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Impaired spare respiratory capacity in cortical synaptosomes from *Sod2* null mice

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Abstract

Pre-synaptic nerve terminals require high levels of ATP for the maintenance of synaptic function. Failure of synaptic mitochondria to generate adequate ATP has been implicated as a causative event preceding loss of synaptic networks in neurodegenerative disease. Endogenous oxidative stress has often been postulated as an etiological basis for this pathology, but has been difficult to test *in vivo*. Inactivation of the superoxide dismutase gene (*Sod2*) encoding the chief defense enzyme against mitochondrial superoxide radicals results in neonatal lethality. However, intervention with an SOD mimetic extends the lifespan of this model, and uncovers a neurodegenerative phenotype providing a unique model for the examination of *in vivo* oxidative stress. We present here studies on synaptic termini isolated from the frontal cortex of *Sod2* null mice demonstrating impaired bioenergetic function as a result of mitochondrial oxidative stress. Cortical synaptosomes from *Sod2* null mice demonstrate a severe decline in mitochondrial spare respiratory capacity to physiological demand induced by mitochondrial respiratory chain uncoupling with FCCP or plasma membrane depolarization induced by 4-aminopyridine treatment. However, *Sod2* null animals compensate for impaired oxidative metabolism in part by Pasteur effect allowing for normal neurotransmitter release at the synapse, setting up a potentially detrimental energetic paradigm. The results of this study demonstrate that high throughput respirometry is a facile method for analyzing specific regions of the brain in transgenic models, and can uncover bioenergetic deficits in subcellular regions due to endogenous oxidative stress.

Keywords

Superoxide dismutase 2 (sod2); Synaptosome; Bioenergetics; Mitochondria; Respiration; Oxidative Stress

Introduction

Pre-synaptic nerve terminals require a constant supply of ATP for the proper maintenance of active physiological events such as neurotransmitter release and reuptake. In order to meet these ATP demands at the synaptic bouton, mitochondria are physically located at the terminus. There has been a growing body of evidence that synaptic dysfunction is a critical

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event in a number of neurodegenerative diseases, including Alzheimer's and Parkinson's disease [1,2], dementia [3,4] and Huntington disease [5,6]. It has been postulated that one possible mechanism underlying such synaptic dysfunction, is a depletion of ATP at the terminus, mediated by an 'ATP crisis'. This concept has been referred to as the "spare respiratory capacity" hypothesis, and proposes that when the bioenergetic reserve present in a neuron is exceeded by energetic demand outpacing supply, a lack of ATP results, with potential neurodegenerative consequences [7]. This may result in an escalating sequela of pathologies as the synaptic network breaks down [2]. Most evidence for impaired synaptic function due to mitochondrial abnormalities has been provided by *in vitro* tissue culture studies, that rely on impairing mitochondrial function through the addition of mitochondrial toxins such as rotenone, to directly impair the respiratory chain, thereby mimicking an endogenous respiratory defect [8,9]. However, use of a genetic model of mitochondrial deficiency affords the opportunity to test bioenergetic hypotheses in the brain in a more direct fashion. The *Sod2* null mouse is one such model of endogenous mitochondrial dysfunction.

Superoxide dismutase 2 (SOD2) is arguably one of the most important oxidative stress defense enzymes in the cell. SOD2 is exclusively located in the mitochondrial matrix, and scavenges superoxide radicals, a by-product of mitochondrial oxidative phosphorylation. Constitutive lack of SOD2 in mice on multiple genetic backgrounds mediated via homologous recombination results in an embryonic (C57BL/6J) or neonatal lethal (CD1, DBA/2J, and B6D2F1) phenotype [10,11]. *Sod2* null mice on a CD1 background treated with antioxidants from an early age present with pronounced neurodegeneration [12,13]. This demonstrates the importance of controlling endogenous levels of superoxide anion in the mitochondria, and facilitates study of the consequences of endogenous mitochondrial oxidative stress on synaptic function. Neurological phenotypes that have been reported in constitutive *Sod2* null mice include seizures, spongiform encephalopathy, tremor, and neurodegeneration [12–15]. Biochemical characterization of mitochondria isolated from the forebrains of *Sod2* null mice show profound respiratory chain defects including reductions in the catalytic activity of complexes I, II, III, and IV, as well as TCA cycle enzymes [16]. Further, gene expression profiling studies have also revealed a transcriptional signature indicative of neurodegeneration involving abnormal glutamate neurotransmitter metabolism [17]. The neurodegeneration and spongiform encephalopathy reported in *Sod2* null mice were uncovered after therapeutic treatment with catalytic antioxidants, which have the ability to extend the lifespan of these mice by up-to 4-fold [12–14]. Treatment of *Sod2* null mice with such an antioxidant, EUK189, at either "high" or "low" doses, prevents or allows a spongiform encephalopathy, with accompanying neurodegeneration. Without any antioxidant treatment, *Sod2* null mice expire in the first week of life [11,12,14]. Therefore, the coupling of *Sod2* null mice with antioxidant treatment encapsulates a system that can be exploited to investigate the consequences of endogenous mitochondrial oxidative stress on the CNS, and in the case of our study, sub-regions within the brain. Mitochondrial function is best evaluated using respiration rate, as quantitating the steady state concentration of ATP alone is not informative with regards to how the mitochondria perform work under energetic load, due to the dynamic nature of ATP supply and demand [18]. The recent introduction of a high throughput respirometer, which in the context of studying synaptosomes, requires as much as 10 fold less biological material than a classical Clarke Oxygen electrode [19–21], allows detailed bioenergetic information to be obtained from synaptosomes prepared from specific brain regions of individual mice as opposed to whole brain homogenates. Other advantages in studying synaptosomes via this method, are a closer approximation of mitochondrial function to their *in vivo* physiology, as mitochondria are evaluated in the synaptic bouton, more closely approximating their cellular environment, while as traditional bioenergetic measurements (oxygen electrode) often use mitochondria purified from total tissue homogenates. We present here direct evidence demonstrating that endogenous

mitochondrial oxidative stress prevents an appropriate bioenergetic response to increased ATP demand within the frontal cortex of *Sod2* null mice, which has mechanistic implications for neurodegenerative diseases in which mitochondrial dysfunction is implicated.

Methods

Reagents

All chemicals used in these experiments were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise indicated.

Animals

Constitutive *Sod2* null homozygous mice on a CD1 background ranging in age from 17 to 21 days old and age matched wild type siblings were used for all experiments as previously described [12,14]. Genotype determination was performed within 3 days of birth, as previously described [14], and either 1 mg/kg or 30 mg/kg of the synthetic antioxidant EUK189 (Dalton Pharma Services, Toronto, ON, Canada) was injected daily via intraperitoneal injection from the 4th day after birth until the day before the animals were euthanized. All mouse procedures were carried out under approved Buck Institutional IACUC protocols.

Preparation of cortical synaptosomes

Mice were euthanized by CO₂ overdose & decapitation, and cortices were removed and subjected to a synaptosome isolation method as previously described [22] with slight modifications. Briefly, frontal cortex was rapidly isolated (about 100 mg/ brain) and rinsed with ice-cold sucrose medium (320 mM sucrose, 1 mM EDTA, 0.25 mM dithiothreitol, pH 7.4) to remove excess blood, transferred to a pre-chilled Dounce glass homogenizer containing 3 ml sucrose medium, and homogenized gently by ten strokes. The homogenate was then centrifuged at 1,000g for 10 minutes at 4°C. The supernatant was carefully layered on top of a discontinuous Percoll gradient (3, 10 and 23% Percoll in sucrose medium) in a 15ml centrifuge tube, and centrifuged at 32,500g for 10 minutes at 4°C in a JA-25.50 fixed angle rotor in a Beckman Avanti J-26 XPI centrifuge. Synaptosomes were isolated and recovered from the band between 10% and 23% Percoll layers. The synaptosomes isolated from the 10–23% interface were diluted into “ionic medium” (20 mM HEPES, 10 mM D-Glucose, 1.2 mM Na₂HPO₄, 1 mM MgCl₂, 5 mM NaHCO₃, 5 mM KCl, 140 mM NaCl, pH 7.4) for measurement of respiration. The final synaptosome pellet was then resuspended in ionic medium, and protein concentration was determined by the Bradford method (Bio-Rad).

Respirometry

For monitoring respiration, 10 µg synaptosomal protein per well was aliquoted into a 24 well of a cell culture microplate (Seahorse Bioscience, North Billerica, MA) coated with a 1:15,000 diluted polyethyleneimine (PEI). The plate was centrifuged at 3,400g for 1 hr at 4°C in an A-4-62 rotor in an Eppendorf 5810R centrifuge. Then, medium was replaced with 700 µl of “seahorse incubation medium” (3.5 mM KCl, 120 mM NaCl, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 1.2 mM Na₂SO₄, 15 mM Glucose, 10 mM Pyruvate, 2 mM MgSO₄, and 0.4% (w/v) fatty acid free bovine serum albumin). The cell culture microplate was incubated and loaded into the Seahorse XF24 extracellular flux analyzer following the manufacturer’s instructions. All experiments were performed at 37°C. All reagents were added at appropriate dilutions in 75µl of seahorse medium. The protocol for the measurement of oxygen consumption and extracellular acidification rate is as previously described [20,21]. Two biological replicates per plate were typical, and experiments (N>50 animals total) were

carried out over a 6 month period. A minimum of five technical replicates were performed in each measurement, greatly increasing the reliability of oxygen consumption measures, and a minimum of 4 mice per group per experiment were used as biological replicates for each measurement to determine statistical difference per treatment. Statistical significance was obtained when the *p* value was lower than 0.05 by two-tailed non-parametric *t*-test.

Measurement of mitochondrial enzyme activities

Synaptosomes were lysed by repetitive freeze-and-thaw, and protein concentration was determined by Bradford method. Five micrograms of lysed synaptosomes were used for colorimetric enzyme activity assays of Complex I, complex II and citrate synthetase as previously reported [23].

Glutamate Release Assay

Calcium dependent and independent glutamate release from synaptosomes was measured using an enzyme linked fluorometric assay [24,25]. A Fluoroskan Ascent microplate fluorometer (Thermo scientific, Waltham, MA) was set to kinetic measurement mode using an excitation of 355 nm and an emission of 460 nm. 100 µg/well of synaptosomes were placed into a final volume of 200 µl/well of 'ionic medium' used in the respirometry experiments without the addition of calcium (see above). The buffer was then supplemented with 1 mM NADP⁺ and either 1.2 mM CaCl₂ or 0.5 mM EGTA to determine the calcium dependent release. Lastly, 8 units per reaction of glutamate dehydrogenase enzyme was added to start the reaction. After a period of baseline measurement, the depolarizing agent 4-Aminopyridine (4-AP) (1 mM) was injected into each well. Each curve generated was normalized to the baseline reading prior to the injection of depolarizing agents. Three synaptosome samples per animal were assayed (n=7), the individual curves from each well were normalized and presented as the mean of all curves. Statistical significance was determined by two-tailed non-parametric *t*-test applied to each time point to determine significant differences between the curves of wild-type and *Sod2* null animals at each time point.

Confocal microscopy for basal ROS in single synaptosomes

For single synaptosome confocal imaging, 3µg of synaptosomes/well were aliquoted into 96-well glass-bottomed multi-well plate (Whatman) and centrifuged at 3400g for 1 hr at 4°C in an A-4-62 rotor in a Eppendorf 5810R centrifuge. For superoxide measurement, synaptosomes were loaded with either 1µM DHE (Invitrogen) for total ROS content or 0.1 µM Mitosox (Invitrogen) for mitochondrial ROS content for 30 min. The fluorescence from fields of synaptosomes was monitored for 3 separate time points over a 15 minute period under basal conditions fields of synaptosomes were monitored in a Zeiss LSM510 confocal microscope with a 40X air objective, using filter sets for emission/excitation according to manufacturer's directions. Images were then analyzed using a custom built MATLAB program (MathWorks, Natick, MA) which aligned and identified each field of synaptosomes from three timepoint observations separated by several minutes, then quantitated the fluorescent signal from each synaptosome in the field (n=2 *sod2* ^{+/+} and n=3 *sod2* ^{-/-} mice treated with 1 µg/kg EUK189) with a minimum observation of 339 synaptosomes, up to maximum of 884 observations within the field.

Measurement of total glutamate content

Measurement of total glutamate content was carried out using Biovision (Mountain View, CA) glutamate assay kit. 10 µg of synaptosomal protein per well was assayed against glutamate standards according to manufacturer's directions. Protein from six animals per treatment were each tested in duplicate.

Results

Impaired mitochondrial enzyme activity and elevated ROS content in synaptosomes isolated from *Sod2* null mice

We previously reported that mitochondria purified from a bulk tissue preparation of cortex of *Sod2* null mice at 3 weeks of age had a number of deficits in the catalytic activity of respiratory chain complexes and TCA cycle enzymes [13]. These studies were carried out on mitochondria isolated from all cellular sources in the tissue, including glia, neurons and other cell types in the brain. This meant that our previous data were generalized to a tissue homogenate, and not specific to any single region or cell type in the brain. In order to better understand the neuronal and subcellular distribution of our prior reported respiratory chain abnormalities, we measured the activity of complex I, complex II, and citrate synthase in synaptosomes isolated from frontal cortex of *Sod2* null mice compared to wild-type controls. Synaptosomes are exclusively derived from the termini of neurons. Hence, we are examining one specific subcellular compartment of the brain, greatly refining our knowledge of the distribution of the any measured parameter. Consistent with our prior work on isolated mitochondria from the forebrains of *Sod2* null mice [13], complex II exhibited only 22% of the activity of wild-type controls. Figure 1B shows that complex II activity is substantially reduced in synaptosomes isolated from *Sod2* null mice treated with either high or low dose EUK189 (a catalytic antioxidant with dismutase activity), relative to the activity in synaptosomes isolated from age-matched wild-type controls (10.5 ± 1 nmoles converted/min/mg protein versus 45.8 ± 5 nmoles converted/min/mg protein, $p < 0.01$). The difference in complex II is not due to differing amounts of mitochondria in synaptosomes between genotypes, as the level of the mitochondrial marker voltage-dependant anion channel (VDAC) was the same in synaptosomes isolated from either wild-type controls, or synaptosomes isolated from *Sod2* null mice (Figure S1). However, in contrast to our previous studies using mitochondria isolated from whole frontal cortex in the *Sod2* null mouse paradigm [13], we found no significant difference in the levels of complex I or Citrate Synthase activity between synaptosomes isolated from controls versus *Sod2* null mice (Figure 1A and 1C). This suggests that the complex I deficit we reported previously in purified mitochondria from *Sod2* null mice is due to either differential cell specificity or subcellular compartmentalization, as synaptosomal mitochondria are derived only from the termini of neurons.

To assess the presence of elevated levels of ROS consistent with the loss of *Sod2* in this mouse model, we examined fields of synaptosomes with reactive oxygen species sensitive dyes (Figure 2A). We observe that with both DHE and MitoSOX dyes there is a significant increase in the signal that the dyes provides from individual synaptosomes in the 1 mg/kg EUK189 treated mice (Figure 2B). The latter dye is reported to have a more specific affinity for mitochondria produced superoxide as this dye's cationic charge selectively partitions the dye to the inner mitochondrial matrix. Overall, these data confirm that there exists an elevated presence of superoxide in the *Sod2* $-/-$ synaptosome under basal bioenergetic conditions both within the mitochondria and in the cytoplasm.

In vivo oxidative stress impairs mitochondrial spare respiratory capacity in *Sod2* mice

In order to fully evaluate synaptosomal respiration isolated from the forebrains of CD1 mice of 17–21 days of age, we used a Seahorse XF24 extra-cellular flux analyzer (Seahorse Bioscience, North Billerica, MA). Figure 3 shows respiration results using the XF24 extra-cellular flux analyzer of synaptosomes isolated from *Sod2* null mice versus wild-type controls treated with 1mg/kg of EUK189 at 17–21 days of age. The dose dependence for the ideal dosage of FCCP was determined to be 4 μ M, a dosage which elicits a response in the wildtype mice, but not in the knockout. The optimal formulation of the media for eliciting

maximum respiration rate in synaptosomes from mice of this age and genetic background was previously determined, and found to be 15 mM Glucose + 10 mM Pyruvate by Choi et al. [20]. Subsequently, all experiments were performed in media with both these substrates.

Synaptosomes isolated from wild-type CD-1 mice aged 17–21 days treated with EUK189 at 1mg/kg showed a basal respiration maintained at approximately 30 nmol O₂/min/mg protein (Figure 4A, grey box). Addition of oligomycin decreased respiration to approximately 20% of the basal OCR (purple box). Addition of FCCP increased mean respiration rate up to 2.8 fold (blue box). This increase in mitochondrial respiration was completely inhibited after the addition of rotenone and myxothiazol (green box). No statistically significant difference in the level of basal respiration was observed between synaptosomes prepared from control wild type animals versus *Sod2* null animals. However, synaptosomes isolated from cortices of *sod2* null mice exhibited a dramatically lower level of induction of maximal respiration rate (Figure 4A). There was no significant induction above basal respiration rates in synaptosomes from *Sod2* null mice after addition of 4 μM FCCP in the presence of 0.04% BSA (blue box, Figure 4A), as opposed to synaptosomes isolated from wild type animals which elicited a large respiratory response. It is unlikely that this impaired response is due to a selective vulnerability of synaptosomes during isolation from *Sod2* null mice compared to wild-type controls, as the basal respiration rate of synaptosomes from *Sod2* null mice were comparable to those from wild-type controls.

Components contributing to the basal respiration were calculated using an established methodology [28,29]. Proton leak (oligomycin insensitive mitochondrial respiration), ATP generation (oligomycin sensitive mitochondrial respiration) and non-mitochondrial oxygen consumption, were expressed as a fraction of the basal oxygen consumption rate for both the *Sod2* nulls and wild-type animals (Figure 4B). Interestingly, *Sod2* null animals show no significant difference in any of these parameters, yet have a clear difference in spare respiratory capacity upon the administration of FCCP.

Calcium dependent neurotransmitter release is sufficient in *Sod2* null animals

FCCP treatment uncovered an impaired spare respiratory capacity in synaptosomes isolated from *Sod2* null mice, however, FCCP treatment *per se* does not mimic physiological load. To further test the hypothesis that mitochondrial oxidative stress impairs synaptic bioenergetic response to energy demand, we employed the K⁺ channel blocker 4-aminopyridine (4-AP) which more closely mimics repetitive firing of action potentials *in vivo* [30,31], and has been previously reported to be effective in the characterization of pre-synaptic exocytosis [32]. Synaptosomes respond to the presence of 4-AP in a dose-dependent fashion, by increasing the mitochondrial oxygen consumption rate through increasing intracellular ATP demand via mimicking the *in vivo* situation of bioenergetic demand [20]. It was previously shown that treatment with 1mM 4-AP induced maximal neurotransmitter release from cortical synaptosomes [33], hence we used a 1mM concentration of 4-AP to induce a maximal respiratory response in the paradigm we report here.

Having established that synaptosomes from *Sod2* null mice treated with 1 mg/kg of EUK189 had a reduced bioenergetic capacity to deal with the increased energy demand imposed by treatment with the uncoupling agent FCCP, we wished to determine if treatment with an agent which mimics synaptic firing would also reveal an impaired bioenergetic response. To test this hypothesis that endogenous mitochondrial oxidative stress resulted in bioenergetic insufficiency, and therefore functional insufficiency, we treated synaptosomes isolated from *Sod2* null mice versus wild-type controls with 4-AP as an energetic challenge to the nerve terminal. We first monitored synaptic function using an enzyme linked fluorescence assay to measure the calcium dependent exocytotic release of glutamate induced by action potential-

like plasma membrane depolarization. Synaptosomes received 1 mM 4-AP, and were monitored post-injection for an increase in NADPH⁺ fluorescence indicative of glutamate release. Figure 5B shows the Ca²⁺ dependent component of 4-AP triggered glutamate release. However, there was no significant difference in rates of neurotransmitter release among the *Sod2* ^{-/-} and *Sod2* ^{+/+} animals (n=8). The measurement of the total pool of glutamate within the synaptosomes also revealed no statistical difference between these groups; revealing that the release rate was not affected by the quantity of glutamate within the synaptosomes prior to depolarization. This indicates that these mice can maintain normal glutamate release under these particular experimental conditions. In other words, synapses derived from the frontal cortex of *Sod2* ^{-/-} mice have functionally normal glutamate release despite a severe bioenergetic deficit upon energetic load.

Mitochondrial oxidative stress alters synaptic metabolic pathways under physiological load

Because the synaptosomes from the *Sod2* ^{-/-} animals demonstrate normal neurotransmitter release despite having mitochondrial metabolic defects, we examined the possibility of a compensatory increase in alternate energy sources, such as the glycolytic activity of synaptosomes. Synaptosomes from *Sod2* null mice were again treated with 4-AP (1 mM) to increase energy demand while simultaneously measuring OCR and the extracellular acidification rate (ECAR) in the X-24 extracellular flux analyzer. The ECAR is a measure of glycolytic activity plus carbonic acid evolution from the krebs-cycle [20]. The 4-AP triggered induction of OCR in the *Sod2* null mice was only 42% of the wildtype immediately after the addition of 4-AP ($p < 0.02$, n=8, non-parametric *t*-test, Figure 6A). Interestingly, the *Sod2* ^{-/-} animals have an induction of the ECAR after the administration of 4-AP (Figure 6B) which is as robust as that of the wildtype animals, and is even slightly although not significantly elevated under basal conditions prior to 4-AP addition. This increased ECAR value in the presence of decreased OCR (thus decreased Krebs-cycle flux) indicates potent and sufficient glycolytic activity, suggesting that one mechanism in which synaptic terminals compensate for the mitochondrial defects of the *Sod2* ^{-/-} mice is by Pasteur effect during repetitive action potential firing.

Taken together, the data we obtained from the assays using FCCP or 4-AP challenge to the cortical synaptosomes from *Sod2* null animals compared to wild-type controls suggests that endogenous mitochondrial oxidative stress results in a limited capacity of the synaptosome to adjust oxygen consumption in response to intracellular ATP demands needed to carry out normal physiological functions. However, synaptosomes from antioxidant treated *Sod2* null mice (1 mg/kg EUK189) are able to at least temporarily, employ alternate energy sources (for example via the Pasteur effect) to compensate for the loss of spare respiratory capacity thus maintaining critical biological functions at the synapse.

Discussion

Oxidative stress has been associated with a number of pathological conditions including aging, ischemic heart disease, cancer, and neurodegenerative disease [3,34]. Although there is widespread debate about the levels of endogenous oxidative stress in multiple tissues and/or cell types, and the particular targets of ROS, it is clear from a variety of genetic studies that mitochondrial oxidative stress in particular has the capacity for eliciting severe broad-ranging phenotypes in mice, and more generally mammals as a whole. The brain is particularly vulnerable to ROS damage [3] in addition to requiring high levels of ATP for optimal function. Therefore, mitochondrial function must be optimally maintained in order to reduce both endogenous ROS, as well as supply ATP for neuronal function. Mice that constitutively lack *Sod2* have been reported to have severe CNS phenotypes including a spongiform encephalopathy, with regional neuropathology, behavioral abnormalities

including seizures and ataxia, synergistic interactions with the deposition of amyloid, hyperphosphorylation of tau, and neurodegeneration [10,12–15,35]. These phenotypes exemplify the importance of controlling endogenous ROS in the brain, as well as illustrate the utility of the *Sod2* null mice in uncovering the role of mitochondrial dysfunction in the brain.

Our prior works in characterizing the consequences of endogenous oxidative stress due to lack of *Sod2* in the brain revealed a number of key features. Firstly, treatment of *Sod2* null mice with synthetic catalytic antioxidants was able to prevent the early neonatal death due to lack of *Sod2*, and greatly extend the lifespan of these mice by up-to 4-fold [12]. Secondly, neurodegenerative phenotypes caused by lack of *Sod2* responded to differential dosing with catalytic antioxidants, with low doses (1 mg/kg EUK-189) of these antioxidants uncovering severe neuropathology and neurodegeneration, and high doses (30 mg/kg) completely rescuing or significantly attenuating the neuropathology and neurodegeneration respectively. In addition to these phenotypes, we also reported a number of mitochondrial and TCA cycle biochemical abnormalities, with a number of electron transport chain complexes (ETC) being severely depressed, most notably complexes I & II, in addition to the TCA cycle enzymes citrate synthase and alpha-ketoglutarate dehydrogenase [13]. Gene expression profiling studies on RNA extracted from the frontal cortex of *Sod2* null mice revealed an expression profile consistent with glutamate mediated excitotoxicity [17]. However, all these prior studies used bulk preparations of cortical tissue, and/or mitochondria purified from the entire frontal cortex. Such preparations have a number of inherent disadvantages. First, mitochondria purified from whole cortical tissue, are derived from both glia and neurons, hence it is difficult to identify neuronal specific deficits. Secondly, mitochondria purified in this fashion are by necessity removed from their cytoplasmic environment; hence there is a possibility of altering mitochondrial function through the purification itself. Therefore, it is desirable to study the effects of mitochondrial dysfunction in the brain, in a manner that maintains the intracellular milieu, while retaining the cellular specificity. Synaptosomal assays afford such advantages, as the mitochondria are retained in their appropriate cellular environment, and are by definition derived from neurons. However, in the past, synaptosomal preparations usually required a great deal of cortical material in order to study mitochondrial function [36], and this often precluded their use in mouse model systems where biological material was either limiting, or multiple assays were required due to biological variation. We have employed the recently developed extracellular flux analyzer [19] to study the consequences of endogenous mitochondrial oxidative stress in the synaptosome. This approach facilitates high-throughput bioenergetic studies which often require less than 2% of the biological material normally required to assess mitochondrial function in synaptosomes [20,21].

In prior studies, we had shown that there are a number of significant biochemical abnormalities in mitochondria derived from neurons and glia of frontal cortex in *Sod2* null mice [13]. Respiratory chain complexes I–IV are variably reduced in catalytic activity from 12% to 88% compared to age-matched controls. In addition, several TCA cycle enzymes were negatively affected including alpha-ketoglutarate dehydrogenase and citrate synthase [13]. Complexes I, III, IV, and citrate synthase were capable of being rescued to wild-type levels via increasing the dose of antioxidant used to treat the mice, while complexes II and KGDH were not. In the current study, we evaluated the catalytic activity of respiratory chain complexes I and II, and the TCA cycle enzyme citrate synthase, in synaptosomes. Consistent with our prior studies, we observed that complex II (succinate dehydrogenase) was most severely affected, while there was not a significant effect on the TCA cycle enzyme citrate synthase, or Complex I. Therefore, our prior observations of mitochondrial deficits in multiple respiratory chain and TCA cycle enzymes implies that these deficits are differentially distributed within the brain, dependent on cell origin, and mitochondrial

distributions within cells. This may have implications for the variability of pathologies seen in certain familial forms of neurodegenerative disease, where despite a genetic lesion being present in all cells, the pathological outcome is specifically associated with a certain cell type, such as dopaminergic neurons in Parkinsons disease. Additionally, we observed in this study that the basal level of reactive oxygen species within cortical synaptosomes is elevated as measured by DHE indicative of total synaptosomal superoxide as well as mitochondrial superoxide sensitive MitoSOX dye. This indicates that under basal metabolic conditions these synapses are undergoing constant mitochondrial oxidative stress which may be linked to the loss of activity in mitochondrial enzymes.

In a broader sense, our observations of an impaired bioenergetic response to a simulated *in vivo* demand by *in vitro* assay by either FCCP uncoupling, or by pharmacologically induced plasma membrane depolarization with 4-AP, demonstrates directly that mitochondrial oxidative stress can impair mitochondrial energy production potentially leading to loss of normal cortical neuronal function. Interestingly, a low dose (1 mg/kg) of EUK189 antioxidant is sufficient to allow for normal calcium dependent release of glutamate neurotransmitter even though there is ongoing neuronal death. The examination of the ECAR of synaptosomes revealed that the nerve terminals from these animals, at least in part, compensate for the loss of mitochondrial respiration at the synapse with glycolytic activity at or above that of wildtype animals. It has been suggested that a shift towards glycolytic energy production may be protective to neuronal tissue in the short term but may eventually lead to bioenergetic deficit and cell death [37,38]. Scott and Nicholls established in a study of guinea pig synaptosomes that anaerobic glycolysis could maintain ATP levels at the synapse and sustain mitochondrial membrane potential under non-depolarizing conditions. However, Anaerobic glycolysis alone during clamped plasma membrane depolarization was unable to maintain cytosolic synaptic ATP levels leading to mitochondrial membrane potential collapse [30]. This observation is supported by the examination of clonal neuronal cultures in which increased flux through alternative metabolic pathways provided a higher degree of protection against amyloid beta toxicity [39]. Therefore the *Sod2* null model of an endogenous mitochondrial defect could provide insight into the bioenergetic changes which occur *in vivo* during neurodegenerative disease.

While there are number of enzymatic, expression and metabolic changes in the frontal cortex of *Sod2* $-/-$ mice, the most significant result of this study is the almost total loss of respiratory capacity in response to bioenergetic challenge. Spare respiratory capacity, the ability of neuronal mitochondria to meet increased energy demand with increased respiration, has been proposed to be a primary factor defining the survival of neurons under conditions of extreme stress [8]. Additionally, partial Complex I restriction [7], 'mild-uncoupling' [45] and adenine nucleotide translocator inhibition following glutathione depletion [46] each exacerbate cytoplasmic calcium dysregulation and necrosis of cultured neurons, while at the level of the single synaptosome restricted electron transport capacity results in a slow progressive bioenergetic failure of a sub-population of vulnerable terminals when challenged with an increased energy demand. The present study demonstrates that this concept may be extended to an *in vivo* model of oxidative stress, where high throughput respirometry provides novel information about the bioenergetic status of subregions within the brain. Our previous studies have demonstrated a neurodegenerative phenotype of this mouse model of mitochondrial dysfunction. This new data presents an intriguing linkage between mitochondrial oxidative stress, synaptic dysfunction, and neurodegeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Mitochondrial enzymology of (A) complex I (B) complex II (succinate:quinone oxidoreductase), and (C) the TCA cycle enzyme citrate synthase

A highly significant decrease in the activity of complex II is seen in *Sod2* null mice (on either 1 or 30 mg/kg EUK189 treatment), $p < 0.001$, but no significant difference is seen in the activity of complex I or citrate synthase. Four independent measurements were made for each group, mean $\pm$ S.E.M. are shown.

Figure 2. Reactive oxygen species under basal conditions in individual synaptosomes

To assess the reactive oxygen species content of the synaptosomes two ROS sensitive dyes were used as an estimation of oxidative stress under basal conditions. (A) sample fields of plated synaptosomes which were stained with DHE or MitoSOX dye. (B) Quantification of hundreds of individual synaptosomes within the microscope image field for ROS content show that there is an elevation in the basal ROS content within the synaptosomes using both dyes, mean $\pm$ S.E.M. are shown. ($p < 0.001$, $N \geq 388$ synaptosomes for each of the biological replicates).

Figure 3. Titration of FCCP for optimal measurement of spare respiratory capacity in both wildtype and *Sod2* null mice

(A) Sample respiration data set of wildtype synaptosomes exposed to a titration of FCCP from 0.2 μ M to 4.0 μ M after measurement of a stable 50 minute baseline. After the injection of FCCP the mitochondrial oxygen consumption rapidly increases peaking at the first data point subsequent to addition of the drug. (B) Titration of FCCP to ensure that the dose of FCCP is not suboptimal for *Sod2* $-/-$ mice. Bars represent fold induction of oxygen consumption rate (OCR) over the basal rate measured prior to FCCP addition. Uncoupled respiration in the *Sod2* $-/-$ mice are unaffected across a range of doses indicating the effect is not a function of suboptimal ionophore dosage.

Figure 4. Synaptosomal oxygen consumption rate from cortices of *Sod2* null mice versus wild type controls

(A) Respirometry graph of *Sod2*^{-/-} and *Sod2*^{+/+} synaptosomes with the sequential addition of mitochondrial metabolic inhibitors (white spaces between colored regions). The graph region bounded by the grey box represents the basal oxygen consumption rate (OCR, a). The pink region represents the oxygen consumption after inhibition of ATP synthase with the addition of 4 μ g/mg oligomycin, (b). The blue box represents the maximal stimulatory capacity of synaptosomes from *Sod2* null mice or wild-type controls in response to the uncoupling agent FCCP (4 μ M), and the green box represents the OCR after the combined treatment of Rotenone (50 nM) and myxothiazol (50 nM) to completely inhibit mitochondrial respiration, (c). Error bars represent S.E.M. (n=7). **(B)** Oxygen consumption which is oligomycin sensitive [100-((a-b)/a)] or insensitive [100-((b-d)/a)], the non-mitochondrial respiration [100-(d/a)], and spare respiratory capacity [100-(c/a)] from the values in a,b,c, and d are indicated in (A)(error bars represent S.E.M, n=7).

Figure 5. Superoxide dismutase 2 null animals do not have impaired glutamate release in response to 4-AP induced depolarization

(A) Total glutamate content per mg protein in cortical synaptosomes is not significantly different between the *sod2* null (Red) and wild-type (Blue) animals as determined by enzymatic assay ($n=6$, mean $\pm$ S.D.). (B) Synaptosomes from *Sod2* null (red trace) or wild-type control mice (blue trace) both treated with 1 mg/kg of EUK189 were challenged with 1 mM 4-AP provoking plasma membrane depolarization and glutamate exocytosis. Glutamate release in *Sod2* -/- vs *Sod2* +/+ animals was not significantly decreased in the quantity post-injection onward as determined by two-tailed non-parametric *t*-test at each timepoint ($N=7$, $p<0.05$).

A)

B)

Figure 6. Synaptosomes from *Sod2* null mice have impaired oxidative phosphorylation and increased glycolysis in response to plasma membrane depolarization

Synaptosomes from *Sod2* null (red squares) or wild-type control mice (blue circles) (both treated with 1 mg/kg of EUK189) were challenged (arrow) with 1 mM 4-AP to provoke an energetic demand. The response of the *Sod2* null synaptosomes over baseline oxygen consumption (A) to this demand is blunted by 50% ($p < 0.02$) comparing the basal point prior to drug addition and the first datapoint after addition indicating a reduced mitochondrial respiration response in the *Sod2* null mice. Simultaneous measurement of the extracellular acidification rate (B) reveals glycolytic activity in the *Sod2* null animals is not impaired and is upregulated after induction with 4-AP as much as the wildtype synaptosomes. data is shown as mean $\pm$ S.E.M., and $n = 8$ per group.