Published in final edited form as:

Adv Drug Deliv Rev. 2009 November 30; 61(14): 1283–1298. doi:10.1016/j.addr.2009.07.015.

THE *ENERGY-REDOX* AXIS IN AGING AND AGE-RELATED NEURODEGENERATION

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Abstract

Decrease in mitochondrial energy-transducing capacity is a feature of the aging process that accompanies redox alterations, such as increased generation of mitochondrial oxidants, altered GSH status, and increased protein oxidation. The decrease in mitochondrial energy-transducing capacity and altered redox status should be viewed as a concerted process that embodies the mitochondrial *energy – redox axis* and is linked through various mechanisms including: (a) an inter-convertible reducing equivalents pool (*i.e.*, NAD(P)⁺/NAD(P)H) and (b) redox-mediated protein post-translational modifications involved in energy metabolism. The energy–redox axis provides the rationale for therapeutic approaches targeted to each or both component(s) of the axis that effectively preserves or improve mitochondrial function and that have implications for aging and age-related neurodegenerative disorders.

Keywords

Mitochondria; Energy-redox axis; Lipoic acid; Thiol/disulfide exchange; Alzheimer's disease; Aging

1. INTRODUCTION

The exponential increase in the occurrence of neurodegenerative diseases with chronological age suggests that although their etiologies are different and likely multi-factorial, aging and age-related neurodegenerative diseases share some common pathological mechanisms that lead to neuronal dysfunction; notably the presence of an energetic deficit or a hypometabolic state, likely a combinatorial effect brought about by factors such as insulin resistance, decreased glucose uptake, and mitochondrial impairment [1–5]. Clinical studies support the notion that depressed cerebral glucose metabolism is the earliest and consistent abnormality seen in

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Alzheimer's disease (AD) and mild cognitive impairment (MCI), before the occurrence of any overt pathology or severe atrophy in the brain [6,7]. This indicates that changes in energy metabolism in the brain are not merely a consequence of neuronal loss, but rather a contributory factor to the progression and development of the disease. Neurons have a high and variable demand for cellular ATP [8] in order to sustain various neuronal processes such as firing of action potentials, neurotransmission, ion homeostasis, etc. As such, age-related decline in metabolism may contribute to the cognitive declines associated with aging [9,10], which may be further exacerbated in selective regions of the brain in Alzheimer's (AD) and Parkinson's diseases (PD) [11,12].

The energy required for all endergonic processes in eukaryotic cells is generated through oxidative phosphorylation in mitochondria. Aside from its role as intracellular power plants, mitochondria play a fundamental role in other important cellular processes such as calcium homeostasis, apoptosis, intermediary metabolism, and cellular signalling through the regulated generation of intracellular oxidants such as hydrogen peroxide (H_2O_2) and to a certain extent nitric oxide (NO) [13,14]. Mitochondria have emerged as central organelles in aging biology as well as over 40 other diseases [15] as they are not only the sites of generation of ATP, but also a significant source of intracellular oxidants – H_2O_2 and NO [13] – of which H_2O_2 and its stoichiometric precursor superoxide anion ($O_2^{\cdot-}$) is suggested to play a role in influencing lifespan in some species such as *Drosophila melanogaster* [16]. However, the relationship between $O_2^{\cdot-}/H_2O_2$ and controlling lifespan is not as clear in rodent models. Modulation of $O_2^{\cdot-}$ and H_2O_2 levels show increase oxidative stress but no reduction in lifespan [17] while data with senescence accelerated mice, SAM, indicate the contrary [18]. These discrepancies highlight the notion that the relationship between oxidant generation and lifespan is not so simple. Regardless, it is apparent that gradual decline in mitochondrial bioenergetic capacity (production of ATP during oxidative phosphorylation) and increase generation of mitochondrial oxidants is a feature of aging mammalian tissues and age-related neurodegeneration [5,19,20]. Mitochondrial $O_2^{\cdot-}$ originates largely from the autoxidation of (a) ubiquinone – a mobile carrier that transfers electrons from complex I / II to complex III of the mitochondrial respiratory chain and from (b) rotenone-sensitive complex I [21,22]. Alterations in mitochondrial $O_2^{\cdot-}/H_2O_2$ in transgenic mice model indicate an important role for mitochondrial $O_2^{\cdot-}/H_2O_2$ in regulation of metabolic status. Modulation of mitochondrial H_2O_2 in peroxiredoxin 3 transgenic mice resulted in improved glucose tolerance reflected in lower blood glucose levels and increased glucose clearance [23]. Recently in SAM mice, an inverse relationship between mitochondrial $O_2^{\cdot-}/H_2O_2$ and whole brain glucose uptake was observed as a function of age [18].

Oxidative stress and altered redox status is a component of aging and age-related diseases [24–26]. Pro-oxidant shift in glutathione redox status in human plasma as well as in rodent tissues was observed as a function of age [24,27]. Longitudinal studies of redox status of human plasma collected from ages 19–85 show a decreased redox status as indicated by the linear oxidation of cysteine/cystine (Cys-SH/CysS-S) and GSH/GSSG (only after 45 years). This linear pro-oxidant shift indicates a *chronic increase* in oxidative events throughout adulthood [27]. Measurement of GSH levels from peripheral lymphocytes showed decrease GSH levels and increased GSSG levels from male AD patients [26]. Oxidation of GSH redox status in AD was reflected by increased glutathionylation of proteins in the inferior parietal lobule in AD patients as compared to matched controls [28]. Although the data are correlative, it may be surmised that increase generation of mitochondrial oxidants ($O_2^{\cdot-}$, H_2O_2 , $ONOO^-$) may modulate the cellular and mitochondrial redox status, of which increased protein post-translational modifications of redox sensitive proteins (*i.e.*, disulfide bond formation, glutathionylation, sulfenic acid formation) are likely consequences.

Overall, aging is associated with decline in energy production in the brain as well as parallel changes in redox status with a pro-oxidant shift which may be due in part through increase mitochondrial generation of $O_2^{\cdot-}$ and H_2O_2 . These aged related declines in energy production and redox status should not be viewed as independent variables that decline with aging, but rather as an *interdependent* relationship reflected in a *mitochondria energy- redox axis* – centered on mitochondrial metabolism and redox pathways –critical for neuronal function (Fig. 1). The *energy* component of the axis entails the entry of glycolytic substrates into the TCA cycle and generation of reducing equivalents (NADH, $FADH_2$) flowing through the respiratory chain and the *redox* component of the axis consists of interlinked mitochondrial redox indicators: glutathione (GSH/GSSG), thioredoxin (Trx(-SH)/Trx-SS), glutaredoxin (Grx), peroxiredoxins (Prx), systems *dependent solely on steady flux of NADPH generated through reduction of $NADP^+$ by NADH by the energetic component of mitochondria*. Typically viewed as parallel changes, decrease in mitochondrial bioenergetic capacity and altered redox status should be viewed as a concerted process, linked through various mechanisms including: (a) inter-convertible reducing equivalent pool (*i.e.*, $NAD(P)^+/NAD(P)H$) and (b) redox mediated post-translational modification of proteins involved in metabolism. Although the relationship might seem unidirectional, the reality is that fluctuations on either component will invariably lead to corresponding changes. Subsequently, the modification of several mitochondrial proteins (e.g., aconitase, complex I) can lead to impairment of mitochondrial function through alteration of their respective enzymatic function. Impairment of mitochondrial protein function may lead to further impairment of mitochondrial bioenergetics, increase in mitochondrial oxidant generation and *sustained* redox post-translational modifications of proteins that can contribute to neuronal dysfunction associated with aging.

The *energy-redox axis* represents a dual pronged approach to look at the changes in mitochondrial function as a function of age and disease. In this review, we emphasize the principle of an *energy-redox axis* critical for defining changes in neuronal and mitochondrial bioenergetics that, when perturbed, play a role in aging and neurodegenerative diseases. Additionally, we will discuss the potential neuroprotective mechanisms elicited by energy substrates and redox modulators that lead to modulation of the energy-redox axis.

2. MITOCHONDRIAL ENERGY METABOLISM IN AGING

1.Oxidative Phosphorylation

NADH produced by the tricarboxylic acid cycle (TCA) is oxidized by the mitochondrial respiratory chain, a sequence of multi-subunit complexes embedded in the inner mitochondrial membrane. The terminal electron acceptor of the respiratory chain, cytochrome oxidase, is responsible for 95% of oxygen consumption in mammalian cells. The energy required for ATP synthesis is garnered through the proton motive force generated by the translocation of protons across the inner mitochondrial membrane as electrons flow through the complexes [8,19]. The operational concepts of mitochondrial metabolic states and respiratory control were defined by Chance and Williams in 1955: state 4 (controlled or resting respiration), with the availability of respiratory substrates but not ADP and state 3 (active respiration) with ample respiratory substrate and ADP availability. The latter, is considered the maximal physiological rate of ATP production and O_2 consumption. Through measurement of mitochondrial O_2 uptake in state 4 and state 3, as well as O_2 uptake in tissue slices and perfused organs, it was estimated that under physiological conditions mitochondria in mammalian tissues are mostly in state 4 (~ 65%), whereas the rest are in state 3 (~ 35%). This indicates that under non-stimulated conditions, there is about 35% utilization of ATP-producing capacity [13].

The effects of aging on respiration are tissue-specific and are more prominent in tissues whose parenchyma contains mostly post-mitotic cells such as brain, heart, and skeletal muscle rather than tissues that contain slowly dividing cells, such as liver. Partial loss in the capacity for

energy generation has been well documented from mitochondria isolated from aged animals and has been attributed not only to changes in protein activities but also at the transcriptional level. Decline in mitochondrial state 3 respiration, respiratory control ratios, and uncoupled respiration rates using either NAD- or FAD-linked substrates and mitochondrial membrane potential ($\Delta\psi_m$) was observed in various aging models [3,29,30], all of which indicate impaired mitochondrial respiration. Increase in mitochondrial state 4 respiration and decreased membrane potential suggest increased membrane permeability to H^+ and therefore increased H^+ leakage back into the mitochondrial matrix, thereby decreasing the efficiency of mitochondrial energy transduction [31]. Most tissues have a constant ATP turnover; however for neurons and muscle cells that have a high and variable energy demand, their mitochondria require a spare ATP-generating capacity to handle peak demands and to prevent energy deficits [8]. Complex I, III, and IV activities decreased with age in mitochondria isolated from numerous organs, including the brain (see [19] for Review, [3,32]). Mitochondria isolated from synapses show that complex I, III, and IV activities had to be decreased by approximately 25, 80 and 70% respectively before any significant changes in ATP synthesis and oxygen consumption were detectable [33], although non-synaptic mitochondria show higher thresholds for inhibition of the respective complexes, indicating heterogeneity of mitochondrial populations within neurons [34]. Best-fit stoichiometrical organization of the respiratory chain in heart mitochondria was determined as, 1 complex I: 1.3 complex II: 3 complex III: 6.7 complex IV [35]. Low threshold for complex I inhibition and stoichiometric organization likely places the kinetic control of oxidative phosphorylation on complex I. This probably explains why decreases in mitochondrial respiration on NAD-linked substrates manifests in brain aging where a 35% decrease in complex I activity in brain and liver of old rats has been observed [19]. Decrease in mitochondrial respiratory complex activities is further compounded by morphological changes in mitochondria such as increase in mitochondrial size observed during aging in rat brain, increased mitochondrial fragility, and already lower mitochondrial mass present in neurons, thus leaving aged neurons unable to respond to increasing ATP demands [31,32]. Morphological and functional impairment of mitochondria maybe further exacerbated by low mitochondrial turnover in brain, thereby leading to increased accumulation of dysfunctional mitochondria with respect to aging [19].

2. Tricarboxylic acid cycle

In aerobic organisms, all catabolic pathways converge on the tricarboxylic acid cycle where fuel molecules are oxidized to generate reducing equivalents, NADH and $FADH_2$ for ATP synthesis [36]. NADH generated by the TCA cycle serves to donate electrons to complex I for oxidative phosphorylation [36] and is also important for the generation of NADPH through the reduction of $NADP^+$ by $NADP^+$ dehydrogenases or $NADP^+$ transhydrogenase; NADPH is the only electron source for the maintenance of glutathione and Trx2 system [37] (discussed in section 3.1). The activities of aconitase, α -ketoglutarate dehydrogenase (α -KGDH) [38], and pyruvate dehydrogenase (PDH) decline as a function of age [39,40]; the latter two, including isocitrate dehydrogenase (IDH), were also decreased in AD [41,42]. Reperfusion-induced decline in NADH-linked respiration in cardiac mitochondria was largely due to inhibition of α -KGDH, which was exacerbated by age; additionally, the supply of NADH to the electron transport chain appears to influence the rate of NADH-linked respiration in this model [43]. Inhibition of oxidant-sensitive α -KGDH is apparently sufficient to alter NADH levels [44]. Alterations in mitochondrial TCA enzymes such as α -KGDH activities could lead to decreases in the levels of NADH within the mitochondria, thereby affecting NADH-linked respiration at complex I.

Additionally recent work in our laboratory demonstrated an age-dependent decrease in Succinyl-CoA: 3-oxoacid Co-A transferase (SCOT) activity, a key mitochondrial matrix enzyme that mediates the rate-limiting step for ketolysis, the only alternative energy source

for the brain during glucose starvation. Loss of SCOT activity as a function of age renders the brain more susceptible to an energy deficit, especially under conditions of impaired glucose utilization such as diabetes or conditions entailing a decline in whole brain glucose uptake [3,10]. However, the relevance and extent of decrease in SCOT activity in neurodegeneration needs to be further confirmed in post-mortem tissues. Impairment of SCOT and PDH in rat mitochondria as a function of age indicates a decrease in substrate (*i.e.*, glucose and ketone bodies) availability into the TCA cycle, likely as a decrease in acetyl-CoA; the condensation of oxaloacetate and acetyl-CoA to yield citrate is the first step in initiating the TCA cycle [45]. Collective impairment of the activity of proteins that supply acetyl-CoA (*i.e.* PDH and SCOT) and TCA enzymes (*i.e.* aconitase, α KGDH) during aging may affect the rate of the TCA cycle and therefore ATP levels as the rate of the cycle is precisely adjusted to meet cellular demand of ATP[45]. Mitochondrial TCA enzymes whose activities are decreased as a function of age are also altered in pathological aging such as AD and PD (*e.g.* PDH). There is considerable overlap in several features that are inherent in aging and play a role in the development of neurodegenerative diseases.

3. THE MITOCHONDRIAL REDOX ENVIRONMENT IN AGING

The redox environment is determined by the sum of the products of the reduction potential and reducing capacity of the linked set of redox couples present [46]. Dependent mainly on the concentration of electron donors, NADPH and NADH, thiol/disulfide redox buffers (GSH) and H_2O_2 removal systems (Trx – dependent peroxiredoxins and GSH dependent peroxidases) [46,47], the redox status serves to maintain mitochondrial redox and thiol status of proteins in a reduced state[48]. Oxidation of the mitochondrial redox status due to increase generation of oxidants can lead to redox mediated post-translational modification of proteins and subsequent modulation of mitochondrial function such as the capacity to generate energy. As such, many age-related pathologies are associated with an oxidized cellular environment [32,49,50].

1. Thiol/disulfide exchange and its regulation in mitochondria

Thiol/disulfide exchange is critical to the maintenance of the mitochondrial redox environment as well as the pyridine nucleotide pool (NADPH), which is the primary reductant for the thiol/disulfide pool. Major players involved in thiol/disulfide exchange are glutathione, thioredoxin, and related enzyme systems.

Glutathione (GSH) is a small hydrophilic molecule that is synthesized in the cytosol from glycine, glutamate, and cysteine in a two step process by γ -glutamylcysteine synthetase and GSH synthase [51]. GSH is ubiquitously present in most cellular compartments and rapidly equilibrates between cytosol and the mitochondria. The mechanism of GSH transport into mitochondria is not well defined but has been shown to be under the governance of mitochondrial metabolic status and membrane potential [52]. As the concentration of GSH in the mitochondria far exceeds that of any other redox couple (~ 100 – $10,000$ greater) [51], the redox status of the mitochondria can be assessed by considering the redox potential for GSH/GSSG. As the most abundant non-protein thiol (~ 5 – 10 mM) [53]), GSH plays a central role in the removal of H_2O_2 generated by the electron transport chain. The role of mitochondrial GSH in protection against oxidative stress is supported by (a) a direct reaction with reactive oxygen and nitrogen species and (b) as an electron donor for enzyme linked systems, peroxiredoxins and glutathione peroxidases [51]. GSH is oxidized to GSSG by the direct reaction of the thiol of GSH with H_2O_2 , as a by product in the degradation of GSNO[54], or through glutathione peroxidases catalyzed reduction of H_2O_2 and alkyl peroxides [55]. GSSG is reduced back to GSH by glutathione reductase, a NADPH dependent enzyme ubiquitously distributed throughout the cell and in tissues [51,55]. Under non oxidative conditions, the concentration of GSSG is negligible at $1/100^{\text{th}}$ of the total GSH pool [46]. Although mitochondria import GSH from the cytosol through transporters, the mitochondrial GSH pool

can apparently function autonomously from the cytosolic GSH pool [53]. This suggests that the mitochondrial GSH/GSSG ratio can vary independently of that of the cytosol, in response to local changes in the production of mitochondrial oxidants [53]. A consequence of altered GSH/GSSG redox status is the formation of protein mixed disulfides (i.e. glutathionylation). Glutaredoxin (Grx2) are general thiol-disulfide oxidoreductases that are capable of reducing protein disulfides or GSH mixed disulfides (glutathionylated proteins) in a coupled system with GSH, NADPH, and glutathione reductase [55]. Glutaredoxins can be thought of as reductants (via GSH) that can control the amount of glutathionylated proteins. Their activity is central in the response to oxidative stress as the biological activities of many proteins are altered by the formation of GSH-mixed disulfides [56,57] and protein disulfides.

Mitochondrial GSH is important in regulating H_2O_2 production from the mitochondrial matrix but not $\text{O}_2^{\cdot -}$ generation from the intermembrane space. Low levels of GSH depletion has shown to have no bearing on H_2O_2 production by mitochondria; however, once a threshold of GSH depletion was reached, GSH loss corresponded linearly to increased H_2O_2 production [48]. To date, caloric restriction is the only regiment proven to extend lifespan in all mammals. Comparison of GSH and GSSG levels in two strains of mice that exhibit markedly different responses to late life caloric restriction demonstrated that a pro-oxidant shift in GSH redox status is a component of aging. Lower GSH:GSSG ratios and higher protein mixed disulfides was apparent in DBA/2 mice that were unresponsive to caloric restriction extension of lifespan as compared to age matched C57Bl/6 mice [58]. Measurements of GSH levels in brain mitochondria did not decrease significantly with age. However, there was a 63% increase in GSSG levels and a slight shift towards a more pro-oxidizing redox potential with respect with age [24].

The mitochondrial thioredoxin (Trx2) system, together with the glutathione - glutaredoxin system work to maintain a reducing environment within the mitochondria [59]. Trx2 is a small protein (~ 12 kDa) that contains two redox-active cysteine residues in the active center, and thus highly efficient at reducing disulfides in proteins and peptides [59,60]. Trx2 undergo redox reactions with multiple proteins via the transient formation of a mixed disulfide between the target protein and Trx2 followed by thiol-disulfide exchange, resulting in the reduction of the target protein and oxidation of Trx2 [60]. Intracellular concentrations of Trx are in the micromolar range in bovine tissue and places them orders of magnitude lower than that of GSH [46]. Oxidized Trx2 can be reduced by Trx2 reductase (TrxR2), a flavoprotein using NADPH as an electron donor [60] [57]. Recently, it has been demonstrated that Trx2 is associated with Peroxiredoxin 3 (Prx3) [61], thiol specific low efficiency peroxidases that relies on Trx2 as the hydrogen donor for the reduction of H_2O_2 and lipid hydroperoxides [62]. Biochemical studies suggest a transfer of electron through the Trx system accordingly; $\text{NADPH} \rightarrow \text{TrxR2} \rightarrow \text{Trx2} \rightarrow \text{Prx3} \rightarrow \text{H}_2\text{O}_2$ for peroxide metabolism in mitochondria [61]. Overexpression of Trx2 or Trx2R in some cell lines did not protect against oxidative stress, suggesting that NADPH supply rather than activities of Trx2 or Trx2R may be limiting for protection against oxidative stress [47]. Redox western analysis of Trx2 from HeLa cells showed only a 65% reduction of Trx2 in control samples, further supporting the notion of an upstream rate-limiting step [61]. Age-dependent decrease in TrxR2 was observed in skeletal muscle and heart, which was normalized upon caloric restriction. The relevance of decrease of TrxR2 was investigated using knockdown of TrxR2 that sensitized myoblasts to ceramide- induced apoptosis. [63]. The molar ratio of NADPH/NADP⁺ decreased with respect to age in kidney mitochondria [38]; this, together with decreases in Trx2-R levels may limit the electron flow through the Trx2 system, affecting the efficiency of peroxidoredoxin-catalyzed H_2O_2 degradation, thus contributing to the increased oxidative stress observed with aging. Trx2 interacts with α -ketoglutarate dehydrogenase and protects it from self inactivation during catalysis at low NAD⁺ levels [64]. Decrease in reduced Trx2 in aging might lead to impairment of this mechanistic interaction between the redox system (Trx2) and energy system (α KGDH) leading

to further loss of NADH levels through inhibition or reduction in turnover efficiency of the TCA cycle. The GSH and Trx systems function in parallel and are non-redundant [59]. These two systems may also overlap as TrxR can reduce both the active site disulfide and the glutathione-glutaredoxin mixed intermediate formed during glutathionylation of proteins at the expense of NADPH, therefore reducing Grx2 [65].

NADPH is the major source of reducing equivalents for reductive biosynthesis and is the only electron source for the reduction of the GSH and Trx2 pools in mitochondria. The redox potential of the NADPH/NADP⁺ couple is ~ 410 to 390 mV [46,66] and its half cell potential is independent of absolute concentrations. The [NADPH]/ [NADP⁺] ratio is approximately 100:1 while the [NADH]/ [NAD⁺] ratio tends to be between 1:10 to 1:1000. These couples are maintained many order of magnitudes away from equilibrium with each other allowing them to fulfill their individual functions [46]. In the mitochondria, the electron donor for the energy axis is NADH and the major electron donor for the redox system is NADPH. Measurement of mitochondrial $\Delta\psi_m$ and NAD(P)H status of brain mitochondria as a function of state 3 and state 4 (on complex I substrates) indicates that mitochondrial metabolic status influences the flux between oxidized and reduced NAD(P)H status and further correlates with H₂O₂ production [67]. The age-dependent decrease in the molar ratio of NADPH/NADP⁺ in kidney mitochondria [38] might affect oxidant production and membrane permeability transition.

2. Mitochondrial oxidant formation, aging, and neurodegeneration

Mitochondrial derived oxidants (*i.e.* O₂⁻, H₂O₂) are important regulators of both mitochondrial and cytoplasmic redox status in cells. Originally construed as “metabolic waste”, haphazard consequences of oxidative phosphorylation, O₂⁻ and H₂O₂ released from mitochondria are now recognized as physiologically important metabolites that partake in specific signaling cascades that collectively regulate numerous cellular processes through redox dependent alterations [68]. Mitochondrial derived H₂O₂ can affect cellular and mitochondrial redox status, evidenced in the alterations in GSH/GSSG redox status and post-translational redox modifications that modulate the activity of proteins [51,69]. Many key metabolic pathways are regulated by mitochondrial derived H₂O₂ through (a) activation/ inhibition of redox-sensitive signaling proteins (*i.e.* JNK, Akt, GSK) that regulate metabolism and/or (b) through post-translation redox modifications of proteins involved in metabolism (*i.e.*, aconitase, PDH). The targets of mitochondrion-derived oxidants are domain specific, thus entailing proteins with specific redox-sensitive cysteine-containing motifs that act as redox sensors [70]. Consequently, O₂⁻ and H₂O₂ released from mitochondria can act as signaling molecules that coordinate functional responses between mitochondria and cytoplasm. However, during aging and neurodegeneration when mitochondria oxidant production is enhanced, dysregulation of various signaling pathways may occur (e.g. JNK) together with random oxidative damage to proteins, lipids, and DNA.

O₂⁻, generated primarily by ubisemiquinone autooxidation at complex III [71–73] and secondarily by the NADH-dehydrogenase segment (complex I) [22,48], is the stoichiometric precursor of mitochondrial H₂O₂ [71,72]. O₂⁻ generated by complex I is vectorially released into the matrix, where matrix Mn-SOD (SOD-2) converts O₂⁻ into H₂O₂. Although glutathione peroxidase and peroxidoredoxins reside in the matrix to reduce H₂O₂, a portion of H₂O₂ generated in the matrix diffuses to cytoplasm, particularly when H₂O₂ production is high. Ubisemiquinone autooxidation at complex III releases O₂⁻ both into the matrix and innermembrane space (IMS); O₂⁻ in the IMS is released from mitochondria through voltage dependent anion channels (VDAC) and regulates the redox status of proteins in the intermembrane space [48,73–75]. The negative charge on O₂⁻ limits its diffusibility across the inner membrane whereas H₂O₂, which is neutral in charge, can diffuse across mitochondrial

inner and outer membrane. Consequently, mitochondria are major intracellular sources of both $O_2^{\cdot-}$ and H_2O_2 that can regulate many signaling pathways in cytoplasm (Fig.2).

Mitochondrial H_2O_2 generation increase with age and this effect is ameliorated by caloric restriction [17,19,76]. In addition, the age associated increase in the rates of mitochondrial generated $O_2^{\cdot-}$ and H_2O_2 was found to be lower in caloric restricted animals [5,16,76]. There are several possible mechanisms by which mitochondrial generation of $O_2^{\cdot-}$ and H_2O_2 increases, including increase in mitochondrial state 4 respiration and redox alterations including post-translational redox modifications to mitochondrial respiratory chain complexes. The mitochondrial production of H_2O_2 is strongly modulated by mitochondrial metabolic state where maximal generation of H_2O_2 occurs in the resting state (state 4) and negligible during oxidative phosphorylation (state 3) [13]. The production of H_2O_2 in state 4 is about 4–5 times higher than in state 3. The redox status of mitochondria (determined by GSH, NADPH, Trx2) is important in regulating H_2O_2 release from mitochondria, and age-related changes to mitochondrial redox (i.e. NADPH, Trx) may contribute to increase mitochondrial H_2O_2 production with age. Glutathionylation of complex I in liver mitochondria resulted in increase $O_2^{\cdot-}$ production from complex I [77]. Since glutathionylation has been reported to increase with age and in AD [55], increased glutathionylation of complex I or other mitochondrial proteins may be involved in the increase generation of $O_2^{\cdot-}$ and H_2O_2 during aging and neurodegenerative diseases.

Altered GSH redox status, inhibition of complex I, and oxidized proteins in Parkinson's and AD all suggest that mitochondrial $O_2^{\cdot-}$ and H_2O_2 may be enhanced [26,28,78]. In PD, monoamine oxidase (MAO) found ubiquitously in the outer mitochondrial membrane represents another important source of H_2O_2 from mitochondria. MAO is a flavo-protein important in oxidative deamination of catecholamines (*i.e.* dopamine, serotonin) which results in H_2O_2 production [79]. About 2.71 nmol/min/mg of H_2O_2 can be produced by MAO during the oxidative deamination of tyramine [80]. Consequently, MAO generation of H_2O_2 in cells when catecholamine levels are high, may play an important role in oxidative damage during PD.

The rapid and reversible activation of a series of linked sequence of reactions by specific agonists and stimuli, and the consequent transmission of precisely regulated and domain specific signals that produces a specific molecular or biochemical outcome is the principle of a signaling pathway [70]. Mitochondrion-derived H_2O_2 fulfils this principle and thus, establishes its role as an important regulator of signal transduction as evidenced by the diverse biological responses. However, during aging and neurodegeneration enhanced mitochondrial oxidant production can result in dysregulation of various signaling pathways (e.g., JNK) and random oxidative damage to macromolecules [19].

Aside from its role as a cellular source of $O_2^{\cdot-}$, mitochondria are important sinks of nitric oxide ($^{\cdot}NO$) [81]. Like H_2O_2 , mitochondria can be sites, as well as, targets of $^{\cdot}NO$ generated in the cytosol, within the mitochondria or extracellularly from inflammatory cells. The three isoforms of nitric oxide synthase (NOS) (inducible or inflammatory (iNOS), neuronal (nNOS), and endothelial (eNOS) are responsible for the production of NO. The amount of $^{\cdot}NO$ generated by each isoenzyme varies with the inducible form (iNOS) generating NO in the micromolar range while nNOS generates NO in the nanomolar range. The differential expression, activation state and cellular and sub-cellular distribution of the NOS isoenzymes determine the concentration and duration of $^{\cdot}NO$ production [82]. The effects of $^{\cdot}NO$ on mitochondria can be achieved through cytosol-generated $^{\cdot}NO$ diffusing to mitochondria or by the activity of mitochondrial nitric oxide synthase (mtNOS). Immunohistochemical [83] and biochemical methods show the presence of mtNOS in most tissues including brain and liver mitochondria from rats [14,84]. In the rat brain the mtNOS appears to be a 144 kDa nNOS variant [85],

voltage-dependent, and likely associated to mitochondrial cytochrome oxidase in the inner membrane [85,86]. The mitochondrial metabolic state is important in the regulation of mitochondrial $\cdot\text{NO}$ generation into the cytosol; [84,87] release of $\cdot\text{NO}$ was higher by 1.7–2.0 times higher in state 4 than in state 3 [87]. Although there is some discrepancy regarding the presence of mtNOS, data from our laboratory as well as others indicates that the presence and activity of this isoform maybe more robust in certain strains of rats and mice [3]. Interestingly, levels of mtNOS has been reported to decrease with aging particularly in brain, and it has been suggested to be a biomarker of brain aging. This may in part impair the biogenesis of new mitochondria during aging [19]. The ability for $\cdot\text{NO}$ to function as a signaling molecule stems from its ability to (a) bind to or react with transition metals or metal-containing proteins and the subsequent activation of signaling cascades - classically exemplified by the binding of $\cdot\text{NO}$ to the iron heme group of guanylate cyclase, yielding cGMP and signaling amplification upon activation of cGMP kinase [82] (b) reversible inhibition of mitochondrial cytochrome oxidase [88], and (c) protein post-translational modifications, either reversibly through S-nitrosylation or glutathionylation of cysteine residues or irreversibly through nitration of proteins [3,51]. The latter two are of particular interest in brain aging and neurodegenerative diseases and establish the principle of a redox-energy axis, critical for cellular function.

At nanomolar concentrations, $\cdot\text{NO}$ reversibly binds to the binuclear center of cytochrome oxidase in competition with O_2 and results in a transient depolarization of the mitochondrial membrane potential [85,89], a consequence of decrease in electron flux and proton extrusion. Inhibition of the electron flux at cytochrome oxidase shifts the electron transport chain towards a more reduced state and favors the formation the generation of $\text{O}_2^{\cdot-}$. Mathematical modeling predicts that $\cdot\text{NO}$ inhibition of cytochrome oxidase during state 3 (reduced) and state 4 (oxidized) have disparate outcomes : in state 4, prevents the *excessive* buildup of mitochondrial membrane potential that elicits rapid production of oxidants and in state 3, increase the efficiency of oxidative phosphorylation by increasing ADP/O ratio [88]. Therefore, suggesting a biological role of $\cdot\text{NO}$ in metabolic adaptability as a function of electron flux, substrate availability and $\cdot\text{NO}$ concentration. The molar ratio of COX over complex I is approximately 8 and the control coefficient of cytochrome oxidase on mitochondrial respiration is low [89], cytochrome oxidase has rarely more than 20% of the total control of ATP production even at peak metabolic demands [90]. This suggests that there is some excess in cytochrome oxidase activity; this is important in maintaining high affinity of mitochondrial respiration towards O_2 s and preventing excessive inhibition of mitochondrial respiration by $\cdot\text{NO}$. This may imply that complex IV levels found in cells appear to be optimized to allow moderate but not excessive overlap between the signaling roles of $\cdot\text{NO}$ and the inhibition of state 3 respiration by $\cdot\text{NO}$ [89]. This overlap may decrease in brain aging where there is increased nitrosative stress [3] and lower mitochondrial mass as compared to other organs[32], thus facilitating increase in $\cdot\text{NO}$ inhibition of mitochondrial respiration within a cellular setting. The increase in state 4 and decrease in state 3 during aging and the age-dependent decrease in mtNOS [30] may likely reduce the stimulatory effect of physiological levels of $\cdot\text{NO}$ on oxidative phosphorylation and may even affect O_2 gradients within tissues [90].

Initial work demonstrated that chronic exposure of $\cdot\text{NO}$ led to persistant inhibition of mitochondrial respiratory chain complexes I and III, without affecting complex II activity. This effect was initially thought to occur due to direct interactions between $\cdot\text{NO}$ and components of the complexes, however, further work utilizing sub mitochondrial particles (inverted mitochondria) demonstrated that long term inhibition of complex I[91] and complex III [81] was dependent upon the secondary formation of ONOO^- . The antimycin like effect of higher concentrations of $\cdot\text{NO}$ favors the formation of ONOO^- through the subsequent reaction of $\cdot\text{NO}$ with $\text{O}_2^{\cdot-}$ at diffusion-controlled rates ($\sim 10^{10} \text{ M}^{-1}\text{s}^{-1}$) yields peroxynitrite ONOO^- [81] which can lead to the selective and persistent inhibition of complex I activity during prolonged exposure of mitochondria to $\cdot\text{NO}$ [91]. As the reaction rate between NO and $\text{O}_2^{\cdot-}$ exceeds those

of $\cdot\text{NO}$ utilization by cytochrome oxidase ($0.8 \times 10^{-8} \text{ M}^{-1}\text{s}^{-1}$) and Mn-SOD-catalyzed disproportionation of $\text{O}_2^{\cdot-}$ in the mitochondrial matrix, the one order of magnitude higher concentration of $\text{O}_2^{\cdot-}$ in mitochondria (10^{-10} M) over the cytosol (10^{-11} M) favors the formation of ONOO^- within the mitochondria [92]. ONOO^- formation in mitochondria constitutes the major pathway for $\cdot\text{NO}$ metabolism in the mitochondria [13].

Localized formation of ONOO^- within mitochondria occurs as indicated by nitration of mitochondrial proteins in the presence of increase $\cdot\text{NO}$ diffusing from the cytosol [3]. Nitration of complex I activity bears significance considering the kinetic control of complex I on mitochondrial respiration with implications for aging and PD [93]. Redox modification of key mitochondrial metabolic proteins such as complex I, provides mechanistic support for the mitochondrial energy-redox axis whereby mitochondrial dysfunction with a shortage of energy supply likely causes brain dysfunction in aged hippocampus. Although most of the ONOO^- ($t_{1/2} = 3\text{--}5 \text{ ms}$) generated in the mitochondria will react with targets at the site of production; based on its $t_{1/2}$ and size of mitochondria ($\sim 2\text{--}3 \mu\text{m}$) the diffusion of ONOO^- in and out of the organelles would be feasible [94]; whether mitochondria are significant sources of ONOO^- *in vivo* remains to be determined.

Excessive production of $\cdot\text{NO}$, as a consequence of neuroinflammation, in the brain has been implicated in aging as well as in a number of neurodegenerative diseases including Alzheimer's and PD [2,5]. During aging and neurodegeneration, alterations in the levels of NOS isoforms, suggests that physiological regulation of mitochondrial function elicited at lower concentrations of $\cdot\text{NO}$ appear to decrease (e.g. mtNOS) and increase in pathophysiological levels of $\cdot\text{NO}$ (*i.e.* nNOS, iNOS) appears to occur in brain. As observed with mitochondria derived reactive oxygen species, specific $\cdot\text{NO}$ signaling is replaced with random $\cdot\text{NO}$ -mediated modifications to proteins during aging. Increase in nitrosative stress due to increase in nNOS expression during aging, led to an increase in nitrosylated proteins in the cytosolic fraction and nitrated proteins within the mitochondria from aged rats [3,26].

3. Post-translational modifications of proteins and the redox status

The release of oxidants ($\text{O}_2^{\cdot-}$, H_2O_2 , $\cdot\text{NO}$) from mitochondria as a function of the mitochondrial metabolic state serves as a coordinated response between the seemingly autonomous organelle and the rest of its intracellular counterparts. Consequently, it becomes clear that the mitochondrial metabolic status regulates not only the bioenergetic profile of neurons but also the redox profile of the cell, which plays an important role in the activation of numerous redox-sensitive signaling pathways through post-translational redox modifications. The biological consequences of changes in the cell redox and energy status during situations such as oxidative or nitrosative stress are the chemical modifications of target proteins that may be associated with either reversible (e.g. S-nitrosylation or glutathionylation) or irreversible (e.g. nitration, carbonylation) loss of protein function[69] (Fig.3). Protein sulfhydryls can undergo reversible oxidation or nitrosation, thus revealing a sensitive mechanism that modulates redox pathways in cell signaling [59]. The sulfhydryl group in the vast majority of protein cysteine residues (cys-SH) have a pK_a of > 8 [55]. However, redox sensitive proteins have cysteines that are ionizable such that thiolate anion can be stabilized by acid base interactions with neighboring amino acid residues, resulting in the lowering of their pK_a values at neutral pH [95]. These cysteine residues are redox sensitive because they are more vulnerable to chemical modifications or oxidation during redox changes in the cellular environment[55]. The principles and targets of redox modifications have been thoroughly reviewed elsewhere (see [55,70]).

4. MITOCHONDRIAL ENERGY-REDOX AXIS IN NEURONAL HEALTH: AN INTERDEPENDENT PROCESS

1. Shuttling reducing equivalents – the role of nicotinamide nucleotide transhydrogenase

Mitochondrial NADPH is formed through three pathways (1) NADP⁺ isocitrate dehydrogenase, (2) malic enzyme, and (3) nicotinamide nucleotide transhydrogenase (NNT) generated NADPH [96]. In the mitochondria NADPH generation by the Δp -driven transhydrogenase, nicotinamide nucleotide transhydrogenase (NNT), catalyzes the reduction of NADP⁺ from NADH ($H^+_{out} + NADH + NADP^+ \leftrightarrow H^+_{in} + NAD^+ + NADPH$). In the presence of a proton gradient, the reaction from left to right is favored on a magnitude of 5–10 fold [37]. In mitochondria, NADPH constitutes 95% of NADPH/NADP⁺ pool of which ~50% is uncoupler sensitive; this suggests that NNT-catalyzed reduction of NADP⁺ by NADH accounts for 50% of the total NADPH pool. *The conversion of NADH to NADPH provides a critical link between the energy component and redox component in the energy-redox axis, through the shuttling of reducing equivalents.* The dependence of NNT activity on membrane potential (i.e. energy metabolism) links energy transduction to the mitochondrial redox system. Therefore, changes in energy generation that occur with aging can result in net changes of NADPH levels, which in turn will alter redox status through limitations in reduction power for the redox systems. Although the reaction catalyzed by NNT is reversible, whereby NADPH hydrolysis is facilitated to maintain membrane potential through H⁺ extrusion, the relatively low free energy available from this reaction suggests that contribution of the reaction to the proton gradient is transient and of little significance [96]. The NNT is largely inhibited at high levels of NADPH/NADP⁺; its maximal rate of conversion of 30 nmol/min/mg mitochondrial protein is reached when the rate of NADPH consumption exceeds the steady-state of the transhydrogenase [37,97]. NADPH is the only source for reduction of the glutathione and Trx2 pool in the mitochondria, and thus may become a limiting factor for the respective systems [37,47,61]. Lower mitochondrial membrane potential due to loss of electron flow through the electron transport chain might affect the rate of conversion of NADH to NADPH and [98] and thus affect the rate of deglutathionylation catalyzed by NADPH dependent Grx 2. Concentrations of ONOO⁻ capable of inhibiting components of the respiratory chain resulted in modulation of NNT activity; ONOO⁻ treatment led to a two fold increase in the k_m values of both NADH and NADPH, which was prevented upon the pre-treatment with NADH [99]. Increase in ONOO⁻ formation within mitochondria as a function of age may affect the generation of reducing equivalents in the mitochondrial matrix through the modulation of NNT activity. The role of NNT in disease is poorly defined but NNT ablation in C57BL/6J mice is associated with a diabetic phenotype: impaired insulin secretion induced by high glucose challenge, [37,96] although the significance of NNT impairment in human diseases needs to be confirmed. Initial work carried out in our laboratory suggests that there is an age-dependent decrease in transhydrogenase activity during aging in mitochondria isolated from rats and mice, which was maintained upon caloric restriction. Decline in NADPH: NADP⁺ molar ratios despite increase in NADP⁺ isocitrate dehydrogenase (suggested to be an important source of NADPH in certain mammalian cells [96]) during aging in kidney mitochondria isolated from mice suggests that NNT dysfunction may play a significant role in NADPH generation.

2. Protein Post Translational Modifications of Metabolic Targets

The release of oxidants (O₂⁻, H₂O₂, ·NO) from mitochondria regulates not only the bioenergetic profile of neurons but also the redox profile of the cell that plays an important role in the activation of numerous redox- sensitive signaling pathways through protein post-translational modifications. As previously discussed, redox mediated post-translational modification of metabolic proteins represents another mechanism by which energy and redox status are linked. Mitochondria have been established as *bona fide* sites for signaling through

reversible phosphorylation; a number of phosphatases and cytosolic kinases such as members of the insulin signaling pathway, glycogen synthase kinase (GSK3 β), protein kinase B (AKT), as well as c-Jun N-terminal kinase (JNK), and other members of the MAP kinase family have been reported to localize to all compartments within the mitochondrion [100]. NO and H_2O_2 diffusing from mitochondria to the cytosol constitutes a pleiotropic signal for a series of cellular processes regulated through the activation of cytosolic kinases [13]. Activation and subsequent translocation of JNK to mitochondria occurred as function of increased intracellular H_2O_2 and which can subsequently lead to modulation of cellular metabolism [39,101,102]. Association of JNK with mitochondria results in the selective phosphorylation of proteins localized within the mitochondrial matrix and inner and outer membrane, including pyruvate dehydrogenase $\text{E}_{1\alpha}$ subunit [103]; the PDH complex serves as a bridge between anaerobic glycolysis and oxidative phosphorylation by catalyzing the conversion of glycolysis derived pyruvate to acetyl-CoA (pyruvate + Hs-CoA + NAD^+ $\rightarrow$ acetyl-CoA + NADH + H^+ + CO_2). Phosphorylation of PDH by JNK in primary cortical neurons resulted in decrease in neuronal ATP levels and accumulation of lactic acid, denoting a shift towards anaerobic metabolism of pyruvate [39]. JNK inhibition of PDH and corresponding metabolic changes occurred as function of age and thus, the energy transduction deficit due to impairment of PDH activity during aging may be mediated through redox regulation of JNK signaling [40]. While age-dependent phosphorylation and inactivation of PDH is not a direct consequence of redox-mediated post-translational modification, it is clearly a process regulated by redox signaling of the JNK pathway. Decrease in PDH activity appears to be an early event in AD and homogenous throughout the brain, regardless of the presence of neuropathology, suggesting that decrease in PDH activity is likely involved in the disease process rather than a consequence of tissue damage [39]. Measurement of PDH activity from patients with autopsy-confirmed AD disease showed the highest correlation between decreased PDH activity and clinical state [41].

Ketone bodies, β -hydroxybutyrate and acetoacetate, are the only significant source of substrates that have been shown to contribute to cerebral metabolism and become exceptionally important when utilization of glucose becomes limited in instances such as during starvation or type II diabetes [104]. In the brain, β -hydroxybutyrate is metabolized efficiently in mitochondria through a three-step process into acetyl-CoA by a series of enzymes of which SCOT catalyzes the rate-limiting step. The catalytic activity of SCOT was compromised as a consequence of tyrosine nitration induced likely thorough increase generation of ONOO^- due to age-dependent of increase nNOS expression [3]. Unlike phosphorylation of PDH, nitration of SCOT is irreversible and thus nitration of SCOT as a function of age renders the brain more susceptible to energy crisis when coupled with PDH inhibition. Redox regulation and modulation of PDH and SCOT activity may result in the collective impairment of substrate entry (i.e. acetyl-CoA) into the TCA cycle, thereby affecting the energy conservation systems. Furthermore, impairment of mitochondrial metabolism may further lead to decrease NADPH production through the NNT (Fig. 4).

Regulation of the TCA cycle is determined largely by substrate availability and product inhibition, mainly at three regulatory points represented by citrate synthase, IDH, and α -KGDH. Decrease in a select subset of TCA enzymes during aging and neurodegenerative diseases have been well documented in animal models as well as brains harvested post-mortem from patients with AD; these enzymes include aconitase, α -KGDH, and IDH with some variability between tissues and disease models [38,41,42]. Catalyzing the stereospecific conversion of citrate to isocitrate via the formation of *cis*-aconitate, the inactivation of aconitase is also often viewed as a marker of oxidative stress in biological systems due to susceptibility of the cubane $[\text{4Fe-4S}]^{2+}$ cluster [36,105] of aconitase to very low levels of reactive oxygen and nitrogen species. Decrease in aconitase activity has been reported to be the main functional target of aging amongst all the other TCA enzymes in mitochondria from mice kidney [38].

Very low doses of reactive oxygen and nitrogen species can inactivate the cubane $[4\text{Fe-4S}]^{2+}$ cluster of aconitase, but higher doses can result in post-translational redox modifications (i.e. nitration, glutathionylation) of other sites on aconitase. Nitration of aconitase has been demonstrated to increase with aging in semimembranosus (type II) muscle [106]. Inactivation of aconitase by ONOO^- challenge resulted in (a) disruption of the cubane structure through the loss of a labile iron at lower concentrations and (b) redox modification of vicinal tyrosine (nitration) and cysteine (glutathionylation) residues at higher concentrations. The redox modulation of aconitase is selectively controlled by mitochondrial citrate levels whereby citrate binding to the Fe-S active site resulted in protection against ONOO^- mediated disruption of the cubane structure. In contrast, allosteric modulation of aconitase activity as a consequence of redox modifications were not regulated by citrate levels [105]. The latter mechanism might be of relevance due to the millimolar concentration of GSH in the mitochondrial matrix and alteration in overall GSH redox status (decrease GSH/GSSG) in aging [24] and neurodegenerative diseases such as AD and PD [26,55]. In intact rat liver mitochondria, the proportions of exposed and reactive thiols are five times higher than that of GSH suggesting that the relationship between exposed protein thiols and GSH play a key role in mitochondrial thiol metabolism through, the formation of protein mixed disulfides [53]. Furthermore, reperfusion induced inhibition and reactivation of cardiac aconitase activity involves the reversible oxidative modification of sulfhydryl residues indicating that glutathionylation of aconitase is a conserved regulatory mechanism [107].

Aside from aconitase, glutathionylation of E_2 subunit of α -KGDH has been demonstrated in the presence of H_2O_2 in isolated cardiac mitochondria [108,109]. Alterations of α -KGDH activity are of particular interest in AD pathology due to the high correlation of decreased α -KGDH activity with the occurrence of AD [41,42]. Tretter *et.al* demonstrated selective susceptibility of aconitase and α -KGDH to H_2O_2 levels, where higher concentrations of H_2O_2 was needed to inhibit α -KGDH activity [44]. The differential susceptibility is likely a result of the potential mechanisms of H_2O_2 mediated through direct interaction with the cubane structure of aconitase versus redox modulation of α -KGDH cysteine residues as the latter has been demonstrated to be resistant to direct interaction with peroxides [108]. Impairment of α -KGDH resulted in limited production of NADH under oxidative stress due to the inability to utilize glutamate to drive a segment of the TCA cycle [44].

Mitochondrial dysfunction associated with partial deficiencies in complex I activity has been implicated to aging [19] as well as neurodegenerative diseases such as PD [5]. Deficiencies in complex I activity can arise at the genetic level through mutations as in the case of Leber's hereditary optic neuropathy [110] or through redox-mediated post-translational modifications of complex I; the latter mechanism leads to increase generation of $\text{O}_2^{\cdot-}$ and ultimately decreased oxidative phosphorylation capacity [77,93,111]. The mechanisms that lead to persistent and transient inhibition of complex I maybe to due to the nature and duration of the oxidant and the redox status of the mitochondrial GSH pool, whereby oxidation or depletion of the mitochondrial GSH pool influenced the rate and reversibility of the redox modification of complex I [111]: (a) either through NO mediated mechanisms through the formation of ONOO^- (through NO induced oxidation of the ubiquinol pool and subsequent increase in $\text{O}_2^{\cdot-}$ formation [91]) [112] or (b) through oxidation of the mitochondrial GSH pool and subsequent formation of protein mixed disulfides as complex I thiols were found insensitive to $\text{O}_2^{\cdot-}$ and H_2O_2 but rapidly reactive with disulfides such as GSSG and S-nitrosothiols [77, 112]. Impairment of mitochondrial complex I activity has been demonstrated to lower the mitochondrial respiratory spare capacity during high energy demands [113] and shift the threshold voltage for permeability transition pore opening close to resting potential [114]. The redox modulation of mitochondrial complex I and the dependence of brain mitochondria largely on the channeling of reducing equivalents through complex I highlights the *energy-redox* axis, critical for neuronal function. This concept is underscored by the observation that

GSH depletion is the earliest reported biochemical change in the substantia nigra of patients with PD and may facilitate inhibition of complex I.

Aside from complex I, complex V (ATP synthase) is also redox modulated through a spectrum of protein post translational modification. Increased nNOS activity during brain aging resulted in the nitration of F₁-ATPase and corresponding decline in activity in animal models. LC/MS/MS analysis revealed nitration at Tyr²⁶⁹, suggesting deficits in ADP binding. The age-dependent decline in F₁-ATPase was at variance with the decline in cytochrome oxidase indicating that the activities of F₁-ATPase may become rate limiting in affecting energy metabolism in the brain [3]. Unpublished work in our laboratory indicates that glutathionylation of F₁-ATPase and subsequent partial inhibition under oxidative stress can be reversibly regulated by reducing the mitochondrial pool. A new concept of the regulation of cellular respiration was recently advanced; it retains the classical notion that energy demands drive respiration but places the kinetic control of both respiration and energy supply on the availability of ADP to F₁-ATPase and O₂ and [•]NO to cytochrome oxidase [115]. Hence, redox modification of F₁-ATPase limits the rate of oxidative phosphorylation and inadvertently alters the membrane potential [116] and the activities of voltage dependent enzymes such as the NNT.

In summary, redox-mediated post translational modifications of mitochondrial proteins may lead to impairment of mitochondrial ability to efficiently act as a site for substrate (i.e. glucose, ketone bodies) catalysis. Unpublished results from our laboratory demonstrate that during nitrosative stress, GAPDH was reversibly inhibited through glutathionylation; loss of GAPDH activity may lead to impairment of substrate availability to the mitochondria through modulation of glycolysis. Changes in substrate availability to the mitochondria through cytosolic events such as redox mediated inhibition of GAPDH and impaired glucose uptake [10] and substrate catalysis (i.e. mitochondrial function), may collectively play a role in a cellular energy deficit present in aging and exacerbated in neurodegenerative diseases (Fig. 5).

5. POTENTIAL THERAPIES TARGETING THE MITOCHONDRIAL ENERGY-REDOX AXIS

The previous section highlighted the role that the *energy-redox* axis plays in overall neuronal metabolic capacity, the consequences of some of the changes that lead not only to energy impairment but also support a pervasive cycle of mitochondrial generation of oxidants that regulate mitochondrial function (a) indirectly through the redox modulation of cytosolic signaling cascades but also (b) directly through redox-mediated post translational regulation of mitochondrial proteins. This decline in the *energy-redox* axis may be an important factor in sensitizing the brain to an energy crisis during aging, especially under conditions of impaired glucose utilization in the case of age-associated type II diabetes or AD where there is severe decrease in glucose uptake and impaired glycolysis. The energy crisis in the brain may deprive neurons of energy necessary for normal function, which may manifest as decreases in cognitive and motor functions, as well as neuronal death. Because impairment of the energy-axis may be a major pathologic component of brain aging and neurodegenerative disorders, an important therapeutic strategy being tested is the supplementation of energy substrates and/or redox modulating agents to overcome the deficits in the energy-redox axis. Because energy and redox are interlinked, energy supplementation may then help increase the reductive potential of neurons through increases in NADPH. Conversely, redox supplements may increase the energy capacity of neurons by increasing the redox potential that would result in decrease steady-state levels of redox post-translational modifications that inhibit metabolic pathways. A flurry of research has begun exploring the utilization of energy supplements including ketone bodies and redox modulating agents, such *N*-acetyl-cysteine (NAC) and lipoic acid, to improve brain functionality and health by modulating the energy-redox axis.

1. Energy and redox modulation in preventing injury in cultured neurons

Energy supplementation to overcome deficits in the energy-redox axis have been supported by cell culture studies that have demonstrated the favorable use of mitochondrial energy substrates such as pyruvate as neuroprotective agents against oxidative stress [117,118], nitrosative stress [119] as well as A β toxicity [117,120]. A β treatment like oxidative and nitrosative stress is associated with redox perturbation, possible through modulation of mitochondrial bioenergetics and reactive oxygen species generation. Addition of pyruvate to H₂O₂ - challenged neurons attenuated mitochondrial membrane depolarization, decreased mitochondrial O₂⁻ production [117], and prevented caspase-3 release during A β toxicity [120]. Initially shown as a non-enzymatic scavenger of H₂O₂ [118], pyruvate has been shown to directly affect mitochondria function through modulating mitochondrial NAD(P) redox status [120], generation of O₂⁻ [117], and increase aconitase activity [121]. Physiologically, neurons may be provided pyruvate in the form of lactate by astrocytes. There is increasing support of the astrocyte-neuronal lactate shuttle that supports the notion of metabolic coupling between astrocytes and neurons in the form of lactate [122]. Recently, a mitochondrial isoform of lactate dehydrogenase was identified, attached to the mitochondrial inner membrane and constitutes part of a mitochondrial lactate oxidation complex with cytochrome oxidase and monocarboxylate transporters 1 and 2 (lactate/pyruvate shuttles) [123]. The presence of a mitochondrial lactate oxidation complex enables neurons to oxidize lactate into pyruvate to maintain mitochondrial generation of ATP. However, the value of lactate could be limited by the fact that it has to be converted to pyruvate first and PDH activity is inhibited in aging as well as AD.

Unpublished work in our laboratory showed that pre-treatment of primary cortical neurons with either malate, pyruvate, or β -hydroxybutyrate protected against 'NO-mediated cell death independent of ATP levels. Aside from the obvious effect of supporting mitochondrial oxidative phosphorylation, mitochondrial energy substrates may maintain neuronal function through other mechanisms such as modulating mitochondrial redox status through the regulation of GSH and NAD⁺ pools. The generation of acetyl-CoA by substrate supplementation leads to the churning of the TCA cycle and the subsequent reduction of mitochondrial NAD⁺ to NADH, thereby maintaining mitochondrial NADP⁺/NADPH ratios. Mitochondria NAD⁺ is important in maintaining cellular viability even when cytoplasmic pools were depleted [124]. Supplementation of cells with substrate may also protect key metabolic enzymes such as aconitase by protecting the active site from reactive oxygen and nitrogen species-mediated damage. Many metabolic enzymes such as aconitase contain redox sensitive prosthetic group in the active site that may be protected from damage by presence of substrates through steric inhibition. Of note, in the presence of the aconitase substrate, citrate, a 100 fold increase in ONOO⁻ concentration was required to elicit half maximal inhibition of aconitase function [105]. The protective effects of citrate can be attributed to steric inhibition of the active site containing the redox sensitive Fe-S cluster from ONOO⁻ mediated damage.

Supplement of redox modulating agents are highly effective in protecting neurons and other cells from a wide range of stresses. Lipoic acid and N-acetylcysteine (NAC), are two commonly used redox-modulating compounds, which have been shown to increase intracellular GSH levels and modulate various redox-sensitive signaling pathways in many cells types including neurons [125,126]. Acetylation of the cysteine residue in NAC, stabilizes the thiol group, preventing its rapid oxidation thereby enhancing its efficacy as an intracellular redox modulator. Lipoic acid has received a good deal of attention as a nutritional supplement due to its antioxidant and redox modulating properties [127]. Lipoic acid and NAC treatment have been shown to protect neurons from hypoxia, oxidative and nitrosative stress, as well as amyloid-induced neurotoxicity [128–132]. NAC treatment prevented neuronal death induced by hypoxia in primary hippocampal neurons and various 'NO donors (DEA/NO, PAPA-

NONOate, SIN-1) in various neuronal cells [129,133]. In SHSY-5Y, the neurotoxic effects of amyloid beta A β ₂₅₋₃₅ could be prevented by pre-treatment with NAC [134]. Similarly, lipoic acid treatment protected neurons isolated from chick embryos from hypoxia (cyanide-induced model) [128], and cholinergic cells from NO mediated death induced by sodium nitroprusside treatment [130]. Lipoic acid also protected primary cortical neurons from H₂O₂- and A β induced neurotoxicity [131], through pathways entailing Akt activation [135]. In addition, lipoic acid pretreatment (1 μ M) was able to protect rat hippocampal neurons from glutamate-induced excitotoxicity [128,132]. Furthermore, lipoic acid is an essential co-factor in α -keto acid dehydrogenase complexes in mitochondria that includes α -KGDH, PDH and IDH. Supplementation of lipoic acid may protect α -KGDH, PDH and IDH from oxidant mediated damage and increase their respective activities; administration of lipoic acid resulted in increase PDH activity in rats [127].

The neuroprotective actions of both NAC and lipoic acid may be due in part to their ability to increase GSH levels and thus the redox potential of cell. *In vitro*, NAC can act as a reducing agent, antioxidant, and cysteine precursor that support GSH synthesis [136]. When transported into cells, NAC is purported to undergo deacetylation to cysteine, the rate-limiting amino acid in GSH synthesis. The intracellular mechanism of action for lipoic acid is complicated by its ability to undergo a unique redox cycle within cells [137]. Due to its unique chemical structure, lipoic acid can act both as radical scavenging agent and an oxidizing agent, due to the presence of a disulfide in the thiolane ring [138]. Lipoic acid may increase GSH synthesis in cells by activating Nrf2, a transcriptional regulator of glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis. Found normally associated with Keap1 in the cytosol, Nrf2 contains redox sensitive thiols that upon oxidation leads to its dissociation from Keap1 and subsequent translocation into the nucleus [139]. Aside from upregulation of GCL expression, Nrf2 is also an important transcriptional regulator of important genes involved in antioxidant defense such as SODs [139]. *In vivo*, lipoic acid treatment increases Nrf2 translocation to nucleus and GSH synthesis, thus suggesting that lipoic acid may oxidize Keap1 to activate Nrf2 and GCL expression in cells [140,141]. Lipoic acid treatment to cells may also increase GSH levels by increasing cysteine transport into cells [137]. In various cells, several reductases and mitochondria have been shown to reduce lipoic acid to dihydrolipoic acid (DHLA), a potential reducing agent [142,143]. Once formed DHLA diffuses from the cell and reduces extra-cellular cystine to cysteine, which is rapidly transported into cells for GSH synthesis by the efficient ASC transport system [137]. Increase cysteine transport via the ASC system and increase Nrf-2 activation may be the mechanisms underlying lipoic acid mediated increase of GSH in cells. Another interesting aspect of lipoic acid is that it contains a medium length fatty acid tail that can undergo β -oxidation to generate ATP and various metabolites in mitochondria [138]. Thus, lipoic acid maybe similar to pyruvate in that it can act both as an energy source and antioxidant in cells. Because of the complex chemistry of lipoic acid, it remains uncertain which properties of lipoic acid are essential in neuroprotection against a wide range of stresses.

2. The use of compounds that regulate energy-redox axis in a clinical setting

The protective effects of metabolic substrates or redox modulating agents in cell culture against a wide range of stresses have lead to many animal studies and clinical trials in patients. The potential of ketone bodies and monocarboxylates such as lactate as therapeutic regiments against AD and in ischemia reperfusion brain injury were recently investigated. [104,144, 145]. The use of ketone bodies has also shown promise in rodent PD models [146,147]. NAC feeding has been shown to improve mitochondria function and delay age-related memory impairment in mice [148] and is the subject of a wide range of clinical trials testing its neuroprotective properties in various pathologies. Similarly, lipoic acid has been heavily investigated in the treatment of brain aging and AD.

In the context of an energy-redox modulator, lipoic acid has received attention as a therapeutic agent for improving cognitive function and metabolism during normal brain aging [125,142]. Although detailed pharmacokinetics data on lipoic acid in brain have not been performed, initial studies suggested that lipoic acid could cross the blood brain barrier [149] [150]. Regardless of whether lipoic acid crosses the blood-brain barrier, lipoic acid feeding strongly modulates many metabolic and antioxidant pathways in brain [125,151], for it has several unique attributes that make it a potent modulator of the energy-redox axis during aging: First, as mentioned above, lipoic acid can increase GSH levels in cells; lipoic acid feeding increased GSH levels in brain and other tissues [152,153] as well as levels of other antioxidants. Lipoic acid feeding increased antioxidant defenses (e.g., glutathione, ascorbate), improved mitochondria respiration, and carnitine acetyltransferase activity as well as decreased oxidative stress in brain of old rats; demonstrating biochemical changes elicited by lipoic acid was accompanied by an improved cognitive function [154–156]. Both aging and Alzheimer's disease are associated with decreased cellular redox status and increased disulfide bonding of proteins, such as glyceraldehyde-3-P dehydrogenase (GAPDH) [28,55]. The oxidative stress component in normal brain aging and Alzheimer's disease [5,49] may be reduced by augmenting the antioxidant defenses upon lipoic acid intake. In addition to increasing redox potential of brain, lipoic acid may directly increase energy production in brain by increasing glucose uptake in neurons. Lipoic acid has been shown to directly bind to the insulin receptor and therefore having *insulin-like effects* [157] by increasing Akt phosphorylation and glucose uptake in various cell lines [149,158]. Because energy hypometabolism always accompanies clinical AD and is the earliest abnormality observable [7,41], the ability of lipoic acid to stimulate glucose uptake may render it a potent therapeutic agent against this neurodegenerative disorder. Not surprisingly, lipoic acid feeding has been shown to prevent mitochondria declines and improve cognitive function in various mice models of brain aging and AD (*i.e.* SAMP8, Tg2576, ApoE4) [159–161]. Lipoic acid supplementation has already been shown to delay cognitive decline in AD in an open label study in patients [162], and is undergoing phase II clinical trials for Alzheimer's treatment. One additional characteristic of lipoic acid that may be beneficial in AD therapy is its ability to reduce inflammation in mice: lipoic acid feeding decreased monocyte activation and LPS-induced acute inflammation *in vivo* by a mechanism entailing activation of Akt and decreased NF- κ B activity and adhesion [163]. Because AD is associated with neuroinflammation and activation of microglia [164] (which associate with senile plaques [165]), the anti-inflammatory properties of lipoic acids may have potentially beneficial effects in the treatment of this disease.

Ketone bodies have been demonstrated to be neuroprotective against other models of CNS injuries in rodent models of global ischemia, hypoglycemia, glutamate toxicity and PD [147, 166]. Examining the ontogeny of brain metabolism indicates metabolic adaptation throughout life: primary dependence on glucose in utero, lactate during weaning, glucose and ketones during suckling to primary reliance of glucose after weaning and a shift to ketones and glucose in middle age in rodent models [166–168]. The metabolism of β -hydroxybutyrate to acetyl-CoA requires three enzymatic steps (localized within the mitochondria) while the conversion of glucose to acetyl-CoA requires 11 biochemical steps. It is probably this unique feature of ketone metabolism that makes it a more "efficient" fuel under pathological situations. Increasing insulin resistance as a function of age –and to a greater extent in AD – coupled to depressed glucose uptake has lead to the use of ketone bodies to alleviate the hypometabolic state in the brain [1]. Clinical trials with AC1202™, a medium chain triglyceride that leads to increase plasma ketone bodies, improved memory and cognitive function in patients with mild-to-moderate AD [145]. The ability for ketone bodies to improve cognitive function and the direct oxidation of ketone bodies in the mitochondria suggests that impaired mitochondrial function plays a central role in cognitive decline inherent in AD. There is some suggestion that the neuroprotective effects of ketosis maybe due to reduced excitatory transmission through decrease glutamate release. Glutaminergic neurons fed only β -hydroxybutyrate had a net

decrease in glutamate release through possible alterations in aspartate-glutamate homeostasis shuttle, where loss of reducing equivalents normally generated during glycolysis leads to short circuiting of the mitochondrial TCA cycle through transamination of oxaloacetate to aspartate [169]. The long-term effects of ketone body-enriched diets still need to be further investigated. Possible concerns with long-term utilization of ketone bodies are the negative feedback inhibition of PDH activity through an increase of acetyl-CoA levels, a “short circuiting” effect of ketone metabolism in the overall generation of reducing equivalents by the TCA cycle as well as ATP in the long run through OAA depletion. Furthermore, there is evidence that infusion of β -hydroxybutyrate resulted in depressed cerebral glucose uptake in humans as well as insulin-dependent glucose uptake in cardiomyocytes [104,170].

6. CONCLUSIONS AND PERSPECTIVES

Understanding this principle of an *energy-redox axis* allows for a dual pronged approach to understanding interdependent redox and energy changes as a function of age and disease. Altered mitochondrial energy metabolism, or mitoenergetic failure, is a feature of aging and also contributes to the pathophysiology of acute brain injury and chronic neurodegenerative diseases, whereby impairment of mitochondrial energy-transducing capacity leads to an energy deficit affecting key energy-dependent processes, such as DNA repair, turnover of oxidatively-damaged proteins, biosynthesis of macromolecules, and decrease of NADPH levels. Redox and energy changes occur simultaneously during aging and age-related neurodegenerative diseases and may work synergistically to modulate neuronal injury through an energetic crisis. Reversible and irreversible redox modification of key metabolic targets in the mitochondria leads to a pervasive cycle that likely facilitates both redox and energy changes. Targeting this energy-redox axis bears therapeutic potential for attenuating age- and pathology-related changes in the brain such as energy hypometabolism, often associated with cognitive declines.

Acknowledgments

The authors would like to thank Ryan Hamilton Ph.D for his help in the preparation of the manuscript. This work was supported by NIH grant RO1-AG16718 (EC) TRDRP grant 16RT-0186 (EC), NIH grant AA016911 (DH), and Zumberge grant (Office of the Provost at the University of Southern California; DH).

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Figure 1. The mitochondrial energy-redox axis in cell function

The mitochondrial *energy-axis* entails an: (a) Energy component - the entry of glycolytic substrates into the TCA cycle and the generation of reducing equivalents (NADH , FPH_2) flowing through the mitochondrial respiratory chain and (b) the mitochondrial redox component - interlinked mitochondrial redox indicators: glutathione (GSH/GSSG), thioredoxin ($\text{Trx}(-\text{SH})/\text{Trx}(-\text{SS})$), glutaredoxin (Grx), peroxidoredoxins (Prx), systems *dependent solely on steady flux of NADPH*. Typically viewed as independent components, the mitochondrial metabolic state and redox status can be viewed as concerted processes, linked primarily through inter-convertible reducing equivalents pool (i.e. $\text{NAD(P)}^+/\text{NAD(P)H}$), catalyzed by the $\Delta\psi_m$ NNT bound to the mitochondrial inner membrane. Perturbations in either mitochondrial metabolism or redox pathways modulates the rate of generation of mitochondrial metabolites (e.g. H_2O_2) that results in domain specific signaling achieved through redox mediated post translational modifications of cytosolic targets (eg. GAPDH).

Figure 2. Production of $\text{O}_2^{\cdot -}$, H_2O_2 and NO by mitochondria

Mitochondria are major intracellular sources of $\text{O}_2^{\cdot -}$, H_2O_2 and in some instances, NO . Mitochondrial $\text{O}_2^{\cdot -}$, originates largely from (a) the autooxidation of ubiquinone in complex III and (b) rotenone-sensitive complex I of the mitochondrial respiratory chain. The majority of the $\text{O}_2^{\cdot -}$, generated in mitochondria is dismutated into H_2O_2 in the matrix by MnSOD (not shown) although a proportion may be formed in the inner membrane space likely through autooxidation of the outer ubiquinone pool at complex III. Voltage-dependent mtNOS is likely associated to mitochondrial cytochrome oxidase in the inner membrane and leads to generation of NO within the mitochondria. The generation of mitochondrial derived $\text{O}_2^{\cdot -}$, H_2O_2 and NO is regulated by the mitochondrial metabolic status. In state 4, where there is

limited ADP, protonic potential is high and the rate of respiration is limited due to inhibition of ATP synthesis due to lack of ADP. This results in longer lived transients of the respiratory chain such as the semiquinone radical, (QH·) and the subsequent reduction of O_2 to $O_2^{\cdot-}$, the stoichiometric precursor of H_2O_2 . Similarly, $^{\cdot}NO$ production increases exponentially in the presence of a high membrane potential, however the mechanisms remains unclear. A fraction of $O_2^{\cdot-}$ released into the mitochondrial intermembrane space can be in turn released into cytosol via a voltage-dependent anion channel (VDAC).

Figure 3. Protein post-translational modifications by mitochondrial oxidants

The biological consequences of changes in the cellular redox and energy status during situations such as oxidative or nitrosative stress are the chemical modifications of target proteins that may be associated with either reversible (e.g. S-nitrosylation or glutathionylation) or irreversible (e.g. nitration) loss of protein function. Increase formation of ONOO^- and/or H_2O_2 can lead to redox modification of proteins through oxidation of cysteine residues to sulfonic acid, disulfide bond formation and glutathionylation, through oxidation of GSH to GSSG. Additionally, ONOO^- formation results in the irreversible nitration of tyrosine residues, considered a hallmark of nitrosative stress. Increase generation of NO may lead to both S-nitrosylation and S-glutathionylation of cysteine residues, the latter a consequence of GSH nucleophilic attack on the S-NO bond of nitrosylated proteins. Mitochondrial generation of oxidants can also lead to the activation of various kinases (e.g. JNK, GSK3 β) and subsequent phosphorylation of downstream targets.

Figure 4. Impairment of PDH and SCOT activities may lead to alterations in the mitochondrial nucleotide pool

Redox mediated post translational modification of SCOT and PDH during aging, leads to decreases in PDH and SCOT activity that may result in the collective impairment of substrate entry (i.e. acetyl-CoA) into the TCA cycle. As condensation of oxaloacetate and acetyl-CoA to yield citrate is the first step in initiating the TCA cycle, decreased acetyl-CoA generation may limit NADH generated during the TCA cycle. NADPH formation - catalyzed by NNT - may be reduced as a consequence of decreased formation of NADH during the TCA cycle and the rapid consumption of NADH by complex I. Changes in membrane potential during aging may also lead to a slower rate of NADPH formation through modulation of NNT activity. Decrease in NADPH may compromise the efficiency of the mitochondrial redox system, resulting in a pro-oxidant shift through limited removal of H₂O₂.

Figure 5. Pathophysiological significance of GAPDH, PDH, SCOT, and ATPase in brain aging-the premise of multi-level failure in energy hypometabolism

In the adult brain, glucose is the primary source of fuel for the synthesis of ATP. Taken up by the brain, glucose is broken down in 11 steps during glycolysis to generate pyruvate, which is then transported into the mitochondria where PDH catalyzes the oxidative decarboxylation of pyruvate into acetyl-CoA. Lactate taken up by neurons, through MCT, can be converted either by cytosolic or mitochondrial lactate dehydrogenase to also generate pyruvate. During starvation or type II diabetes, the brain can metabolize ketone bodies (D-β-hydroxybutyrate, acetoacetate) imported by MCT to generate acetyl-CoA. Unlike glucose, D-β-hydroxybutyrate does not undergo metabolism in the cytosol but is metabolized directly in the mitochondria where SCOT catalyzes the rate-determining step of ketolysis by converting acetoacetate into

acetyl-CoA. Redox mediated inhibition of GAPDH, such as during inflammation which occurs during aging or neurodegeneration, leads to attenuation of substrate availability (i.e. pyruvate) to mitochondria. Impairment of SCOT also results in limited entry of ketone bodies into the mitochondrial TCA cycle. Attenuation of substrate availability may be further compounded by age-related decreases in PDH, and ATPase activity, which limits substrate catalysis by mitochondria. This multi-level failure –as a consequence of cytosolic and mitochondrial events- leads to a bioenergetic crisis leaving neurons more susceptible to damage and unable to respond fluctuations in energy demand.