live neuronal respiration. Figure 4A shows that basal respiration is not significantly lower in 24 month neurons than in 9 month neurons, based on measurements of oxygen concentration with time in a closed chamber containing viable, substrate-attached neurons.

24 month neurons are unable to efficiently upregulate respiration in response to glutamate stress

The stress of glutamate treatment causes progressive damage to neurons initiated by an influx of calcium and sodium, and accompanied by a release of mitochondrial cytochrome C into the cytoplasm, depleting this necessary electron donor to CCO (Atlante *et al.*, 1999). Calcium and sodium influx activate the Ca^{2+} -ATPase (Scully *et al.*, 1982) and the Na^{+} , K^{+} -ATPase (Vandyke & Scharschmidt, 1983) in the plasma membrane, both of which consume ATP, much of which is supplied by oxidative phosphorylation in mitochondria. To correlate the established age-related glutamate susceptibility that persists in cultured neurons (Brewer, 1998) with possible age-related deficits in oxidative phosphorylation, we measured oxygen consumption of 24 month and 9 month neurons challenged with glutamate over time. Figure 4B shows that embryonic and 9 month neurons upregulate respiration two-fold in response to a glutamate stimulus, while 24 month neurons fail to do so. These results measuring age-related deficits in oxygen consumption under glutamate stress support the *in situ* evidence of an age-related deficit in neuronal CCO (Figure 1), but do not discriminate between deficits in CCO quantity, substrate supply, or function.

Respiration by CCO more readily inhibited 24 month neurons than in 9 month neurons

As a third test of whether deficits in CCO play a role in neuronal age-related susceptibility to stress, oxygen consumption was monitored while the, CCO inhibitor, potassium cyanide, titrated on to the *in situ* neurons. If 24 month neurons contained less functional CCO, their respiration should be inhibited more than 9 month neurons. Figure 5a shows that 24 month neurons were more inhibited at lower concentrations of KCN than 9 month neurons. The IC_{50} for 24 month neurons was 10 μM while that for 9 month neurons was 18 μM . An extrapolation of the data into a Lineweaver-Burke plot in Figure 5b shows more clearly that, at maximal concentrations of potassium cyanide, V_{max} of CCO was 2.8 $\mu\text{M O}_2/\text{min}/10^6$ cells in 24 month neurons, similar to 2.7 $\mu\text{M O}_2/\text{min}/10^6$ cells in 9 month neurons. Very low concentrations of KCN were excluded from analysis due to their high variability, possible inefficacy and outlying from the trend of higher concentrations. Apparent K_i for KCN was 39 μM in 9 month neurons and 11 μM in 24 month neurons, a 3.5-fold decrease with age. This *in situ* confirmation of age-related deficits in K_i of CCO for KCN could be due to a decrease in substrate, cytochrome C, or cofactor cardiolipin, or a decrease in enzyme quantity per mitochondrion.

No age-related deficiency in CCO enzyme activity in detergent extracts with excess cytochrome C

Greater susceptibility of 24 month neurons to inhibition by potassium cyanide could be due to less catalytic activity of CCO itself or lack of available substrate, cytochrome C. Conventional enzyme activity in submitochondrial particles extracted from cultured neurons and freeze-thawed in 1% lauryl maltoside was evaluated as a change in absorption of cytochrome C (Murray *et al.*, 2007). Figure 6 reveals that CCO isolated from mitochondria in 24 month (24 month) cultured neurons is equally as active as CCO isolated from mitochondria in 9 month (9 month) cultured neurons when measured with excess cytochrome C.

Estrogen treatment prevents age-related loss of cardiolipin and improves neuronal respiration in response to glutamate

Because cardiolipin is an essential cofactor for CCO to function (Brewer, 1999), a deficiency could lead to enzymatic dysfunction that would be missed in detergent extracts. We measured cardiolipin by its unique binding to the fluorescent probe, nonylacridine orange (NAO) (Mileykovskaya *et al.*, 2001). Figure 7 shows that total NAO fluorescence/cell is depleted with age. Figure 3 shows that mitochondrial area remains the same for 9 month and 24 month neurons, both of which are lower than that of embryonic neurons. However, the measure of cytochrome C concentration per mitochondrion decreases with age similar to the *in situ* activity measured in Figure 1. Previous studies demonstrated that estrogen upregulates expression of anti-apoptotic protein Bcl-2 and prevents age-related calcium dysregulation in rat hippocampal neurons (Brewer *et al.*, 2006; Wu *et al.*, 2005). Pre-treatment of neurons with the pharmacologic dose of 10 ng/mL 17- β -estradiol (estrogen) restored cardiolipin levels in 24 month neurons to those in 9 month neurons (Figure 7). Brewer *et al.* (2006) showed dose-dependent neuroprotective effects of estrogen in a dose as low as 0.1 pg/mL in rat hippocampal neurons. 1 pg/mL proved to be an effective dose in spite of being 10⁴-fold less than a pharmacologic dose, leading to our hypothesis that 1 pg/mL would also be effective in improving respiration in 24 month neurons. Pushing the limits of estrogen treatment efficacy, Figure 8 shows that pre-treatment with 1 pg/mL 17- β -estradiol also improves respiratory response to glutamate in both 9 month and 24 month neurons. 9 month neuronal respiratory response to glutamate is significantly improved by 50% ($p = 0.005$). 24 month neuronal respiratory response to glutamate is brought up to the level of that of untreated 9 month neurons, 100% higher than untreated 24 month neurons, $p = 0.01$.

Depleted cytochrome c levels with age

Cytochrome C is a necessary substrate to provide electrons for the function of CCO. The observed age-related reduction in neuronal CCO activity and failure to observe this effect in the presence of excess cytochrome C could be due to a decline in mitochondrial cytochrome C. Therefore we measured cytochrome C levels in individual neuronal mitochondria by confocal imaging of immunostained cultures. To identify mitochondrial cytochrome C, a mask of Mitotracker Green was overlaid on the red anti-cytochrome C immunostained neurons. We analyzed the density of immunostain as a measure of cytochrome C concentration and simultaneously measured the area stained to double-check mitochondrial area per cell. Figure 3 shows that mitochondrial area per cell remains the same for 9 month and 24 month neurons, both of which are lower than that of embryonic neurons. However, the measure of cytochrome c concentration per mitochondrion decreases with age similar to the *in situ* activity measured in Figure 1.

DISCUSSION

We found no significant age-related difference in basal respiration or mitochondrial counts per neuron between 24 month and 9 month neurons in common culture conditions, isolated from hormonal, vascular and immunological differences in the aging brain. In addition, Patel and Brewer (2003) found that glucose uptake is similar between 9 month and 24 month neurons during basal metabolism. Some normal function is expected, as 24 month people, animals, and cells remain alive. To determine the reason for the bioenergetic decline that occurs in spite of these similarities, we tested enzymatic function of CCO and measured levels of other substances necessary for its function. A restorable age-related decline in CCO activity indicates that multiplication repairs the energetic pathway involving CCO or its cofactors that are deficient in 24 month neurons. It is possible that deficient mitochondria are either replaced with fully active CCO or that only cells with adequate capacity for nuclear DNA replication can replicate. When stressed with glutamate, 24 month neurons were unable to sufficiently upregulate respiration like the two-fold increase seen in 9 month neurons. This result indicates that basal-level respiration is sufficient to generate enough energy for survival, but the 92% deficit in spare respiratory capacity for upregulation of energy production with increased metabolic demand is inadequate to maintain viability in 24 month neurons (Brewer, 1998). Respiration measurements with sequential titration of KCN show that 24 month neurons are more inhibited by lower concentrations of the inhibitor than 9 month neurons, suggesting a 50% deficiency in the enzyme quantity or function, its substrate, or its cofactors intrinsic to the whole neuron. The 3.5-fold month lower K_i for KCN in 24 month neuron respiration may be explained if CCO's binding site for KCN is already partially occupied by nitric oxide, a naturally occurring inhibitor of CCO (Zhao *et al.* 1994). Decreased cardiolipin may also reduce the assembly efficiency of CCO with other complexes, or may alter the holoenzyme conformation to reduce the affinity of the binding site for KCN. Damage to cardiolipin or CCO may occur in an increasingly oxidized environment with age (Parihar and Brewer 2007a).

To further explore the possibility that there is a substrate deficiency in 24 month neurons, we tested the activity of CCO in mitochondria from cultured cells in excess of substrate per unit protein and found that there was no significant difference in activity in 24 month neurons relative to 9 month neurons. These results suggest that there is a deficiency in cytochrome C in 24 month neurons or in the neurons' ability to utilize it.

Morphometric analysis of hippocampal synaptic mitochondria in the adult rat brain has revealed similarities in mitochondrial number, mitochondrial volume, and mitochondrial volume density with age (Bertoni-Freddari *et al.*, 2004). However, cytochemical evaluation of CCO activity as the ratio of area occupied by synaptic mitochondria per area of cytochemical precipitate due to CCO activity in brain tissue extracted from the cerebellar granule layer of 24 month rats declined 26% (Fattoretti *et al.*, 2006). Similarly, Figure 1 indicates that CCO activity *in situ* declines 29% in 24 month compared to 9 month neurons. Compared to our method of *in situ* measurement of viable neurons, isolated brain mitochondria suffer oxyradical damage during the extraction process, as evidenced by a two-fold higher V_{max} and 15% greater respiratory control ratio in synaptosomes isolated with the antioxidant cocktail SCAVEGR™ compared to traditional extraction without antioxidants (Brewer *et al.*, 2004). In other types of neurons, quantitative mitochondrial deficits have also been observed and proposed as a cause of an age-related energy decline (Adams & Jones, 1982; Monteiro, 1991; Vidal *et al.*, 2004). Adams and Jones (1982) observed a decrease in mitochondrial volume at the presynaptic terminal in rat cortical neurons while Monteiro (1991) recorded a linear relationship between increasing age and decreased mitochondrial volume in rat neocerebellar Purkinje neurons. Vidal *et al.* (2004) showed a 10% decrease in rat brain mitochondrial volume. In our cultures of whole

hippocampal neurons, mitochondrial area and counts per neuron are maintained with age, but mitochondrial metabolic performance is compromised under glutamate stress. Curti et al. (1990) found a 25% decrease in CCO activity with age in synaptic mitochondria isolated from rat cortical homogenates similar to the 33% age-related decrease in CCO activity in cultures of whole, isolated neurons in Figure 5. The age-related deficit in CCO activity observed in neurons is not restricted to the brain. In skeletal muscle, age-related loss of CCO function and increased deletion mutations in the mitochondrial DNA lead to sarcopenia (Herbst *et al.*, 2007; Tamilselvan *et al.*, 2007). These data imply that loss of bioenergetic function leads to cellular degeneration and may also be responsible for the neurodegeneration seen in age-related diseases such as Alzheimer's Disease.

According to the free radical theory of aging, organisms age as macromolecules in the body suffer free radical attacks (Harman, 1956). However, more recent studies show that free radical attacks do not always compromise bioenergetic function. Activity of CCO measured in isolated rat brain mitochondria declines only marginally as ROS production increases with age (Sen *et al.*, 2007). Sen et al. (2007) found a stronger correlation between aging and a decline in cardiolipin content. Our studies show that the decrease in cardiolipin persists in neurons after regeneration in culture. This suggests an inherent defect in turnover of 24 month mitochondria or epigenetic changes in 24 month neurons that prevent a return to youthful levels needed for demand-driven oxidative phosphorylation. The rejuvenation of CCO activity that we observed following 24 month neuron multiplication also supports a hypothesis of restored turnover or epigenetic changes.

Our *in situ* measures do not explicitly determine maximum mitochondrial capacity. In our respiration measurements, glutamate was titrated to maximize metabolic demand. Although there is no significant difference in basal respiration between 9 month and 24 month neurons, we find that, under glutamate stress, 24 month neurons are unable to sufficiently upregulate respiration to the level of 9 month neurons. This result is supported by findings of an age-related decline in NMDA and AMPA receptor subunits in the rat dentate gyrus (Newton *et al.*, 2007) and age-related declines in NMDA currents in hippocampal neuron cultures maintained under the same conditions as the cultures reported here (Cady *et al.*, 2001). The efficacy of an NMDA receptor antagonist, memantine, in the treatment of Alzheimer's Disease supports the hypothesis that an age-related susceptibility to glutamate excitotoxicity contributes to neurodegeneration (Barber *et al.*, 1978; Padmaja & Panikkar, 1989; Pieta *et al.*, 2007). Caloric restriction early in a rat's life, a well-established model to extend longevity, eliminates the age-related decline of NMDA and AMPA receptor subunits (Shi *et al.*, 2007).

Proteomic analysis of mitochondrial subunits has revealed age-related changes in the assembly of the rat CCO complex (Dencher *et al.*, 2007). Alterations in complex assembly may affect the enzyme's efficacy as well as its ability to access the cytochrome C pool. These data potentially explain the result that CCO is more readily inhibited by lower concentrations of potassium cyanide in 24 month neurons than in 9 month neurons, and that CCO activity is restored in excess of cytochrome C. In addition, we found decreased total cytochrome C levels in aged neurons. In Frataxin animal models, which exhibit abnormal neurodegeneration, cytochrome C levels and CCO activity are also depleted (Napoli *et al.*, 2006).

Brewer et al. (2006) found estrogen to be neuroprotective in concentrations as low as 0.1 pg/mL. Estrogen treatment protected against glutamate and beta-amyloid toxicity, preventing calcium dysregulation in 24 month neurons. Our rejuvenation studies explored the effect of estrogen treatment on age-related cardiolipin loss and neuronal response to glutamate. Previous studies have shown that androgen administration to aged male mice increased

cognitive performance (Frye *et al.*, 2007). Nilsen *et al.* (2007) observe that, following *in vivo* treatment with estrogen (30 µg/kg), there is a 2.5-fold increase in CCO mRNA expression, a 30% increase in CCO protein expression, a 45% increase in CCO activity, and a 26% increase in respiratory control ratio in permeabilized non-synaptic brain mitochondria. Estrogen is intricately linked to cell signaling in the brain and has proven effective against neurodegeneration in animal models (Mannella and Brinton, 2006; Garcia-Segura *et al.*, 2007). Other consequences of aging in our culture model with hippocampal neurons include a decline in NADH, redox ratio, mitochondrial membrane potential, and an increase in reactive oxygen species production (Parihar and Brewer 2007b; Parihar *et al.*, 2008). Brewer *et al.* (2006) show that estrogen treatment ameliorates calcium dysregulation in 24 month neurons. If estrogen improves CCO function, the efficacy of estrogen treatment in restoring cardiolipin levels and response to glutamate in 24 month neurons to that of 9 month neurons contributes to understanding the molecular mechanisms of estrogen neuroprotection. Because NAO staining may not always be specific for cardiolipin (Gohil *et al.*, 2005), future studies will test the effects of estrogen treatment on cardiolipin using mass spectrometry. These findings encourage appropriate hormonal treatment of age-related neurodegenerative disease such as Alzheimer's Disease (Brewer *et al.*, 2006; Napoli *et al.*, 2006).

In summary, while mitochondrial quantity and basal cellular respiration are maintained with age, alterations in other key functions and quantities make neurons susceptible to the pathology of age-related neurodegenerative disease. Age-related decreases in cytochrome C (figure 3), cardiolipin (figure 7), CCO function (figures 1,5), and glutamate response (figure 4) render cells less capable of responding to stress. Estrogen is a promising treatment in restoring neuron function with age.

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Fig. 1. Age-related deficiency of CCO activity restored by neuron multiplication

A. In E18 rat hippocampal neurons, density increases linearly with time as cytochrome C oxidase activity occurs. **B.** *In situ* cytochrome C oxidase activity assay reveals an age-related deficit in activity that can be corrected by multiplication. Neurons in culture for 7–10 days show a significant decline in cytochrome C oxidase activity relative to age, but activity is restored in 24 month neurons by harvesting the neurons followed by replating and culture for 5 days under conditions that promote neuron multiplication. The number of neurons measured for each condition is indicated below the x axis.

Fig. 2. Mitochondrial counts do not decline with age

Neurons in culture stained with Mito-Tracker Red™ and counted digitally reveal the same number of mitochondria per cell in 24 month neurons as in 9 month neurons. Inset is an 24 month neuron, imaged at 8 days in culture. n = 4 neurons.

Fig. 3. Cytochrome c concentration decreases with age

Mitochondrial area (solid bars) is the same in 9 month and 24 month neurons, but immunoreactive cytochrome C concentration (hatched bars) is decreased in 24 month neurons relative to 9 month neurons. $n = 4$ neurons

Fig. 4. 24 month neurons are unable to sufficiently upregulate respiration in response to glutamate

A. There are no significant differences in basal respiration among 3 ages of rat cortical neurons. Cortical neurons cultured for 7 – 12 days on glass coverslips were inserted into a closed respiratory chamber. Oxygen consumption was measured and normalized for cell counts. $n = 13$ independent neuronal cultures of each age. Open bars represent non-mitochondrial oxygen consumption, or oxygen consumption remaining after cells have been maximally inhibited by potassium cyanide. **B.** 24 month neurons are unable to significantly upregulate respiration in response to glutamate stress. The maximum increase in respiration was titrated with glutamate. $n = 5 - 6$ independent neuronal cultures of each age.

Fig. 5. CCO has decreased functional capacity with age

A. CCO is inhibited more by lower concentrations of potassium cyanide in 24 month neurons than in 9 month neurons. B. Lineweaver-Burke analysis indicates 50% less respiration in 24 month neurons inhibitable by potassium cyanide with R^2 values for the linear fits of 0.965 and 0.980 for 9 month and 24 month neurons, respectively. Two points from 9 month cultures with low rates at low KCN concentrations were omitted in the calculation of linear regression. This result suggests less functional capacity of the enzyme in 24 month neurons, a decrease in enzyme substrate cytochrome C or cofactor cardiolipin, a decrease in enzyme quantity per mitochondrion, or a decrease in mitochondrial density. K_i is

13-fold lower in 24 month neurons compared to 9 month neurons. n = 6 cultures for each age.

Fig. 6. CCO activity deficits restored with excess cytochrome C

In detergent and excess cytochrome C, CCO in mitochondria isolated from 24 month cultured neurons is equally as active in oxidizing cytochrome C as from 9 month cultured neurons. Control values for the assay without neuron extracts were subtracted from the final values shown in the graph. $n = 6$

Fig. 7. Age-related cardiolipin loss restored by estrogen

A. NAO-stained 24 month neurons show low cardiolipin content. B. NAO-stained 24 month neurons pretreated with 10 ng/mL 17-β-estradiol (estrogen) show increased cardiolipin content. C. Age-related decline in NAO fluorescence suggests loss of cardiolipin/cell with age. Cardiolipin is a necessary cofactor for CCO activity. The loss can be reversed by pretreatment of the neurons for 2 days with 10 ng/mL 17-β-estradiol (estrogen). The number of cells analyzed for each age and condition is listed below the x axis.

Fig. 8. Respiratory response to glutamate rejuvenated by estrogen treatment

Treatment with 1pg/mL 17-β-estradiol causes increased respiration in 24 month and 9 month neurons maximally stimulated by glutamate. No changes in basal respiration were observed (data not shown). n = 4–6 neuron cultures.