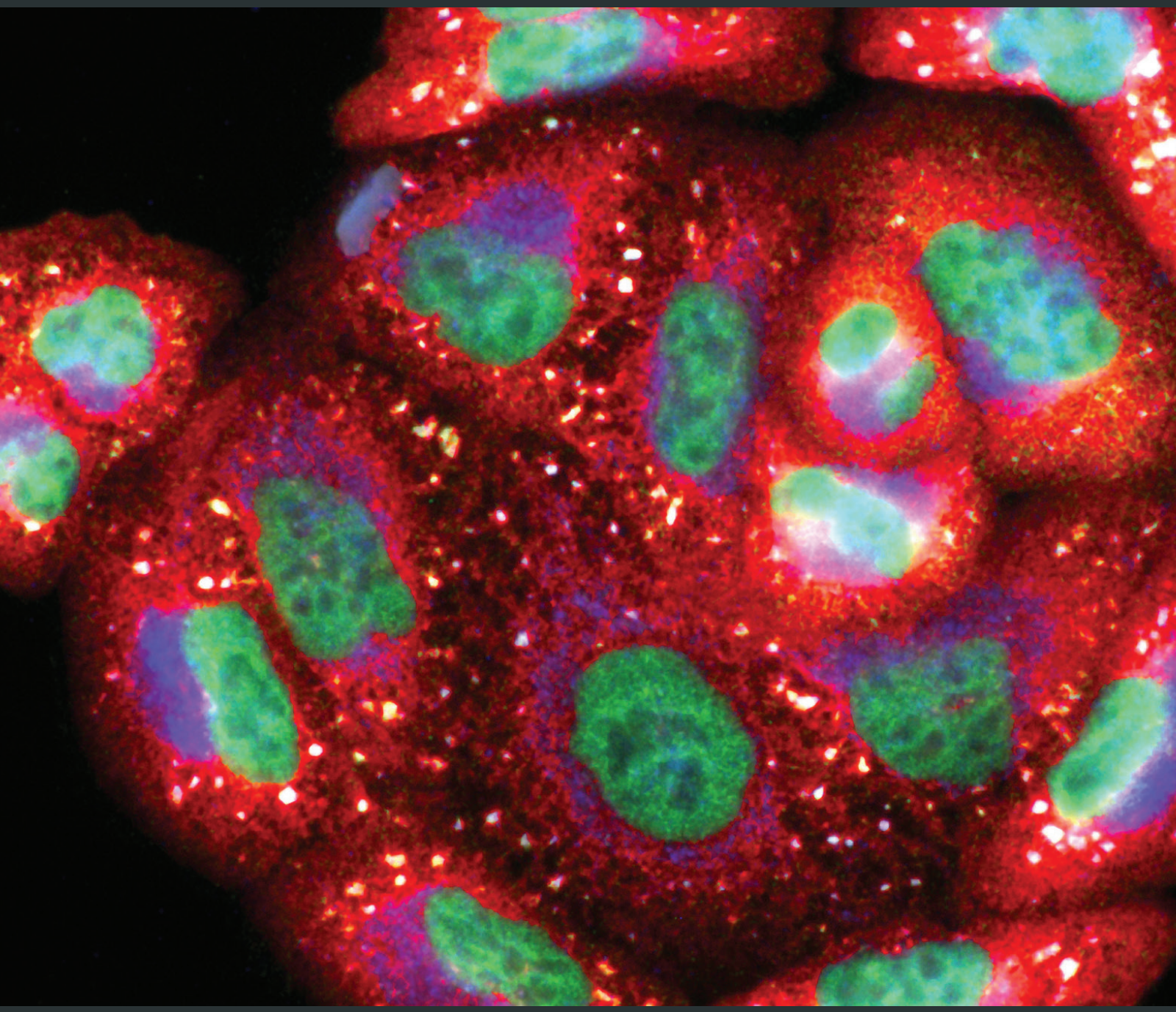
Oxidative Medicine and Cellular Longevity

# Redox Status and Aging Link in Neurodegenerative Diseases 2015

Guest Editors: Verónica Pérez de la Cruz, Sathyasaikumar V. Korrapati, and José Pedraza Chaverrí



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# Editorial Board

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*Editorial*

# Redox Status and Aging Link in Neurodegenerative Diseases 2015

**Verónica Pérez de la Cruz,[1] Sathyasaikumar V. Korrapati,[2] and José Pedraza Chaverrí[3]**

[1]*Departamento de Neuroquímica, Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, S.S.A., 14269 México, DF, Mexico*
[2]*Maryland Psychiatric Research Center, University of Maryland School of Medicine, Baltimore, MD 21228, USA*
[3]*Departamento de Biología, Facultad de Química, Universidad Nacional Autónoma de México, 04510 México, DF, Mexico*

Correspondence should be addressed to Verónica Pérez de la Cruz; veped@yahoo.com.mx

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Aging is a multifactorial degenerative process and is characterized by progressive deterioration in physiological functions and metabolic processes, changes that drive numerous age-related disorders. Within cellular alterations are found oxidative stress, inflammation, and mitochondrial dysfunction, factors that converge in the aging and conduce to cognitive decline and other pathologies. Specially, the brain is susceptible to alterations in the redox environment due its own properties (high concentrations of polyunsaturated fatty acids, high oxygen demand, and its poor antioxidant system compared with other organs). During the aging, the activity of antioxidant enzymes decreases and oxidative markers are elevated in various organs, particularly in the brain. This special issue contributes to understanding of the mechanism involved during the aging process and provides the recent findings in this paradigm.

The original research article by N. Izuo and coworkers shows us that superoxide dismutase-2 (SOD2) knockout mice have an increase in oxidative markers and alteration in the mitochondrial complex II that was associated with the development of neurodegeneration. These findings strongly suggest that SOD2 plays an important role in cellular defense against oxidative damage and could be a potential target in the aging considering that SOD2 is a mitochondrial antioxidant enzyme.

The review by M. Luca et al. is focused on the role of oxidative stress in Alzheimer's disease and vascular dementia. The authors also show the recent evidence regarding the use of antioxidant therapy and discuss new therapeutic strategies, such as the potential of inhibitors of heat shock proteins in the prevention and treatment of these pathologies.

Too many factors are involved during the aging; one of these important factors involves the mitochondrial function, which is a key for the energetic metabolism and its impairment could drive to oxidative stress and cell death. A. Schloesser and coworkers showed here that the diet with tocotrienols complexed with $\gamma$-cyclodextrin improves the mitochondrial membrane potential and ATP concentrations in the brains of aged mice. With these findings, many possibilities are open to study, which may lead to evaluating the mechanisms by which this diet improves mitochondrial parameters evaluated.

An interesting review by L. Szalárdy and coworkers also enhances the importance of mitochondrial function and describes step by step how mitochondria participate in the ROS production and the vicious circle with oxidative phosphorylation when mitochondria are injured. The authors also describe the potential therapeutic relevance of PGC-1$\alpha$ in neurological disorders.

Finally, the original research article by M. Rubio-Osornio and coworkers shows the effect of epicatechin on the oxidative and behavioral damage induced by MPP+, considering that this polyphenol possesses scavenging properties. Their data show a neuroprotective effect of epicatechin in this neurotoxic model and suggest that the protective effect may be due,

at least in part, to the induction of an increase of Cu,Zn-SOD activity.

All the items of this special issue have included an emphasis on mitochondrial function, oxidative stress during the aging, and related diseases. We need to maintain in mind that all these factors are interconnected and cannot be easily separated from each other; for this reason the manuscripts in this special issue are relevant to clarify and understand them.

*Verónica Pérez de la Cruz*
*Sathyasaikumar V. Korrapati*
*José Pedraza Chaverrí*

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*Research Article*

# Brain-Specific Superoxide Dismutase 2 Deficiency Causes Perinatal Death with Spongiform Encephalopathy in Mice

**Naotaka Izuo,[1] Hidetoshi Nojiri,[2] Satoshi Uchiyama,[3] Yoshihiro Noda,[3] Satoru Kawakami,[3] Shuji Kojima,[4] Toru Sasaki,[5,6] Takuji Shirasawa,[3,7] and Takahiko Shimizu[1,3]**

[1]*Department of Advanced Aging Medicine, Chiba University Graduate School of Medicine, Inohana, Chuo-ku, Chiba 260-8670, Japan*
[2]*Department of Orthopedics, Juntendo University Graduate School of Medicine, Bunkyo-ku, Tokyo 113-0033, Japan*
[3]*Molecular Gerontology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173-0015, Japan*
[4]*Department of Radiation Biosciences, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan*
[5]*Department of Medical Engineering and Technology, Kitasato University School of Allied of Health Science, Kitasato, Sagamihara, Kanagawa 252-0373, Japan*
[6]*Redox Biology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173-0015, Japan*
[7]*Department of Ageing Control Medicine, Juntendo University Graduate School of Medicine, Bunkyo-ku, Tokyo 113-0033, Japan*

Correspondence should be addressed to Takahiko Shimizu; shimizut@chiba-u.jp

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Oxidative stress is believed to greatly contribute to the pathogenesis of various diseases, including neurodegeneration. Impairment of mitochondrial energy production and increased mitochondrial oxidative damage are considered early pathological events that lead to neurodegeneration. Manganese superoxide dismutase (Mn-SOD, SOD2) is a mitochondrial antioxidant enzyme that converts toxic superoxide to hydrogen peroxide. To investigate the pathological role of mitochondrial oxidative stress in the central nervous system, we generated brain-specific SOD2-deficient mice (B-*Sod2*$^{-/-}$) using *nestin-Cre-loxp* system. B-*Sod2*$^{-/-}$ showed perinatal death, along with severe growth retardation. Interestingly, these mice exhibited spongiform neurodegeneration in motor cortex, hippocampus, and brainstem, accompanied by gliosis. In addition, the mutant mice had markedly decreased mitochondrial complex II activity, but not complex I or IV, in the brain based on enzyme histochemistry. Furthermore, brain lipid peroxidation was significantly increased in the B-*Sod2*$^{-/-}$, without any compensatory alterations of the activities of other antioxidative enzymes, such as catalase or glutathione peroxidase. These results suggest that SOD2 protects the neural system from oxidative stress in the perinatal stage and is essential for infant survival and central neural function in mice.

## 1. Introduction

Regulation of redox balance is essential throughout life, and dysfunction in the mechanisms underlying the redox balance is believed to be involved in various disease states, including neurodegeneration. Although it remains unclear whether oxidative stress is a major cause or merely a consequence of the cellular dysfunction associated with neurodegenerative diseases [1], accumulating evidence suggests that impaired mitochondrial energy production and increased mitochondrial oxidative damage are early pathological events that lead to neurodegeneration [2]. Superoxide ($O_2^{\bullet-}$), one of the reactive oxygen species (ROS) generated by mitochondrial respiration, is involved in a variety of biological processes in central nervous system [3]. The superoxide dismutases (SODs) are enzymes that catalyze the conversion of $O_2^{\bullet-}$ to hydrogen peroxide ($H_2O_2$) and help prevent the build-up of toxic $O_2^{\bullet-}$. Three SOD isoforms are expressed in mammalian cells: copper/zinc SOD (CuZn-SOD, SOD1), which is located in the cytoplasm [4]; manganese SOD (Mn-SOD, SOD2), which is localized in the mitochondrial matrix [5]; and extracellular SOD (EC-SOD, SOD3) [6]. Together, these

enzymes are responsible for protecting cells and tissues from ROS generated from endogenous and exogenous sources. Mitochondria are both a major source of ROS and a major target of ROS-induced cellular injury. Thus, mitochondrial SOD2 is thought to play an important role in cellular defense against oxidative damage by ROS.

In previous studies, two groups independently reported SOD2-deficient mice [7, 8]. The SOD2 deficiency on a CD1 background resulted in neonatal death by day 10 from severe dilated cardiomyopathy, liver dysfunction, and metabolic acidosis [7]. The other SOD2-deficient mice on a mixed genetic background (C57BL/6 and 129/Sv) died by day 18 with fatty liver and neuronal degeneration, particularly in the basal ganglia and brainstem [8]. Therefore, it was impossible to investigate the pathological consequences of oxidative damage in adult tissues using totally SOD2-deficient mice. The authors of these studies also argued that the phenotypes are too complex to sequester the specific aging processes in each tissue of mutant mice *in vivo*. In our previous studies, we have established several types of tissue-specific SOD2-deficient mice to define the phenotypes observed in these systemic SOD2-knockout mice [9–14]. We reported that heart/muscle-specific SOD2-deficient mice (H/M-*Sod2*$^{-/-}$) show dilated cardiomyopathy involving the excess generation of ROS by mitochondria [12, 15].

In the present study, we successfully generated brain-specific SOD2-deficient mice (B-*Sod2*$^{-/-}$) using the Cre-loxP system under the control of the nestin promoter and established a murine model for neurodegeneration induced by mitochondrial oxidative stress.

## 2. Results

*2.1. Generation of B-Sod2*$^{-/-}$. In order to investigate the physiological and pathological role of SOD2 in the brain, we generated B-*Sod2*$^{-/-}$ using the Cre-loxP system. We used nestin-Cre transgenic mice for the selective expression of Cre protein in the neurogenic lineage, such as in neurons, astrocytes, and oligodendrocytes, which are mainly in brain [16], in early prenatal stage in coordination with nestin expression from E7 [17]. As shown in Figure 1, crossbreeding homozygous SOD2-flox mice, which were set as control, with nestin-Cre transgenic mice gave rise to B-*Sod2*$^{-/-}$. Genomic DNA extracted from brain tissues was analyzed by PCR to detect the deleted fragment from the genomic SOD2 gene. Corresponding to the deletion allele, a 401 bp DNA fragment was specifically amplified by PCR from the brains of B-*Sod2*$^{-/-}$ and heterozygous mice, while no fragment was amplified in control mice (Figure 1(a)). Western blot analyses further showed a specific loss of SOD2 expression in the brain, but not in the liver, kidney, or heart of B-*Sod2*$^{-/-}$ compared with control (Figure 1(b)). The slight expression of SOD2 protein in the brains of B-*Sod2*$^{-/-}$ could be derived from nonneurogenic lineage cells, such as microglia or endothelial cells. B-*Sod2*$^{-/-}$ were born in normal Mendelian ratio and in the neonatal stage, and we were unable to find any differences in the visible appearance or body size between B-*Sod2*$^{-/-}$ and control (data not shown). However, at 10 days of age, B-*Sod2*$^{-/-}$ began to exhibit a delayed increase in body weight (Figure 1(c)), and the brain weight of B-*Sod2*$^{-/-}$ was significantly lower than that of control at the age of 16–19 days (Figure 1(d)), probably caused by growth retardation. Finally, the B-*Sod2*$^{-/-}$ began to die from 12 days of age (Figure 1(e)), and all of B-*Sod2*$^{-/-}$ died by 25 days of age, with a median survival rate of 19.6 ± 3.6 days (Figure 1(e)). These results suggest that SOD2 expression in brain is required for the postnatal survival of mice.

*2.2. B-Sod2*$^{-/-}$ *Exhibited a Distinct Spongiform Encephalopathy Associated with Gliosis.* Next, we prepared brain coronal sections from three-week-old B-*Sod2*$^{-/-}$, conducting the histochemical analysis. Hematoxylin and eosin staining revealed vacuolization similar to spongiform encephalopathy selectively in cerebral motor cortex, hippocampus, and brain stem in B-*Sod2*$^{-/-}$, but not in control (Figure 2). This abnormal vacuolization probably resulted from neuronal degeneration due to the susceptibility to oxidative stress. In addition to the vacuolization, we observed that the number of glial fibrillary acidic protein (GFAP) positive cells was increased in cerebral cortex (Figure 3) and hippocampus (data not shown) in B-*Sod2*$^{-/-}$ by immunohistochemical analysis, thus suggesting astrocyte activation. These results suggest that the biological function of SOD2 is essential for preventing neurodegeneration and astrocyte activation.

*2.3. B-Sod2*$^{-/-}$ *Showed Specific Defects of Mitochondrial Respiration.* To better understand the biochemical alterations involved in pathogenesis of the brain in the mice, we examined the mitochondrial respiratory functions in the brain of B-*Sod2*$^{-/-}$ and compared them with control. By an enzymatic histochemical analyses of succinate dehydrogenase (SDH), we assessed the biochemical activity of electron transport complex II in mitochondria on sagittal brain sections from three-week-old B-*Sod2*$^{-/-}$ and control. In the brains of B-*Sod2*$^{-/-}$, mitochondrial complex II activity was hardly detected (Figure 4(c)), while strong staining indicating SDH activity was detected in the brains of control (Figure 4(d)). We also assessed the activity of mitochondrial complexes I (NADH dehydrogenase (NADHD)) and IV (cytochrome c oxidase (COX)) in the brain (Figures 4(a), 4(b), 4(e), and 4(f)) of the mice. The enzymatic activity of mitochondrial complexes I and IV in B-*Sod2*$^{-/-}$ showed strong staining that was comparable to that in the control (Figures 4(a), 4(b), 4(e), and 4(f)). These data clearly suggest the selective loss of enzymatic activity of mitochondrial complex II in the brains of B-*Sod2*$^{-/-}$.

*2.4. Antioxidant Enzyme Activities, Glutathione Levels, and Lipid Peroxidation Levels.* To investigate whether there was a compensatory mechanism for SOD2 deficiency, we measured the activities of antioxidant enzymes in the tissues from B-*Sod2*$^{-/-}$. Although the SOD2 activity was completely depleted, the compensative increase in SOD1 activity was

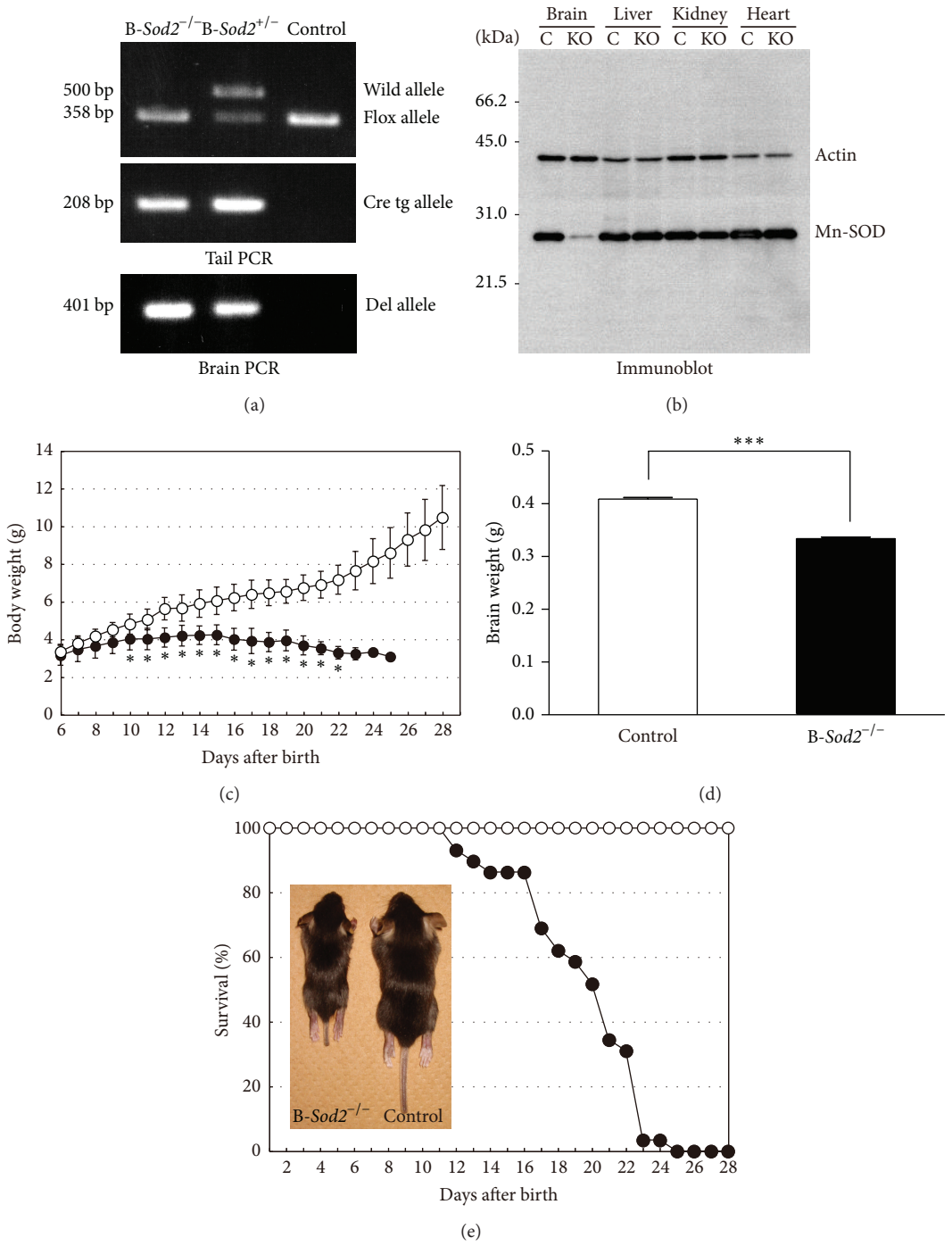


FIGURE 1: Generation of B-*Sod2*$^{-/-}$. (a) DNA fragments amplified from the wild (500-bp) or lox allele (358-bp) were detected in tail DNA. The Cre transgene was confirmed by PCR with Cre primers. A 208 bp DNA fragment was detected in knockout and heterozygous mice using Cre PCR primers. A 401 bp DNA fragment amplified from the deletion allele was detected in the brains of knockout and heterozygous mice. (b) The results of Western blot analysis of SOD2 protein in two-week-old B-*Sod2*$^{-/-}$ and control. Protein extracts from the brain, liver, kidney, and heart of B-*Sod2*$^{-/-}$ or control were immunoblotted with anti-SOD2 or anti-actin antibodies. (c) The growth rate of body weight increase in B-*Sod2*$^{-/-}$ ($n = 24$), indicated by closed circle, progressively decreased compared with control ($n = 29$, $^{*}P < 0.05$), indicated by open circle. (d) The brain weight of B-*Sod2*$^{-/-}$ at 16–19 days of age ($n = 24$) was significantly lower than that of brains from age-matched control ($n = 22$, $^{***}P < 0.005$). (e) The cumulative survival of B-*Sod2*$^{-/-}$ ($n = 29$), indicated by closed circle, and control ($n = 29$), indicated by open circle.

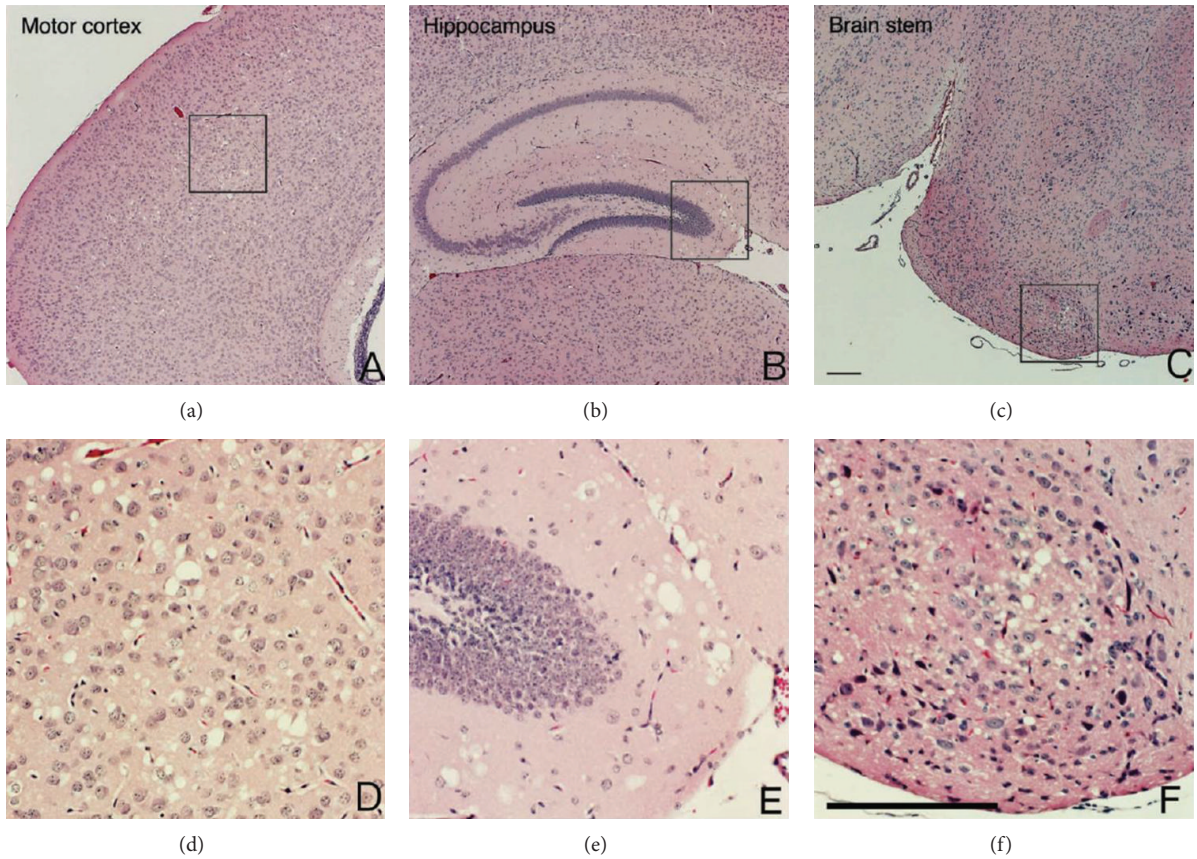


FIGURE 2: B-*Sod2*$^{-/-}$ exhibited distinct spongiform encephalopathy in the brain. (a–f) Coronal sections of brains from three-week-old B-*Sod2*$^{-/-}$. Motor cortex (a, d), hippocampus (b, e), and brain stem (c, f). A large number of vacuoles were morphologically observed in B-*Sod2*$^{-/-}$. Higher power views (inset) showed neuronal degeneration and malformation with cytoplasmic vacuolization, as well as pleomorphic nuclei (d, e, and f). The scale bars indicate 200 $\mu$m.

minimal (Figures 5(a) and 5(b)). We next measured the activities of $H_2O_2$ reductases, such as catalase and glutathione peroxidase (GPx), as well as the levels of glutathione, an endogenous antioxidative substance, but found no significant change in the activities of these enzymes or in the levels of the antioxidants (Figures 5(c)–5(e)). We also measured the amount of malondialdehyde (MDA) as a marker of lipid peroxidation by $O_2^{\bullet-}$ in brain lysates. A higher level of MDA was detected in brain extracts from B-*Sod2*$^{-/-}$ than that from control (Figure 5(f)), indicating that oxidative stress-associated damage accumulated in the brain as a result of SOD2 deficiency.

## 3. Discussion

Deregulation of the redox balance is thought to be involved in aging and in the pathogenesis of various diseases. We recently reported that brain slices from global SOD2-knockout mice exhibited increased amount of ROS after hypoxia-reoxygenation stress, suggesting that SOD2 plays a central role in ROS elimination in *ex vivo* model [18]. In order to elucidate the significance of SOD2 in redox homeostasis *in vivo*, many studies on SOD2-deficient mice have been ongoing in our lab and by other groups. In previous studies, two groups independently developed SOD2-deficient mice, and both lines were reported to show perinatal death, suggesting the physiological and pathological importance of SOD2 [7, 8, 19, 20]. So far, although several lines of tissue or cell-type specific SOD2-knockout mice generated by means of Cre-loxP system have been analyzed [9–14], the cause(s) of the early-stage death was unclear. Since SOD2-deficient mice were reported to manifest lipid accumulation and atypical patterns of glycogen deposition in the liver [7, 8], we generated liver-specific SOD2-knockout mice (liver-*Sod2*$^{-/-}$) by crossbreeding with SOD2-flox mice and albumin-Cre transgenic mice, in which the Cre transgene is controlled by the enhancer/promoter of the albumin gene so that Cre recombinase is specifically expressed in hepatocytes [9, 10]. Unexpectedly, however, liver-*Sod2*$^{-/-}$ did not show any obvious morphological changes or early death [9, 10]. Next, we generated and analyzed H/M-*Sod2*$^{-/-}$, in which SOD2 was selectively depleted in muscle tissues. H/M-*Sod2*$^{-/-}$ exhibited cardiac enlargement similar to the total SOD2-deficient mice and die by 6 months of age [12]. However, the median survival was about 4 months [12], which was longer than the life span of SOD2 totally depleted mice. Furthermore, we

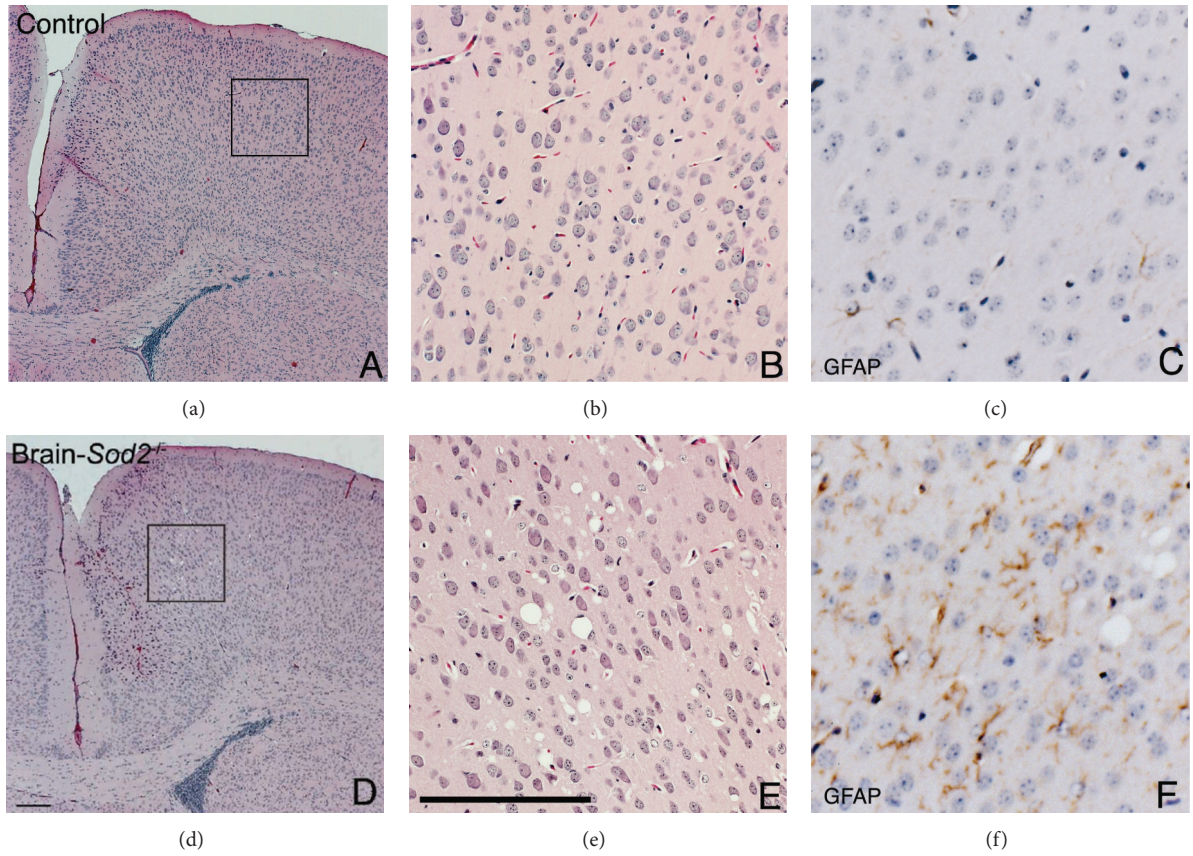


Figure 3: Gliosis was detected in the cortex of B-*Sod2*$^{-/-}$. Coronal sections of the brains from three-week-old control (a, b) and B-*Sod2*$^{-/-}$ (d, e) with hematoxylin and eosin staining. Higher power views (inset) showed neuronal vacuolization in B-*Sod2*$^{-/-}$ (e) but not in control (b). Immunohistochemical staining of sections with anti-GFAP antibody (c and f) revealed enhanced gliosis in B-*Sod2*$^{-/-}$ (f) but not in control (c). The scale bars indicate 200 $\mu$m.

produced skeletal muscle-specific SOD2-knockout utilizing Cre-loxP system under the control of human skeletal actin promoter [13]. These mutant mice exhibited no shortening of life span in spite of severe disturbance in their exercise activity [13]. Although SOD2 total knockout mice showed severe phenotypes in the liver, heart, and skeletal muscle, our findings suggested that there is no direct involvement of SOD2 depletion in these tissues in perinatal death. In this study, we generated B-*Sod2*$^{-/-}$ by using nestin-Cre transgenic mice, in which Cre protein is selectively expressed in neurogenic lineage [16]. We found that B-*Sod2*$^{-/-}$ exhibited perinatal death, reproducing the phenotype of the total knockout mice. This suggests that SOD2 in the brain is essential for perinatal survival via protection against oxidative stress.

However, the mechanisms of perinatal death induced by brain-specific SOD2 deletion have not been understood. It is possible that the spongiform encephalopathy observed in B-*Sod2*$^{-/-}$ brain could cause early death. For example, in cases of prion disease, including bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease (CJD) in humans, spongiform neurodegeneration was observed, which was followed by death [21, 22]. In a genetically manipulated animal model, Attractin (Atrn) or Mahogunin mutant mice also developed hippocampal progressive spongiform degeneration, but this was not fatal [23, 24]. The involvement of spongiform neurodegeneration in death is controversial. In the brains of B-*Sod2*$^{-/-}$, spongiform neuronal loss was observed in restricted area, such as brain stem, cerebral cortex, and hippocampus. Among these regions, brain stem includes the central control for autonomic nervous system. Therefore, spongiform neurodegeneration in brain stem might cause critical dysfunction of autonomic nervous system, leading to perinatal death. However, we cannot exclude the possibility that spongiform encephalopathy is unrelated to the death of the mice. Several studies on heterozygote SOD2-knockout mice, which do not exhibit neuronal loss, showed impaired neuronal transduction [25]. This suggests that the oxidative stress induced by SOD2 deficiency may lead to neuronal dysfunction in some region(s) that are indispensable for survival. The production and analysis of region-specific or stage-specific SOD2 conditional knockout mice could provide a powerful tool for detailed investigations of the mechanism(s) underlying the perinatal death induced by B-*Sod2*$^{-/-}$.

B-*Sod2*$^{-/-}$ were born in normal Mendelian ratio and exhibited no abnormalities in visible appearance, suggesting

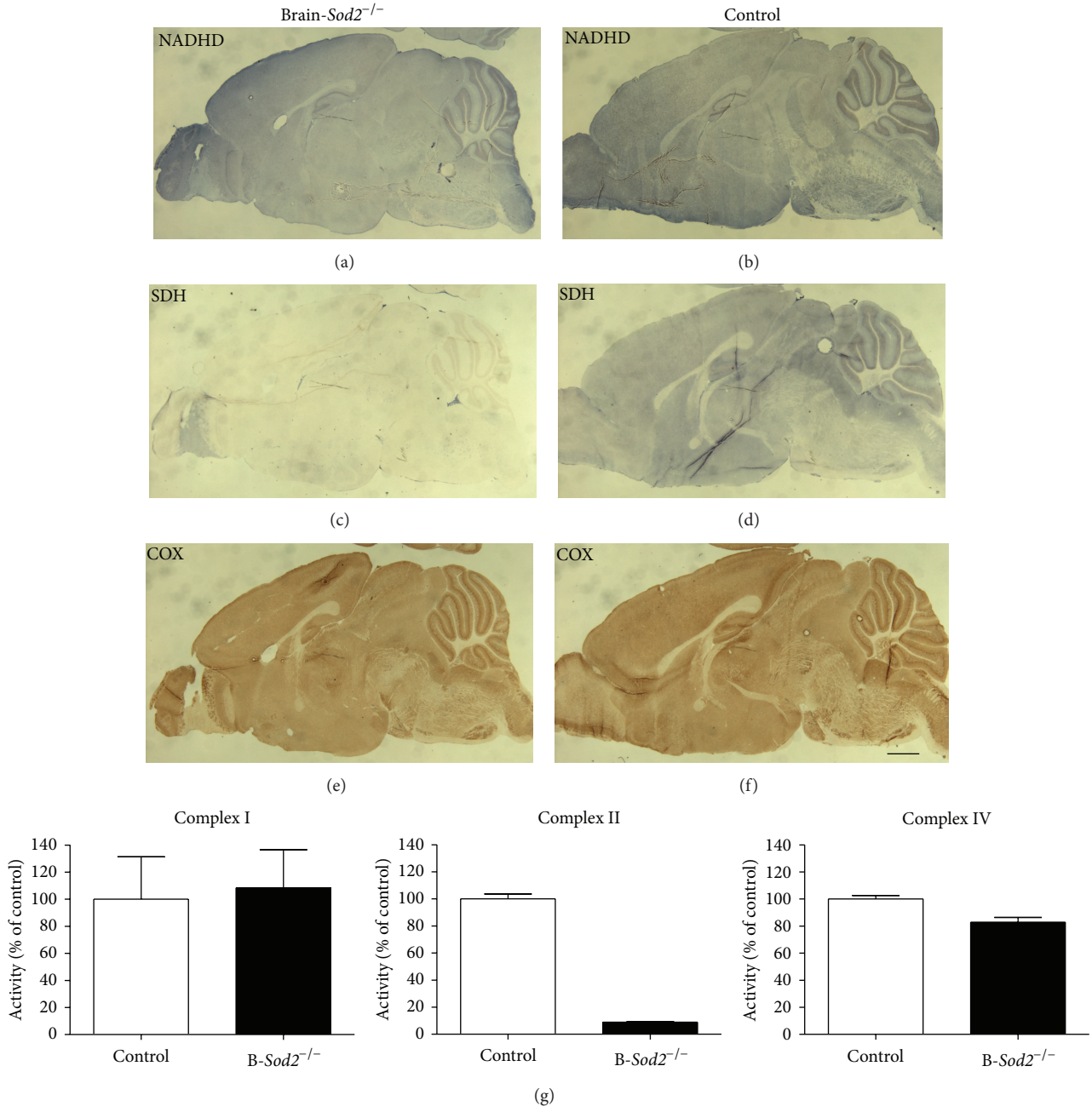


Figure 4: Impaired mitochondrial respiratory activities. Enzymatic histochemical staining for NADHD (top panels) (a, b), SDH (middle panels) (c, d), and COX activities (bottom panels) (e, f) in sagittal sections of brain from three-week-old mice of the indicated genotypes. The scale bars indicate 1 mm. (g) Quantification of enzymatic reactivities of mitochondrial complexes I, II, and IV.

that SOD2 deficiency in brain has no influence on prenatal or neonatal stage. This could be because oxygen supply for infants through placenta is of lower level than that through respiration after birth. Although B-*Sod2*$^{-/-}$ exhibited severe spongiform degeneration in the brain, no structural abnormalities were observed. This suggests that SOD2 in the brain plays essential roles in the perinatal stage, but not in the early developmental stage. Interestingly, neuronal degeneration was observed in selected regions, such as cerebral motor cortex, hippocampus, and brain stem, although mitochondrial dysfunction resulting from SOD2 deletion was observed in the whole brain. Misawa et al. previously generated postnatal motor neuron-specific SOD2-knockout mice by crossbreeding SOD2-flox mice and VAChT-Cre transgenic mice, in which Cre expression is restricted to the postnatal somatomotor neurons [11]. Compared to B-*Sod2*$^{-/-}$, motor neuron-specific SOD2-knockout mice showed the loss of SOD2 expression specifically in a subset of somatomotor neurons and, surprisingly, showed no reduction of the number of neurons up to one year

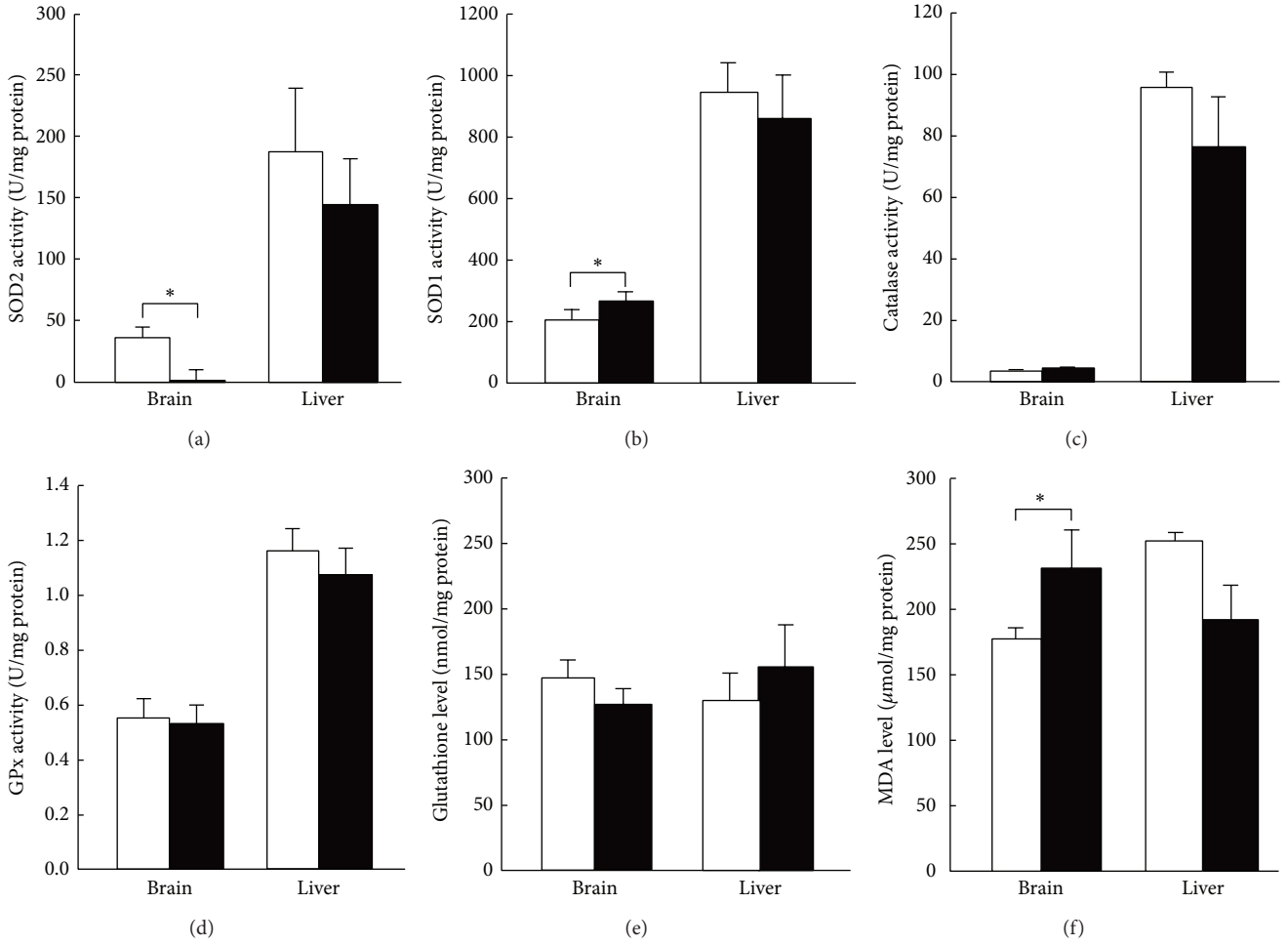


FIGURE 5: The antioxidative enzyme activities and glutathione and lipid peroxidation levels in B-*Sod2*$^{-/-}$ indicated by closed column, and control, indicated by open column. (a) SOD2, (b) SOD1, (c) catalase, (d) GPx activities, (e) glutathione levels, and (f) MDA levels were measured in brain and liver lysates of B-*Sod2*$^{-/-}$ ($n = 5$) and control ($n = 5$) at 3 weeks of age. White and black columns indicate control and B-*Sod2*$^{-/-}$, respectively ($^{*}P < 0.05$).

after birth, even though extensive histological examinations were performed [11]. These data suggested that postnatal motor neurons are resistant to mitochondrial oxidative stress. On the other hand, we found that dopaminergic neuron-specific SOD2-deficient mice, generated by crossbreeding SOD2-flox mice and tyrosine hydroxylase (TH) Cre mice, exhibited severe loss of dopaminergic neurons accompanied by motor dysfunction (unpublished results). Dopaminergic neurons are highly sensitive to oxidative stress, because these neurons contain reactive quinone, which is converted from intrinsic dopamine through autooxidation [26, 27]. Together, these findings suggest that regional selectivity of spongiform neurodegeneration in B-*Sod2*$^{-/-}$ reflects the local vulnerability to mitochondrial superoxide.

Since abnormal glioses were observed in the brains of B-*Sod2*$^{-/-}$, there is a possibility that astrocyte activation induced neurodegeneration. Astrocytes have more potent defense systems against oxidative stress, including NF-E2-related factor 2- (Nrf2-) antioxidant response element (ARE) pathway, than neurons [28]. Astrocytes that are exposed to oxidative stress at physiological levels activate Nrf2-ARE pathway to increase the productions of antioxidants, such as glutathione [28]. Glutathione generated from astrocytes protects not only astrocytes themselves, but also neurons as a secreted protective mediator [28]. On the other hand, under the pathological conditions, such as exposure to excessive levels of oxidative stress or exposure to amyloid $\beta$, astrocytes are activated and produce neurotoxic cytokines [29]. In the B-*Sod2*$^{-/-}$, pathological levels of oxidative stress could induce astrocyte activation and the subsequent secretion of large amounts of toxic cytokines, thus damaging neurons. Furthermore, Liang et al. reported that SOD2 heterozygous knockout mice exhibited epilepsy via astrocyte dysfunction [30]. They showed that a reduction of the SOD2 level induced the downregulation of glutamate transporter 1 (GLT-1) and glutamate-aspartate transporter (GLAST), both of which are glutamate transporters that clear glutamate from synapse, which increased the levels of glutamate at the synapse and led to epileptic conditions [30]. This is in agreement with the evidence that patients suffering from genetic mitochondrial

disease often have epilepsy. In the brains of B-*Sod2*$^{-/-}$, the potently downregulated expression of GLT-1 and GLAST could induce a resultant increase in the glutamate concentration at synapse, leading to excitotoxicity.

In this study, the activity of mitochondrial complex II was dramatically decreased by SOD2 deficiency, in spite of the fact that there were no obvious changes in the activities of complex I or IV in the brain. Importantly, the selective downregulation of SDH activity has also been observed in cardiac and somatic muscle tissues in H/M-*Sod2*$^{-/-}$ [12, 13], suggesting that mitochondrial complex II plays a role as an oxidative sensor localized on electron transport system, as well as citrate cycle, considering that SDH couples electron transport system and citrate cycle. Of note, SOD2 deficiency induced only weak levels of compensatory activity in the antioxidant system. These results suggest that most of the mitochondrial oxidative stress is sequestrated in mitochondria and only a little of it leaks to the cytoplasm and is cleared by SOD1, which is localized in cytosol.

## 4. Experimental Section

*4.1. Animals.* The generation of SOD2-flox mice was described previously [9]. The SOD2-flox mice were backcrossed to C57BL6/CrSlc mice for five or six generations. In order to investigate the physiological role of SOD2 in the central nervous system, we crossbreed SOD2-flox mice with nestin-Cre transgenic mice (Jax #3771, The Jackson Laboratory, Bar Harbor, USA). In nestin-Cre transgenics, the Cre transgene is controlled by the promoter and intronic enhancer elements of the rat nestin gene, so that Cre recombinase is specifically expressed in embryonic neural progenitors [16]. The crossbreeding of SOD2-flox mice with nestin-Cre mice gave rise to B-*Sod2*$^{-/-}$. Genotyping of the Cre transgene and the SOD2-flox allele was performed by PCR using genomic DNA isolated from the tail tip. The primers used to identify carriers of the nestin-Cre transgene were 5′-TTC CAG CTA GAG AGA CTG AGT CCC-3′ and 5′-TCG ACC AGT TTA GTT ACC C-3′ and those used to identify SOD2-flox allele were 5′-TTA GGG CTC AGG TTT GTC CAG AA-3′, 5′-CGA GGG GCA TCT AGT GGA GAA-3′, and 5′-AGC TTG GCT GGA CGT AA-3′. The deleted alleles were confirmed as described in a previous study [9]. Mice were maintained and studied according to protocols approved by the Animal Care Committee of the Tokyo Metropolitan Institute of Gerontology.

*4.2. Western Blot Analysis.* Western blot analysis was performed with brain, liver, kidney, and heart homogenates (5 $\mu$g each) as previously described [9, 10]. Antibodies against SOD2 (1 : 10,000; #SOD-111, Enzo Lifesciences (StressGen), Victoria, Canada) and actin (1 : 100; #A2066, Sigma-Aldrich, St. Louis, MO, USA) were used.

*4.3. Histological and Histochemical Studies.* The organs were dissected, fixed in a 20% formalin neutral buffer solution (Wako, Osaka, Japan) overnight, embedded in paraffin, and sectioned on a microtome following standard techniques. Hematoxylin and eosin staining was performed as described previously [12]. Immunohistochemical staining of brain sections was performed with anti-GFAP antibody (#G9269, Sigma-Aldrich) and VECTASTAIN ABC Elite kit (#PK-6101, Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol.

For enzymatic histochemical staining, tissues were frozen in isopentane in liquid nitrogen and embedded in OTC compound on dry ice. Sections were cut into 8 $\mu$m thick sections and were mounted on silane-coated slide glasses. Frozen sections were dried and incubated in 0.1 M Tris-Cl (pH 7.4), 1 mg/mL nitro blue tetrazolium, and 0.1 mg/mL $\beta$-NADH to assess NADHD (mitochondrial complex I) activity; 50 mM phosphate buffered saline (PBS) (pH 7.4), 84 mM succinate acid, 0.2 mM phenazine methasulfate, 2 mg/mL nitro blue tetrazolium, and 4.5 mM EDTA to assess SDH (mitochondrial complex II) activity; or 50 mM PBS (pH 7.4), 1.0 mg/mL 3,3′-diaminobenzidine, 24 U/mL catalase (Wako), 1 mg/mL cytochrome *c* (Wako), and 75 mg/mL sucrose to assess COX (mitochondrial complex IV) activity. These incubations were performed in the dark at room temperature for 20 min. Quantification was performed by image analyzing software, Leica QWin V3.

*4.4. Determination of SOD, Catalase, GPx Activities, and Glutathione Content.* Tissues from two-week-old mice were homogenized in 0.1 M PBS, pH 7.5, containing 5 mM EDTA, and were centrifuged at 15,000 ×g for 30 min. The supernatant was used to assess the antioxidant enzyme activities. SOD activity was measured by NBT assay [31]. The enzymatic activity was expressed in U/mg of protein. To determine SOD2 activity, lysates were treated with 1 mM KCN to inactivate SOD1. The protein concentration was measured by Lowry method using BSA as a standard. For catalase activity assay, the supernatant was incubated with 9 mM $H_2O_2$, 0.25 mM EDTA, 50 mM Tris-HCl (pH 7.5), and 50 mM PBS at room temperature. Catalase activity was measured by the decrease in $H_2O_2$ absorbance at 240 nm and was expressed as U/mg protein. For GPx activity assay, the supernatant was incubated with 1 U/mL glutathione reductase, 2 mM glutathione, 0.2 mM NADPH, 70 $\mu$M t-butyl hydroperoxide, 0.05 mM EDTA, and 10 mM Tris-HCl (pH 7.5) at room temperature. GPx activity was measured by the decrease of NADPH absorbance at 340 nm and was expressed as U/mg protein.

To determine the glutathione content, the supernatant was added with an equal volume of 10% trichloroacetic acid (TCA) to remove acid-insoluble materials. The acid-soluble fraction was extracted with ether to remove residual TCA. The fraction (25 $\mu$L) was mixed with 250 $\mu$L of 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 733 $\mu$L of 0.3 mM NADPH, and 10 $\mu$L of 2 U/mL glutathione reductase. The rate of change in the absorbance was measured at 412 nm. The glutathione concentration was calculated as nmol/mg protein.

*4.5. Measurement of Lipid Peroxidation.* Lipid peroxidation in tissues homogenates was measured as MDA by the

thiobarbituric assay [32]. The MDA level of homogenates was calculated as $\mu$mol/mg protein.

*4.6. Statistical Analysis.* We analyzed the data using the unpaired $t$-test and considered $P$ values $< 0.05$ to be statistically significant. The data are expressed as the means plus or minus SD.

## 5. Conclusions

SOD2 null mice have been known to have short survival. However, it was not clear why they died during the perinatal phase. Our findings suggest that SOD2 protects the neural system from postpartum ROS injuries in neonates, and SOD2 has essential roles in perinatal survival and in the central neural functions in mice. Furthermore, we propose that spongiform encephalopathy, along with inflammation, is the major cause of death in B-*Sod2*$^{-/-}$, and the activity of neuronal SOD2 could not be compensated for by other antioxidative systems. These results suggest that accumulation of intrinsic ROS produced from mitochondria induces severe damage to neuronal system.

## Conflict of Interests

The authors declare no conflict of interests.

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*Review Article*

# Electron Transport Disturbances and Neurodegeneration: From Albert Szent-Györgyi's Concept (Szeged) till Novel Approaches to Boost Mitochondrial Bioenergetics

**Levente Szalárdy,[1] Dénes Zádori,[1] Péter Klivényi,[1] József Toldi,[2,3] and László Vécsei[1,3]**

[1]*Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Center, University of Szeged, Semmelweis u. 6, Szeged 6725, Hungary*
[2]*Department of Physiology, Anatomy and Neuroscience, University of Szeged, Közép Fasor 52, Szeged 6726, Hungary*
[3]*MTA-SZTE Neuroscience Research Group, Semmelweis u. 6, Szeged 6725, Hungary*

Correspondence should be addressed to László Vécsei; vecsei.laszlo@med.u-szeged.hu

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Impaired function of certain mitochondrial respiratory complexes has long been linked to the pathogenesis of chronic neurodegenerative disorders such as Parkinson's and Huntington's diseases. Furthermore, genetic alterations of mitochondrial genome or nuclear genes encoding proteins playing essential roles in maintaining proper mitochondrial function can lead to the development of severe systemic diseases associated with neurodegeneration and vacuolar myelinopathy. At present, all of these diseases lack effective disease modifying therapy. Following a brief commemoration of Professor Albert Szent-Györgyi, a Nobel Prize laureate who pioneered in the field of cellular respiration, antioxidant processes, and the roles of free radicals in health and disease, the present paper overviews the current knowledge on the involvement of mitochondrial dysfunction in central nervous system diseases associated with neurodegeneration including Parkinson's and Huntington's disease as well as mitochondrial encephalopathies. The review puts special focus on the involvement and the potential therapeutic relevance of peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1$\alpha$), a nuclear-encoded master regulator of mitochondrial biogenesis and antioxidant responses in these disorders, the transcriptional activation of which may hold novel therapeutic value as a more system-based approach aiming to restore mitochondrial functions in neurodegenerative processes.

## 1. The Man Amused by the Dance of Electrons

> *"The fuel of life is electron, or more exactly, the energy it takes over from photons in photosynthesis, and gives up gradually while flowing through the cellular machinery."*

This imagination originates from Professor Albert Szent-Györgyi, a Hungarian physician and biochemist, former chair of the Department of Medical Chemistry and the Department of Organic Chemistry at the University of Szeged from 1930 and 1935, respectively, until the end of World War II in 1945. His early research activities in Groningen and later in Cambridge conducted on biological combustion, cellular respiration, and energy production of plants lead to the discovery of a reducing substance called "hexuronic acid," a substance that is able to lose and regain hydrogen atoms and capable of protecting plants from "browning," an injury that he characterized as oxidative damage due to the excessive activity of an enzyme, peroxidase. This "antioxidant" substance was later proved by Szent-Györgyi to be equivalent with a potent antiscorbutic (antiscurvy) agent and was given the name ascorbic acid, currently widely known as vitamin C, which is most abundant in citrus fruits and paprika, an emblematic vegetable of Szeged. At this time, performing ongoing research in the field of biological respiration, Szent-Györgyi discovered and identified the catalysis of fumaric acid among other steps of the tricarboxylic acid cycle (also referred to as Szent-Györgyi–Krebs cycle, citric acid cycle), an essential component of cellular respiration

that provides reducing equivalents for terminal oxidation and thereby energy production from metabolic products of dietary macromolecules. "*For his discoveries in connection with the biological combustion processes, with special reference to vitamin C and the catalysis of fumaric acid,*" Albert Szent-Györgyi was awarded Nobel Prize in Physiology or Medicine in 1937. In addition to his pioneering work in muscle research—including the discovery of actin and myosin proteins and the mechanism of their joint function—as well as the discovery of vitamin P (flavanone), his subsequent research interests focused on the interactions of proteins and free radicals and their role in regulating cell division and cancer development, and he published a number of books and papers about his findings and scientific theories of bioenergetics and bioelectronics and their roles in health and disease.

Following the imaginations of our honored predecessor, this paper reviews the concepts on the role of impairments in mitochondrial respiration and subsequent excessive oxidation in degenerative central nervous system (CNS) disorders, with special attention to recent findings related to alterations in transcriptional regulation of mitochondrial biogenesis and bioenergetics, and their potential therapeutic relevance.

## 2. Mitochondrial Respiration: The Proper Function of a Dangerous System

Mitochondria are membrane-bound intracellular organelles evolutionary originating from the endosymbiosis of an ancient aerobic alpha-proteobacterium with an early eukaryotic host cell [1]. Harboring their own maternally inherited, double-stranded, circular genome (mtDNA), supplemented by the presence of several ancillary, structural, and regulatory proteins encoded by the nuclear DNA (nDNA), mitochondria host a number of molecular processes essential for cellular life and death. These include processes related to the production of biologically utilizable energy, adaptive thermogenesis via the uncoupling of energy production, as well as the regulation of cellular calcium homeostasis, cell-cycle, and programmed cell death.

Energy production in the mitochondria is performed through the coupled function of pyruvate dehydrogenase complex (PDC), $\beta$-oxidation (processes essential in glycolytic and ketogenic metabolism, resp.), the Szent-Györgyi–Krebs cycle, and the terminal oxidation and oxidative phosphorylation (OXPHOS). While PDC, $\beta$-oxidation, and the Szent-Györgyi–Krebs cycle take place within the mitochondrial matrix, terminal oxidation and OXPHOS are linked to the function of respiratory complexes I–V (electron transport chain, ETC) embedded in the inner mitochondrial membrane. The process of energy production has been extensively reviewed elsewhere [2, 3]. Briefly, glucose is catabolized in the cytosol to yield pyruvate through multiple enzymatic steps of the glycolysis, which is then translocated into the mitochondria and metabolized to acetyl-coenzyme A (acetyl-CoA) by PDC. On the other hand, fatty acids are oxidized during the $\beta$-oxidation to acetyl-CoA entirely within the mitochondria (or in case of longer-chain fatty acids, initially within the peroxisomes). Mitochondrial acetyl-CoA subsequently enters the Szent-Györgyi–Krebs cycle to form reduced coenzyme NADH and succinate in multiple steps, which in turn provide electrons for respiratory complex I (NADH dehydrogenase) and complex II (succinate-ubiquinone oxidoreductase), respectively, using FMN and FAD as prosthetic groups, respectively. The electrons are then transported from both complexes via the mobile carrier coenzyme Q (ubiquinone) to complex III (ubiquinol-cytochrome *c* oxidoreductase) and flow through cytochrome *c* to reach complex IV (cytochrome *c* oxidase) to be oxidized by the final electron acceptor, oxygen. Notably, reducing equivalents produced during the glycolysis (in form of NADH) can also be translocated to the mitochondria via the malate/aspartate shuttle and the glycerol phosphate shuttle to provide NADH and $FADH_2$, respectively, which in turn, similarly to NADH and $FADH_2$ produced during $\beta$-oxidation, feed the ETC at complex I and coenzyme Q, respectively. Within the ETC, respiratory complexes are arranged in an electrochemical order, corresponding to their gradually increasing redox potential and electronegativity. The flow of electrons through the respiratory complexes provides energy used to pump out protons through complexes I, III, and IV (cytochrome *c* oxidase) to the intermembrane space. At the end of the downstream flow of electrons, molecular respiratory oxygen as the final electron acceptor is reduced by complex IV to form water molecule in a process known as terminal oxidation. The release of protons from the matrix develops a gradient of protons between the matrix and the intermembrane space also referred to as mitochondrial membrane potential (negative inside) as well as an electrochemical gradient (alkaline inside). Being impermeable to protons, the inner membrane works as a capacitator and an electric insulator. Therefore, the electrochemical drive to equalize the concentration of protons can be satisfied by the reentry of protons through respiratory complex V ($H^+$-ATP synthase), the subsequent activation of which leads to the formation of ATP from ADP in a process called OXPHOS. The produced ATP represents a biologically available form of electrochemical energy, serving as the main energy-provider for eukaryotic cells (Mitchell's chemiosmotic hypothesis) [4]. The energy stored in mitochondrial membrane potential (also known as "proton motive force") can also be utilized to generate heat or to import calcium or proteins into the mitochondrion via uncoupling the transport of electrons from ATP production, which processes serve adaptive purposes. The proportion of dietary calories burnt within the mitochondrion and allocated to energy production is referred to as "coupling efficiency."

In multicellular organisms, the ability to adaptively regulate and activate mitochondrial biogenesis and functions in response to a variety of conditions is essential to maintain energetic homeostasis and cellular viability. Several lines of evidence obtained in the past decade suggest that peroxisome proliferator-activated receptor-gamma (PPAR$\gamma$) coactivator 1-alpha (PGC-1$\alpha$), a nuclear-encoded coactivator of a wide range of transcriptional factors, plays a key role in the transcriptional cascade of such adaptive processes. PGC-1$\alpha$-mediated coactivation of genes such as nuclear respiratory

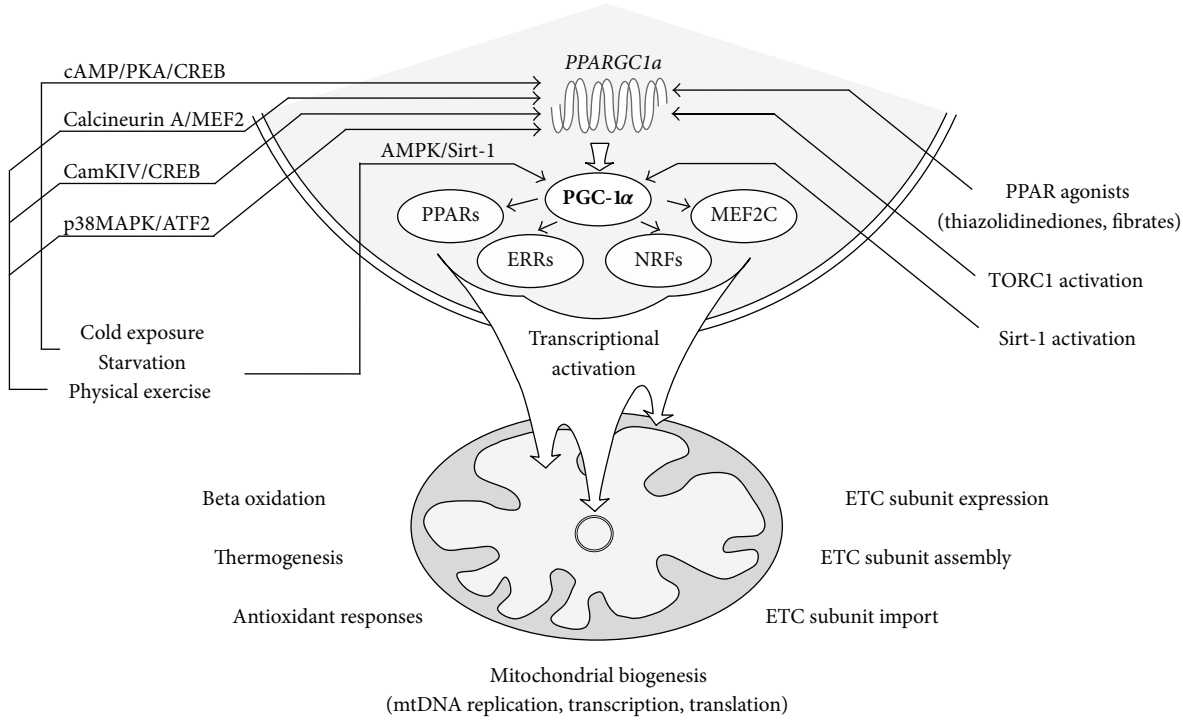


Figure 1: The PGC-1$\alpha$ cascade. Transcriptional upregulation or posttranslational activation of PGC-1$\alpha$ due to fasting, physical exercise, cold exposure, or pharmacological manipulations leads to the transcriptional activation of several nuclear-encoded proteins involved in mitochondrial functioning at multiple levels, including mitochondrial biogenesis, adaptive metabolism, antioxidant responses, and proper ETC assembly/import.

factor 1 and 2 (NRF-1, -2), PPARs, estrogen-related receptors (ERRs), and myocyte-specific enhancer factor 2C (MEF2C) leads to an increased expression of a wide range of proteins involved in mitochondrial transcription, replication, and the import and assembly of a number of nuclear-encoded respiratory complex subunits; furthermore, it boosts OXPHOS and thermoregulation in a tissue-dependent manner, enhances gluconeogenesis and fatty acid oxidation [5], and increase oxidative stress defense [6] (Figure 1). The inducing effect of *physical exercise* (mediated by calcineurin A-linked MEF2 activity, calcium/calmodulin-dependent protein kinase IV- (CaMKIV-) linked cyclic AMP (cAMP) response element-binding protein (CREB) activity, and a p38 mitogen-activated protein kinase- (MAPK-) linked activating transcription factor 2 (ATF-2) activity), *cold exposure* and *starvation* (mediated by catecholamine- and glucagon-induced cAMP elevation and a subsequent phosphorylation and activation of CREB by protein kinase A (PKA)) on PGC-1$\alpha$ expression is well documented [5]. Furthermore, *energy deprivation* through a high AMP/ATP ratio leads to an increased AMP-activated protein kinase (AMPK) activity and a subsequent phosphorylation of PGC-1$\alpha$ protein, priming PGC-1$\alpha$ for subsequent deacetylation and thereby activation by silent information regulator 2 homolog 1 (Sirt-1) [7, 8], the expression of which is also increased in conditions with energy shortage, such as, starvation or exercise, due to a high $NAD^+$/NADH ratio [9]. These posttranslational modifications on PGC-1$\alpha$ play pivotal roles in adaptive mitochondrial biogenesis. The roles of impaired mitochondrial function and more recently a decreased function of the PGC-1$\alpha$ cascade in the pathogenesis of degenerative CNS disorders are of extensive research interest.

## 3. Mitochondrial Dysfunction, Reactive Oxygen, and Nitrogen Species

Free radicals are molecules possessing unpaired electrons in their outer orbit. This renders them highly reactive towards organic macromolecules such as carbohydrates, nucleic acids, proteins, and lipids, which suffer "injury" during such a reaction. The main routes of free radical production and subsequent toxic insults are represented in a schematic depiction in Figure 2. Under physiological conditions, the efficiency of reducing oxygen during terminal oxidation is approximately 97–99%, while 1–3% undergo incomplete reduction to superoxide ($O_2^{\bullet -}$), a highly reactive free radical. $O_2^{\bullet -}$ can be transformed into hydrogen peroxide ($H_2O_2$) both spontaneously and through a reaction catalyzed by mitochondrial manganese superoxide dismutase (Mn-SOD) in the matrix. $H_2O_2$ normally undergoes degradation by glutathione peroxidase (GPX) and catalase (CAT) enzymes, yielding water. In case of an impaired function of the mitochondrial ETC, leakage of excess electrons from complexes I and III leads to a higher amount of $O_2^{\bullet -}$ and subsequent $H_2O_2$ production, which when exceeding the degradative capacity of the mitochondria can be transformed into the extremely toxic hydroxyl radical ($HO^{\bullet}$), through reaction with transition metals ($Fe^{2+}$ and $Cu^{2+}$; Fenton reaction).

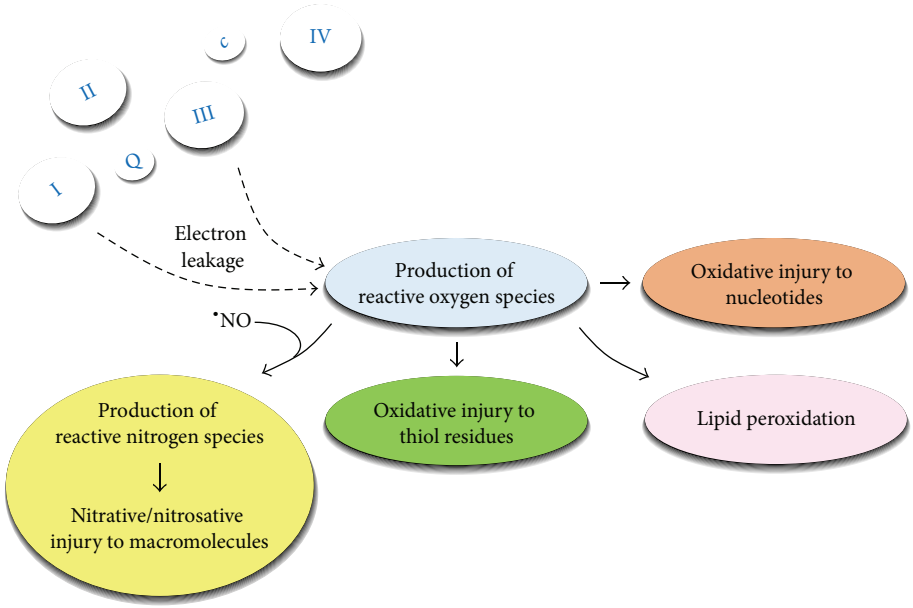


Figure 2: Schematic representation of the generation and the effects of free radicals within the mitochondria. The leakage of electrons from the mitochondrial ETC at complexes I and III results in the formation of superoxide anion. The high reactivity of this molecule evokes a harmful cascade mechanism including the formation of reactive oxygen and nitrogen species. The cascade mechanism deteriorates the functional groups of major components of all kinds of biomolecules (carbohydrates, lipids, proteins, and nucleic acids). In case of pronounced electron leakage or deficient antioxidant protection, a vicious circle of mitochondrial dysfunction develops.

$HO^{\bullet}$ in turn can react with nucleic acids and phospholipids, yielding the formation of further toxic radicals and consequent severe functional impairments of the affected macromolecules. Furthermore, $HO^{\bullet}$ can also react with thiol residues, yielding the formation of multiple reactive free radicals. In addition, $O_2^{\bullet-}$ can also react with nitric oxide ($NO^{\bullet}$) generated from L-arginine by mitochondrial nitric oxide synthase (mtNOS) enzyme [10]; however, other sources of mitochondrial $NO^{\bullet}$ associated with nitrite reductase activity under hypoxic conditions have also been identified [11–13]. The reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$ yields the highly toxic peroxynitrite anion ($ONOO^-$) in a reaction that is three times as fast as the dismutation of $O_2^{\bullet-}$ by Mn-SOD. $ONOO^-$ can evoke injury to proteins via nitration of tyrosine, tryptophan, and phenylalanine residues by nitronium ion ($NO_2^+$) generated through metal-peroxynitrite ($ONOO^-Me^nX$) complexes. Nitration of tyrosine can also be achieved by serial reactions of further pathways involving $ONOO^-$ and $NO^{\bullet}$ (see Figure 3 for detailed description). Nitrosylation of thiol, secondary amine, alcohol, and alkane residues are performed via their reaction with nitrosonium ion ($NO^+$) or indirectly with dinitrogen trioxide ($N_2O_3$), highly reactive radicals also derived from $NO^{\bullet}$. Furthermore, $NO^{\bullet}$ can directly impair the mitochondrial ETC and OXPHOS via competing with oxygen for the oxygen-binding site of complex IV [14], which leads to a further increase in free radical production.

The toxic radicals described above are known as reactive oxygen species (ROS) and reactive nitrogen species (RNS), and their damaging effects on macromolecules are referred to as oxidative and nitrative/nitrosative stress, respectively. Figure 3 presents a detailed overview on the molecular background of toxic processes related to ROS and RNS.

With mitochondrial ETC being the main source of ROS and RNS production, macromolecular components of the mitochondria are extremely exposed to injury due to oxidative/nitrative/nitrosative stress. Of note, proteins that underwent such a damage are highly susceptible to proteolytic cleavage and degradation [15]. The injury to mitochondrial respiratory complex subunits by impaired efficacy of the ETC has two main consequences: (1) it leads to decreased energy production due to impaired OXPHOS, and (2) it decreases the efficacy of terminal oxidation, which results in increased production of ROS/RNS, generating a vicious circle.

The proximity to the main source of free radical production and the relatively high proportion of coding sequences render the mitochondrial genome particularly sensitive to ROS/RNS-mediated injury [16]. Indeed, the mutation rate of mtDNA relative to nDNA is approximately 10 : 1 [17]. Furthermore, as the ability to cope with oxidative/nitrative/nitrosative stress declines with aging [18], the rate of mtDNA mutations further increases in the elderly [19].

Excessive ROS and RNS accumulation can trigger the opening of mitochondrial permeability transition pores (mtPTP), which on the one hand decreases the mitochondrial membrane potential further aggravating the initial OXHPHOS impairment, and, on the other hand, leads to the release of proapoptotic factors (including apoptosis-inducing factor, procaspase-9, and cytochrome *c*) from the intermembrane space to the cytosol. This is in severe cases followed by cellular death that can be either apoptotic or necrotic, depending on the severity of the initial insult and subsequent energy deprivation [20–22]. It should be noted, however, that being among the most ancient signals between mitochondria and their host cells, both ROS and RNS might

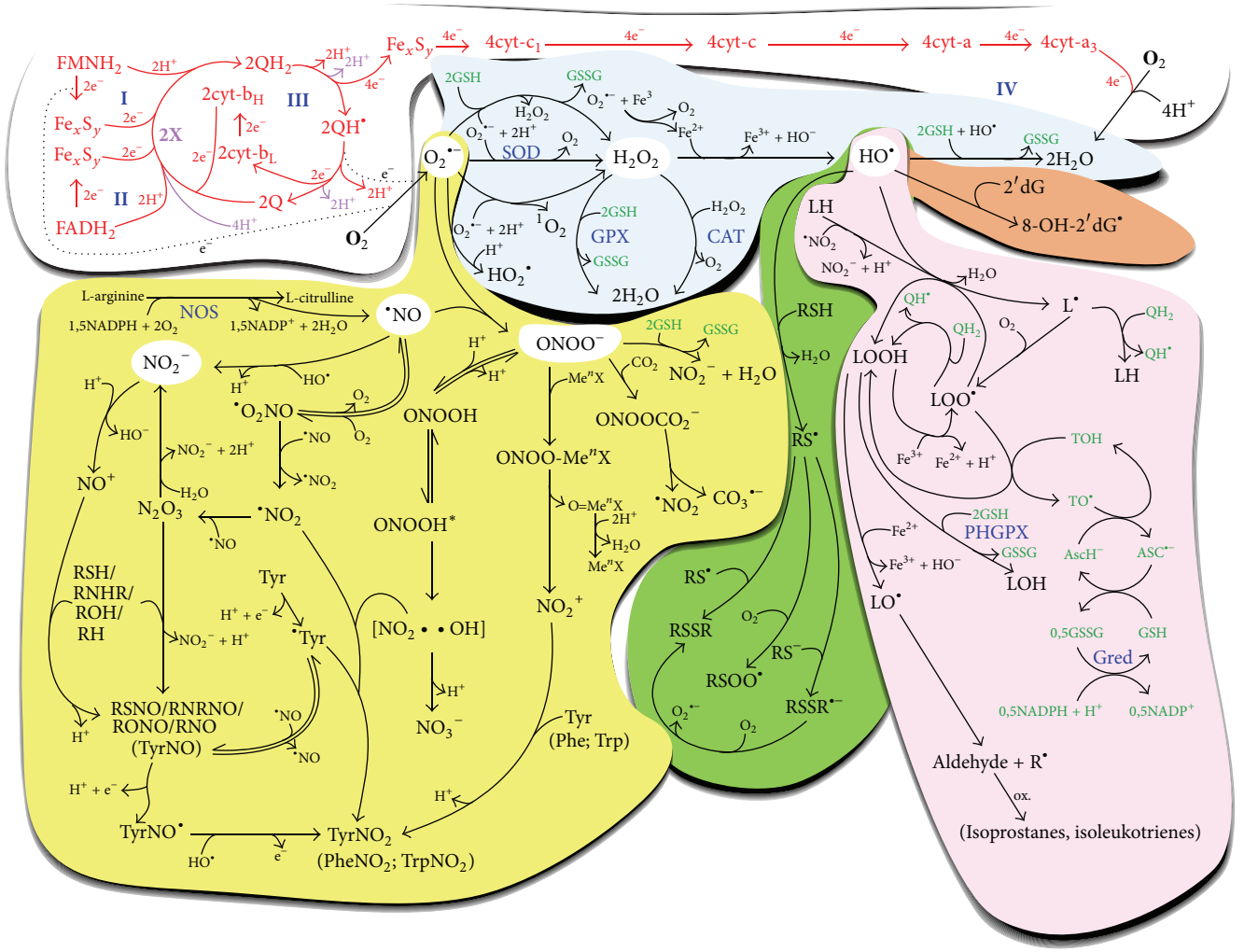


FIGURE 3: The detailed depiction of the chemistry of mitochondrial oxidative and nitrative/nitrosative stresses. The contents of the colored bubbles correspond to the processes indicated in the bubbles of the respective color in Figure 2. Figure 3 presents the chemical processes of ETC and the terminal oxidation (white bubble; blue Roman numbers represent the site of the respective respiratory complexes) together with those involved in the generation of ROS (light blue bubble) and RNS (yellow bubble). An overview is given on the most representative molecules involved in oxidative injury to nucleic acids (brown bubble), lipids (pink bubble), and molecules with thiol residues (green bubble), as well as in nitrosylation/nitration of organic macromolecules (yellow bubble). For detailed explanation of oxidative/nitrative/nitrosative (black) and antioxidant (green) processes as well as the function of the ETC (red), we refer to the corresponding sections within the text and the Abbreviations section.

have essential physiological functions under physiological conditions.

Defensive processes of the mitochondria to counteract excessive free radical production involve low molecular weight antioxidants (LMWAs), an enzymatic redox apparatus to clear ROS/RNS (e.g., SOD, CAT, GPX, and peroxiredoxin), and an nDNA-encoded repair machinery.

Ubiquinol and tocopherols represent the main groups of lipid-associated LMWAs. Since these molecules are transformed to semiquinone radicals upon reduction of toxic free radicals, the immediate restoration of antioxidant capacity is essential. This process depends on the standard redox potential of LMWAs. For example, reduction of the semiquinone form of lipid-associated tocopherol requires ascorbic acid (vitamin C, identified by Albert Szent-Györgyi) and the subsequent reduction of the produced ascorbic acid radical by glutathione. Therefore, at the end of the process, no free radicals are present. However, there is a need for the restoration of the reduced glutathione, which is mediated by the enzyme glutathione reductase (Gred). Reduced glutathione also participates in antioxidant functions associated with the activities of SOD and GPX (including the phospholipid-associated form (PHGPX) as well), and it is responsible for the detoxification of $HO^{\bullet}$ and $ONOO^{-}$. The proper function of this armatory requires the appropriate load of reducing equivalents (NADH + $H^{+}$, NADPH + $H^{+}$). The above mechanisms are depicted in Figure 3.

A number of evidence link PGC-1$\alpha$ to the regulation and activation of mitochondrial antioxidant responses. In a comprehensive study of St-Pierre et al. [6], the expression

of PGC-1$\alpha$ significantly increased after $H_2O_2$ challenge in 10T1/2 cells, which effect was recapitulated by Irrcher et al. on $C_2C_{12}$ muscle cells [23]. This effect is in correspondence with our recent findings of significantly increased PGC-1$\alpha$ expression in the CNS of mice intoxicated with the neurotoxin 3-nitropropionic acid, an irreversible inhibitor or complex II [24]. Furthermore, RNAi against PGC-1$\alpha$ reduced the baseline expression of copper/zinc (Cu/Zn)-SOD, Mn-SOD, and GPX in 10T1/2 cells, whereas the expression of Cu/Zn-SOD, Mn-SOD and peroxisomal CAT was found reduced in the heart and brain of PGC-1$\alpha$-deficient mice [6]. Similarly, overexpression of PGC-1$\alpha$ in $C_2C_{12}$ myotubes displayed increased expression of Mn-SOD and GPX in association with a decreased amount of ROS production [25]. Moreover, PGC-1$\alpha$-deficient fibroblasts exhibited blunted response to ROS challenge and an increased sensitivity to oxidative stress, which was in correspondence with an increased sensitivity of PGC-1$\alpha$-deficient mice to intoxication with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an irreversible inhibitor of mitochondrial respiratory complex I, as well as to that with the excitotoxin, kainate [6].

Mitochondrial repair is now widely-acknowledged as an existing phenomenon, comprising a group of processes that aim to repair deleterious alterations in mtDNA, predominantly due to oxidative injuries. These include enzymatic apparatuses for (1) the hydrolysis of oxidized deoxyribonucleotide triphosphates to prevent mismatch errors, (2) different mechanisms of single- and double-strand break repair, (3) multiple mechanisms of base excision repair, and (4) the degradation of unrepairable mtDNA. The latter is a unique mitochondrion-specific mechanism in eukaryotes, which is enabled by the redundance of mtDNA within the organelle [26]. Certain evidence suggest that, similarly to that seen in nDNA repair, poly(ADP-ribose) polymerase-1 (PARP-1) might play central role of the epigenetic regulation of nDNA-encoded proteins involved in mtDNA repair mechanisms [27].

In case the antioxidant defense and repair systems prove insufficient to protect the organelle, severely damaged mitochondria can be sensed and degraded by a process under the regulation of PINK1 and parkin (mitophagy) [28, 29].

## 4. The Central Role of Mitochondrial Dysfunction in Neurodegenerative Diseases

A number of general observations and considerations explain the special susceptibility of the CNS to suffer injuries due to mitochondrial disturbances. Indeed, the CNS has an especially high energy demand as it represents merely 2% of the total body mass and accounts for some 20% of bodily oxygen consumption [30]. Besides, unlike astrocytes, neurons store low amounts of glycogen and have a poor ability to enhance glycolysis under conditions when mitochondrial respiration is impaired [31]. Therefore, neurons depend on the constant availability of oxygen and glucose to maintain their functions. Furthermore, the CNS contains high amounts of polyunsaturated lipids, which are highly susceptible to oxidative injury by means of lipid peroxidation, and the antioxidant capacity of neurons is known to be relatively poor [32, 33]. The high sensitivity of neurons as opposed to the relative resistance of astrocytes to oxygen or glucose deprivation is well known; however, recent studies suggest that oligodendrocytes are among the most sensitive cell types within the CNS to mitochondrial stress, exceeding the vulnerability of neurons [34, 35], a feature that may have implications for the pathogenesis of characteristic myelinopathies in chronic conditions with mitochondrial dysfunction, including aging, and mitochondrial encephalopathies.

Another CNS-specific mechanism leading to an increased sensitivity to mitochondrial dysfunction is excitotoxicity due to glutamate, the major excitatory neurotransmitter in the brain [36]. In an event of energy deprivation, neurons undergo partial membrane depolarization, which removes magnesium ions that block the ionophore of *N*-methyl-D-aspartate-sensitive (NMDA) glutamate receptors. This leads to a persistent activation of NMDA receptors by glutamate even if it is present in physiological levels [37]. Hyperactivation of NMDA receptors results in an influx of calcium into the cytosol. The persistent increase in intracellular calcium level leads to an increased mitochondrial sequestration of calcium, which in pathological extents evokes the opening of high-conductance mtPTPs [38], resulting in mitochondrial swelling and a decreased mitochondrial membrane potential, with subsequent OXPHOS impairment and ROS overproduction [39]. These culminate in the release of proapoptotic factors eventually triggering cell death [40] by apoptotic or necrotic mechanisms, based on the severity of the event [20, 21]. It has also been postulated, however, that the mechanism and the channel (i.e., NMDA receptor) through which calcium ions get into the cell and not the calcium overload itself may play the pivotal roles in excitotoxic cell death [41]. Indeed, NMDA receptors are functionally and spatially linked to neuronal NOS (nNOS) by postsynaptic density protein of molecular weight 95 kDa (PSD-95) that synthesize $NO^{\bullet}$ in a toxic amount while calcium ions enter into the cell during NMDA receptor overactivation [42]. Notably PSD-95 attaches to the NR2B subunit, which is in correspondence with the observation that glutamate excitotoxicity is predominantly mediated by NR2B subunit-containing NMDA receptors [43], which are mainly located extrasynaptically [44]. In line with these, activation of extrasynaptic NMDA receptors is regarded as neurotoxic, whereas that of the synaptic NMDA receptors appears to be neuroprotective [45, 46], indicating a role of volume transmission in NMDA receptor-mediated neurotoxicity. A central role of nNOS in excitotoxic injury was suggested by earlier studies as well [47, 48]. The role of excitotoxicity in association with mitochondrial dysfunction as well as the possible therapeutic relevance of approaches aiming to counteract glutamatergic overactivation in neurodegenerative diseases, including pharmacological manipulations with the kynurenine system, have recently been extensively reviewed [49, 50] and are not within the scope of this review.

The following subsections emphasize the relevance of mitochondrial dysfunction in the pathomechanism of degenerative CNS disorders through a detailed overview on the involvement of impaired OXPHOS and mitochondrial bioenergetics in Parkinson's disease, Huntington's disease, and

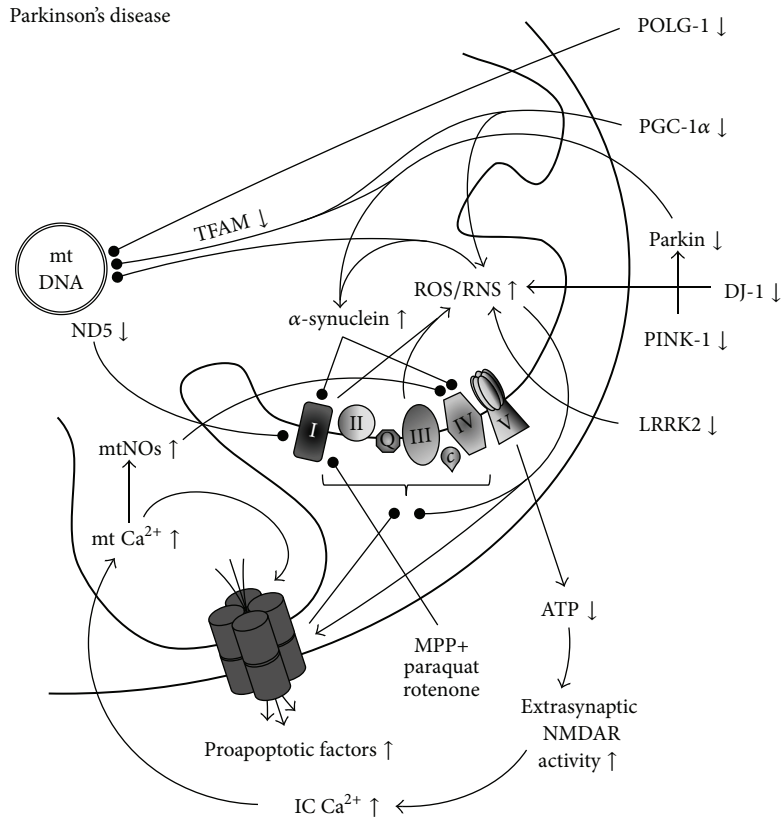


Figure 4: The involvement of mitochondrial dysfunction in Parkinson's disease. Complex I deficiency, the predominant electron transport disorder in sporadic PD has long been linked to the deleterious effects of $\alpha$-synuclein aggregation, a pathognomonic alteration in PD, and inhibitors of complex I (such as MPTP, rotenone, and paraquat) are used in experimental modeling of the disease. Since then a number of genes have been associated with familial forms of the disease, many of them having direct implications in mitochondrial dysfunction. Disturbed OXPHOS in the affected cells can lead to the development of a vicious circle, eventually leading to cell death. Novel findings link PGC-1$\alpha$ dysfunction to the pathogenesis of sporadic PD, the restoration of which may hold therapeutic value. ($\uparrow$ = increased presence/expression/activity; $\downarrow$ = decreased presence/expression/activity; arrow = promotion; bulb-headed arrow = inhibition/deterioration.)

mitochondrial encephalopathies, with special focus on the involvement and therapeutic relevance of PGC-1$\alpha$.

*4.1. Parkinson's Disease.* Parkinson's disease (PD) is a progressive, chronic neurodegenerative disorder, the pathognomonic alterations of which include loss of dopaminergic neurons, and the presence of Lewy bodies in the substantia nigra pars compacta (SNpc), with a subsequent decrease in striatal dopamine levels [51]. Leading clinical symptoms include bradykinesia, rigidity, resting tremor, and postural instability [52, 53], eventually evolving into severe akinesia, dementia, and eventually death. The development of sporadic PD is linked to a complex interplay of genetic and environmental factors, which have multiple implications for mitochondrial involvement (Figure 4). The first implication for the role of mitochondrial dysfunction in PD came from serial cases of intoxication by the side-product of a synthetic illicit drug, MPTP, which evokes parkinsonian symptoms and recapitulates the majority of PD-related pathologies [54]. Its active metabolite 1-methyl-4-phenylpyridinium ($MPP^+$) selectively and irreversibly impairs the function of mitochondrial complex I in dopaminergic neurons [55, 56], and since its discovery, systemic MPTP or intraventricular $MPP^+$ intoxication became the most widely applied *in vivo* toxin models of PD. Similar effects can be achieved by known environmental chemicals including the herbicide paraquat and the insecticide rotenone [57]. Corresponding with the ability of complex I inhibitors to evoke parkinsonism, a decreased activity and/or expression of respiratory complex I has been detected in the SNpc [58, 59], striatum [60] frontal cortex [61], platelets [62, 63], and skeletal muscle [64, 65] of sporadic PD patients, suggesting a systemic impairment of mitochondrial functions in this disease. Less consistent reports have been published regarding the involvement of other respiratory complexes, which indicates a predominant involvement of complex I in sporadic PD. In line with these, a decreased activity of complex I, an elevated production of ROS, an energy impairment, and an increased sensitivity to $MPP^+$ intoxication can be detected in PD cybrids [66, 67]. The increased presence of oxidative damage has also been reported in post mortem SN of PD patients [68, 69].

In the past decades, genetic and, more recently, genome-wide association studies (GWAS) have identified over 20 loci in causative association with familial PD [70], many of them having direct implications in mitochondrial dysfunction. Among them, leucine-rich repeat kinase 2 (LRRK2) protein

is known to colocalize with membrane bound intracellular structures including mitochondria [71]. Its autosomal dominantly inherited mutation, G2019S, the most frequent underlying genetic alteration in both familial and sporadic PD cases, has been associated with mitochondrial dysfunction and morphological alterations in PD tissue samples [72], abnormal mitochondrial dynamics and increased ROS production in murine primary cortical neurons [73], as well as with an increased neuronal vulnerability to rotenone and paraquat in a nematode model of PD [74].

The autosomal dominantly inherited mutation of SNCA gene (A53T) leads to mitochondrial accumulation of $\alpha$-synuclein, the main constituent of Lewy bodies, resulting in the inhibition of respiratory complex I [75, 76]. The protein appears to play pivotal roles in modulating oxidative stress, as its transgenic overexpression leads to enhanced sensitivity against intoxication with paraquat and MPTP [77], whereas $\alpha$-synuclein-deficiency leads to resistance against intoxication with MPTP, 3-nitropropionic acid and malonate in mice [78] (the latter two are inhibitors of complex II and serve as toxin models for Huntington's disease). Accordingly, cell lines transfected with mutant $\alpha$-synuclein exhibit increased levels of oxidation products, decreased glutathione levels, and a markedly increased cell death in response to toxic insults including $H_2O_2$ and $MPP^+$ exposure [79].

Among genes associated with an autosomal recessive inheritance of familial PD, parkin, a ubiquitin E3 ligase, is responsible for the polyubiquitin tagging of toxic protein aggregates for proteasomal degradation [80]. In addition, parkin appears to be involved in antioxidant functions through regulating SOD activity and glutathione levels [81] and may play important roles in mitochondrial transcription via its association with mitochondrial transcription factor A (Tfam) [82]. Accordingly, parkin-deficient mice display decreased expression of complex I and IV subunits accompanied by a diminished antioxidant capacity and enhanced oxidative damage [83], whereas parkin-deficient flies develop abnormal mitochondria and exhibit an increased vulnerability to paraquat [84]. Among *in vitro* conditions, overexpression of wild-type parkin reduced, whereas that of the mutant allele aggravated cell death induced by different oxidative stimuli including $H_2O_2$ and $MPP^+$ intoxication, which aggravation was accompanied by increased levels of oxidative stress markers and a decreased amount of glutathione [85]. In line with these, transfection of cell lines with mutant parkin leads to an increased presence of markers of oxidative and nitrative injuries [86]. Notably, however, the potential of wild-type parkin overexpression to protect against oxidative insults *in vitro* has been challenged [87], and another study found no increase in vulnerability to different regimens of MPTP exposure in parkin-deficient mice [88].

Parkin appears to share common pathway with phosphatase and tensin homologue- (PTEN-) induced putative kinase 1 (PINK1), another protein associated with autosomal recessive PD, with PINK1 acting upstream of parkin [89]. Indeed, both proteins are involved in the regulation mitochondrial dynamics [28, 29, 90, 91], and phenotypes associated with PINK1-deficiency have been repeatedly reported to be rescued by parkin [89, 92–94]. In experimental models, PINK1-deficiency has been associated with impaired mitochondrial respiration (most consistently complex I deficiency) [95, 96], decreased energy production [92, 93, 97, 98], elevated ROS production [98, 99], impaired mitochondrial calcium handling [98–100], mitochondrial morphological alterations [28, 29, 89–91, 93, 98], and an increased susceptibility to mPTP [99–101]. Furthermore, PINK1-deficiency exacerbates neurodegeneration evoked by $MPP^+$ *in vitro* and MPTP *in vivo* [102]. In addition to parkin, tumor necrosis factor (TNF) receptor-associated protein 1 (TRAP1), a mitochondrial molecular chaperone also known as heat shock protein 75 (Hsp75), has been postulated to be another possible downstream target of PINK1, through which PINK1 activity can prevent the release of cytochrome *c* and a subsequent apoptosis [103]. The functional association between TRAP1 and PINK1 has gained further support by more recent studies [104, 105], consistently suggesting that TRAP1 acts downstream of PINK1 and in parallel with parkin when mediating amelioration in mitochondrial dysfunction.

Mutations of DJ-1, an oxidative stress sensor capable of modulating glutathione metabolism and mitochondrial transcription under mitochondrial stress [106], leads to autosomal recessive familial PD. The protein is suggested to function in parallel with PINK1/parkin pathway in maintaining mitochondrial function among oxidative conditions [107]. At the experimental level, DJ-1-deficiency has been associated with increased ROS production [108–110], impaired mitochondrial respiration [109] or energy production [110], mitochondrial morphological abnormalities [109], increased opening of the mPTP [110], as well as an increased sensitivity to oxidative stressors including MPTP [111], paraquat [112], and $H_2O_2$ [112].

Apart from genes identified in monogenic familial PD, a number of genes have been associated with the development of sporadic PD as modifying or susceptibility factors, including genes involved in mitochondrial functions such as mtDNA polymerase gamma 1 (POLG1) [113, 114] and complex I subunit ND5 [115]. In addition to these, an increasing body of evidence suggests that PGC-1$\alpha$ may add important contributions to the pathogenesis of PD. Indeed, a comprehensive genome-wide meta-analysis found a set of 425 PGC-1$\alpha$-responsive nuclear-encoded mitochondrial genes underexpressed in sporadic PD, representing pinpoint defects in glucose metabolism and mitochondrial ETC [116]. Furthermore, associations of single nucleotide polymorphisms (SNPs) of PGC-1$\alpha$ have been reported with the risk of PD, the age of onset and the longevity [117]. These appear to be in correspondence with decreased expression of PGC-1$\alpha$ and its target gene NRF-1 in the SN and striatum of PD patients as well as in the midbrain of conditional parkin knockout mice [118]. In line with decreased ATP production and impairments in mitochondrial OXPHOS [119] and antioxidant responses [6], PGC-1$\alpha$-deficient mice display enhanced susceptibility to MPTP toxicity [6]. Corresponding to observations that mitochondrial dysfunction can promote the aggregation of $\alpha$-synuclein [120], reduced expression of PGC-1$\alpha$ *in vitro* lead to enhanced $\alpha$-synuclein oligomerization [121]. This effect was, however, not confirmed in PGC-1$\alpha$-deficient mice, suggesting a more complex scenario for mitochondrial

dysfunction-induced $\alpha$-synuclein aggregation *in vivo* [122]. Supporting a potential therapeutic relevance in PD, overexpression of PGC-1$\alpha$ demonstrated neuroprotection against $\alpha$-synuclein- and rotenone-induced toxicity *in vitro* [116] and in a parkin interacting substrate (PARIS) overexpression model of PD *in vivo* [118]. In line with these, transgenic overexpression and resveratrol-induced activation of PGC-1$\alpha$ (via deacetylation by Sirt-1) both rendered neuroprotection against MPTP toxicity in mice [123]. Similarly, the administration of pioglitazone, an agonist of PPAR$\gamma$ that enhances the activity and expression of PGC-1$\alpha$ [124], was also protective in MPTP studies [125, 126]. These altogether suggest that a deficient expression and/or function of PGC-1$\alpha$ and its target genes may play important roles in the development of sporadic PD, which may be of therapeutic relevance in the future. Notably, however, contrasting results have also been published reporting that adenoviral overexpression of PGC-1$\alpha$ aggravated MPTP-mediated damage in mice [127] and was ineffective against mutant $\alpha$-synuclein-mediated toxicity in rats [128] and that a sustained overexpression of PGC-1$\alpha$ to high levels *per se* evoked the degeneration of nigral neurons in rats [128]. These findings necessitate further investigations and draw the attention to the possibility that a sustained overactivation of mitochondrial biogenesis aiming to restore mitochondrial functions may also have deleterious consequences on the long run. This issue needs to be clarified in the future.

The search for effective neuroprotective compounds capable of modifying the disease course in PD is still extensive; as molecules targeting mitochondrial dysfunction in PD though provided promising results in experimental models of PD [129–131], they were ineffective in clinical trials [132]. In line with the data on a decreased function of PGC-1$\alpha$ in PD and on the therapeutic potential of its activation, there is a hope that transcriptional activation of mitochondrial biogenesis and antioxidant responses via PGC-1$\alpha$ activation may hold therapeutic value. A phase II safety and futility clinical trial with the PPAR$\gamma$ agonist pioglitazone on patients with early PD has recently been completed, and the results are about to be published in the near future (NCT01280123).

*4.2. Huntington's Disease.* Huntington's disease (HD) is a monogenic, progressive neurodegenerative disease of autosomal dominant inheritance. The genetic alteration is the expansion of CAG trinucleotide repeat sequence on the interesting transcript 15 (IT15) gene on chromosome 4 encoding huntingtin, with increasing number of repeat associating with earlier onset and more rapid progression [133, 134]. The disease onset is usually between 40 and 50 years of age, presenting with behavioral alterations and hyperkinesia in the early stages, subsequently associating with pyramidal symptoms, dystonia, dementia, and psychosis. The pathognomonic alteration is the preferential loss of the striatal $\gamma$-aminobutyric acid (GABA)-ergic medium-sized spiny projection neurons (MSNs) and the presence of intracytoplasmic and intranuclear protein inclusions of mutant huntingtin widely distributed in neuronal as well as extraneuronal tissues. The characteristic decreased activity of respiratory complex II, especially in the striatum, has early linked HD to mitochondrial dysfunction [135] (Figure 5). Since then, deficiency in complex II is the most consistent and predominant alteration reported in HD; however, the involvement of other respiratory complexes has also been suggested [136, 137]. The concept of mitochondrial dysfunction mediating the pathological process induced by mutant huntingtin is consistent with an increased presence of oxidative stress [137–141], which is well-reflected by the increased amount of mtDNA mutations observed in HD patients [142]. Further alterations supporting the primary role of mitochondrial dysfunction in mediating the effects of mutant huntingtin include a transcriptional [143] and/or functional [144] repression of PGC-1$\alpha$, disturbances in mitochondrial trafficking [145], a gradually decreasing mitochondrial number [146], and an impairment of mitochondrial calcium handling [147] with an enhanced sensitivity to calcium-induced opening of mPTP and cytochrome *c*-mediated cell death [148, 149].

In line with the predominant biochemical alteration, irreversible inhibition of complex II by 3-nitropropionic acid effectively recapitulates most of the clinical and histopathological characteristics of HD, including the preferential neuronal loss of GABAergic MSNs within the striatum [150, 151]. Similar alterations can be evoked via reversible complex II blockade by malonate [152]. The relevance of complex II dysfunction in HD is highlighted by the fact that mutant huntingtin leads to decreased expression (at the protein level) of the 30 kDa iron-sulfur (Ip) subunit and the 70 kDa FAD (Fp) subunit of complex II in the striatum of HD patients [153] and *in vitro* lentiviral models [153, 154]. Rather similar pattern of alterations was found to underlie complex II deficiency in transgenic HD mice and in a lentiviral model of HD in rats [155]. Complex II dysfunction appears to be causative in HD as its overexpression demonstrated marked restorative effect in these models [153–155]. Alterations in complex II assembly were accompanied by reduced mitochondrial biogenesis in transgenic animals, prompting the authors to suggest the possible contribution of PGC-1$\alpha$ repression [155]. Indeed the expression of PGC-1$\alpha$ has been found to be downregulated in the striatum of HD patients [143, 144, 146, 156], in transgenic HD animals [24, 143, 144, 157–159], and in *in vitro* HD models [143, 156, 159, 160]. Correspondingly, the decreased expression of several PGC-1$\alpha$ target genes has been identified in the striatum of HD patients [144, 146] and transgenic HD mice [144, 157, 158]. A possible mechanism through which mutant huntingtin can lead to the downregulation of PGC-1$\alpha$ can be secondary to its effect to enhance the expression [161] and activity of NR2B subunit-containing NMDARs [162], features characteristic of transgenic HD mice [163], which in turn results in a decreased striatal CREB signaling [163], and a subsequent downregulation of PGC-1$\alpha$ [164]. In addition, a decreased expression of transducer of regulated CREB-binding protein 1 (TORC1), an activator of CREB-mediated PGC-1$\alpha$ expression, has been found in *post mortem* HD striatum, in transgenic HD mice, and in an *in vitro* HD model, which may contribute to the downregulation of PGC-1$\alpha$ expression in HD [160], whereas others suggest that PGC-1$\alpha$ repression may be secondary to the downregulation of PPAR$\gamma$ in HD [159]. Supplementing the alterations in PGC-1$\alpha$ expression in HD, Johri et al. reported decreased protein

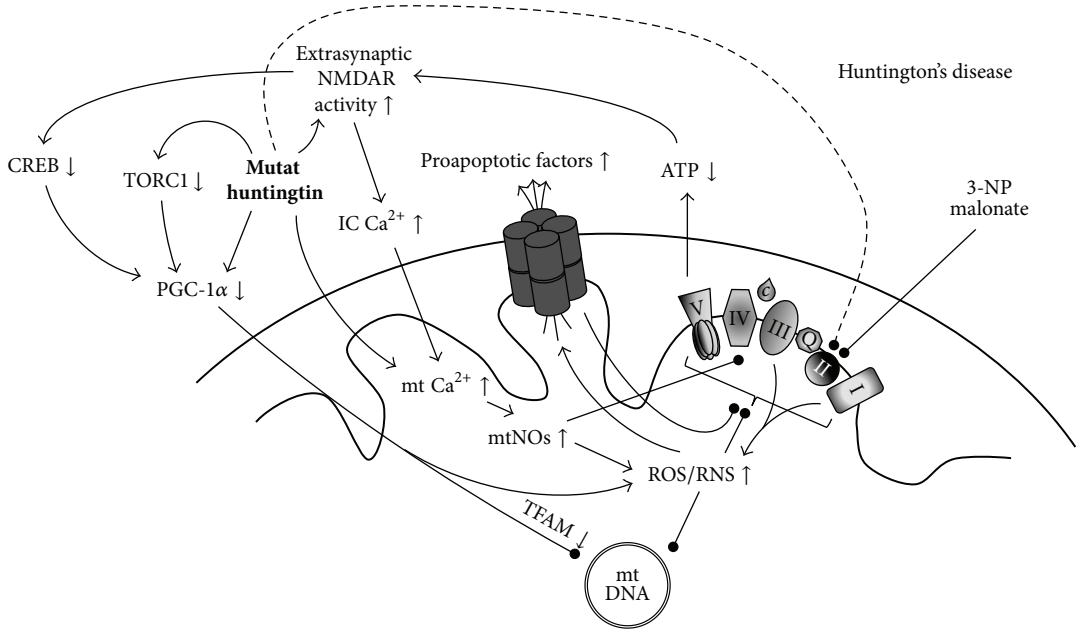


Figure 5: The involvement of mitochondrial dysfunction in Huntington's disease. Complex II deficiency, the predominant electron transport disorder in HD has long been linked to the deleterious effects of mutant huntingtin aggregation, a pathognomonic alteration in HD, and inhibitors of complex II (such as 3-nitropropionic acid (3-NP) and malonate) are used in experimental modeling of the disease. Disturbed OXPHOS in the affected cells can lead to the development of a vicious circle, eventually leading to cell death. Novel findings link PGC-1$\alpha$ dysfunction to the pathogenesis of HD at multiple levels, the restoration of which may hold therapeutic value. ($\uparrow$ = increased presence/expression/activity; $\downarrow$ = decreased presence/expression/activity; arrow = promotion; bulb-headed arrow = inhibition/deterioration.)

level of the functionally active N-truncated splice variant of PGC-1$\alpha$ (NT-PGC-1$\alpha$) in the striatum of low-grade HD patients and young asymptomatic transgenic N171-82Q and R6/2 HD mice, whereas its level was found to be elevated in high-grade HD patients and in older symptomatic transgenic HD mice [156]. This pattern has been recently supported by our study on transgenic N171-82Q HD mice at the mRNA level, revealing a significantly upregulated expression of NT-PGC-1$\alpha$ in both the striatum and overlying cortex of older symptomatic HD mice compared to wild-type and young HD counterparts [24]. These changes were accompanied by a decreased expression of full-length PGC-1$\alpha$ in the striatum and cortex of young transgenic mice, corresponding to prior observations. A main novelty of this study included a previously unidentified consistent elevation of both the full-length and the N-truncated isoforms of PGC-1$\alpha$ in the cerebellum of transgenic HD mice, which may underlie the relative resistance of cerebellar neurons to degeneration in HD [24]. This study further provided evidence for a consistent striatal upregulation of both the full-length and the N-truncated isoforms of PGC-1$\alpha$ following acute but not chronic injury due to 3-nitropropionic acid intoxication in mice [24]. This possibly compensatory elevation corresponds to prior findings of PGC-1$\alpha$ upregulation in response to ROS challenge *in vitro* and highlights the role of PGC-1$\alpha$ in concerting antioxidant responses [6, 23]. Supporting a potential therapeutic relevance, a line of evidence suggests that mechanisms associated with the upregulation of PGC-1$\alpha$ can exert neuroprotection in experimental models of HD. Indeed, the administration of PPAR$\gamma$ agonist thiazolidinediones (such as rosiglitazone and pioglitazone) was proven to be protective in transgenic HD mice [158, 159, 165], in an intrastriatal quinolinic acid-induced rat toxin model of HD [166], in 3-nitropropionic acid-induced murine toxin model of HD [167], and in *in vitro* HD models [159, 165, 168]. Similarly, the pan-PPAR agonist bezafibrate exerted protection in transgenic HD mice [169]. Furthermore, TORC1 activation displayed protective and restorative effects on viability and mitochondrial functions in a striatal HD cell line model exposed to 3-nitropropionic acid [160]. The protective effect of resveratrol, a polyphenol with potent Sirt-1/PGC-1$\alpha$-activating properties, has also been demonstrated in transgenic murine and nematode models [170], in a 3-nitropropion acid-induced murine model [171], and in an *in vitro* model of HD [170]. Notably, however, the potency of resveratrol to significantly elevate the expression of PGC-1$\alpha$ and its target genes within the striatum has recently been questioned [172], which necessitates further investigations. Considering that early attempts with mitochondria-targeted molecules being neuroprotective in experimental HD models [131, 173, 174] provided little success at the clinical level [132], the transcriptional activation of mitochondrial respiration and biogenesis may hold novel therapeutic potential as a more system-based approach. A phase III clinical trial with resveratrol is just about to recruit its participants (NCT02336633).

The corresponding set of evidence implicating a potential pathogenetic role of PGC-1$\alpha$ repression in mitochondrial dysfunction in HD as well as early observations of striatal alterations in PGC-1$\alpha$-deficient mice [175, 176] suggested that such animals may serve as experimental models for HD;

however, findings of a recent detailed neuropathological evaluation of mice lacking the expression of full-length PGC-1$\alpha$ has indicated that it might not indeed be the case and turned our attention to another group of diseases where systemic mitochondrial dysfunction is pathognomonic [122].

*4.3. Mitochondrial Spongiform Leukoencephalopathies.* Mitochondrial diseases are a group of multisystemic disorders where the characteristic pathologies affecting organs with high energy demand (i.e., brain, liver, heart, skeletal muscle, and kidney) are due to mitochondrial dysfunction as a consequence of a genetic alteration either in the mtDNA or in the nDNA. The deleterious loss of functions may affect several components of proper mitochondrial functioning, including genes encoding respiratory complex subunits, proteins responsible for mtDNA transcription/translation, mitochondrial tRNAs and rRNAs, and nuclear-encoded ancillary proteins of mitochondrial function [177]. The diseases are distributed to characteristic syndromes based on the clinical manifestation and the observed neuropathological alterations, including Kearns-Sayre syndrome, Leigh syndrome, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibres (MERRF), neuropathy, ataxia, retinitis pigmentosa (NARP), and mitochondrial neurogastrointestinal encephalopathy (MNGIE) [178–181]. In these diseases, impaired ATP production with various defects in respiratory complexes and excess ROS production in the affected tissues has widely been documented and has excessively been reviewed [182]. Though in somewhat different patterns, mitochondrial encephalopathies are collectively characterized by various degrees of tissue vacuolation in the white and gray matter of the CNS, accompanied by region-selective reactive astrocytosis with or without neurodegeneration.

A number of genetically modified murine strains have been developed to model diseases with mitochondrial defects, however, with variable outcomes [122]. On the one hand, many of the genetic modifications lead to embryonic or early postnatal mortality due to multisystemic insufficiency (e.g., complete knockouts of CREB [183], Tfam [184], NRF-1 [185], NRF-2 [186], ERR$\gamma$ [187], POLG1 [188], synthesis of cytochrome *c* oxidase 2 (SCO2) [189], and optic atrophy 1 (OPA1) [190]). On the other hand, a remarkable proportion of viable models, surprisingly, does not have any pathological changes in the CNS (e.g., complete knockouts of adenine nucleotide translocator 1 (ANT1) [191], PPAR$\gamma$ [192], ERR$\alpha$ [193], and SURF1 [194]; $\Delta$mtDNA Mito-Mice [195]; and Twinkle mutant "Deletor" mice [196]). Genetic models exhibiting a neuropathology closely reminiscent of human mitochondrial leukoencephalopathies include mice deficient in Mn-SOD [197], in thymidine phosphorylase and uridine phosphorylase (TP/UP) [198], and in NADH dehydrogenase [ubiquinone] iron-sulfur protein 4 (NDUFS4) [199]. In addition to these, our recent neuropathological analysis on mice deficient in the expression of full-length PGC-1$\alpha$ revealed widespread spongy vacuolation predominating in the white matter of the striatum, thalamus, cerebellum, and the brainstem, accompanied by moderate to severe reactive astrogliosis in the pontomedullary brainstem and the cerebellar nuclei, corresponding to a pattern of alterations characteristic of the spongiform leukoencephalopathy seen in Kearns-Sayre syndrome [122]. This is especially interesting in light of the facts that experimental animals used for modeling cardiomyopathy in Kearns-Sayre syndrome are the tissue-specific knockouts of Tfam [200, 201], a gene under the regulation PGC-1$\alpha$, and the expression of which is severely downregulated in PGC-1$\alpha$-deficient mice [119, 175]. Notably, no indirect or direct signs indicative striatal neuronal degeneration and/or loss were observed in our study [122], which corresponds to the independent observations of Lucas et al. [202], both publications drawing the conclusion that PGC-1$\alpha$-deficiency *per se* is not sufficient to evoke HD-like pathology, contrasting to what had previously been suggested.

Considering the spectrum of roles of PGC-1$\alpha$ in regulating and promoting mitochondrial functions and the fact that a number of genes involved in disease-causing mutations and/or that involved in modeling mitochondrial disease have direct or indirect interactions with PGC-1$\alpha$ (e.g., ANT-1, POLG1, Tfam, NRF-1, NRF-2, PPARs, ERRs, Mn-SOD, and CREB), the rationale for PGC-1$\alpha$ induction to provide symptomatic benefit in these currently intractable groups of diseases can be accepted [203]. Indeed, transgenic overexpression or bezafibrate-induced expression of PGC-1$\alpha$ delayed the onset of symptoms in a cytochrome *c* oxidase-deficient murine model of mitochondrial myopathy [204]. Similarly, transgenic overexpression of PGC-1$\alpha$ ameliorated the phenotype and increased the activity of mitochondrial respiratory complexes in POLG1 mutant "Mutator" mice [205]. Furthermore, adenoviral overexpression of PGC-1$\alpha$ partially restored respiratory deficits in fibroblasts obtained from patients with mitochondrial disease of various origin (though to different efficacy) and in MELAS cybrids [206]. These altogether suggest a potential therapeutic relevance of boosting mitochondrial biogenesis via PGC-1$\alpha$-mediated approaches in diseases with genetic mitochondrial disorder.

## 5. Concluding Remarks

Since the revelation of the essential role electrons, originating from reducing equivalents that arise from cytosolic and/or mitochondrial metabolic processes, in cellular bioenergetics via their flow through metal-containing electron transport complexes in the mitochondrial inner membrane, an armada of evidence has accumulated linking the impaired function of this system to degenerative diseases of the CNS. While initial attempts to compensate for such alterations showed as much promise at the experimental level as deep disappointment they caused at the clinical level, novel strategies with more system-based approaches aiming to render protection via improving mitochondrial bioenergetics at a transcriptional level may open up new therapeutic perspectives and boost pharmacological research. With more and more evidence linking PGC-1$\alpha$ and its target genes to the pathogenesis of neurodegenerative diseases including PD, HD, and mitochondrial disorders, pharmacological manipulations to

restore and/or activate PGC-1$\alpha$ may provide valuable tools in the therapy of these currently intractable diseases.

## Abbreviations

I: First complex in the mitochondrial ETC
II: Second complex in the mitochondrial ETC
III: Third complex in the mitochondrial ETC
IV: Fourth complex in the mitochondrial ETC
$FMNH_2$: Reduced flavin mononucleotide
$FADH_2$: Reduced flavin adenine dinucleotide
$Fe_xS_y$: Iron-sulfur cluster
cyt-a: Cytochrome a
cyt-$a_3$: Cytochrome $a_3$
cyt-$b_H$: Cytochrome bH
cyt-$b_L$: Cytochrome bL
cyt-c: Cytochrome c
cyt-$c_1$: Cytochrome $c_1$
$O_2^{\bullet-}$: Superoxide anion
Q: Coenzyme Q10 (oxidized)
$QH^{\bullet}$: Coenzyme Q10 (semiquinone)
$QH_2$: Coenzyme Q10 (reduced)
GSH: Glutathione (reduced)
GSSG: Glutathione (oxidized)
SOD: Superoxide dismutase
GPX: Glutathione peroxidase
CAT: Catalase
$^1O_2$: Singlet oxygen
$HO^-$: Hydroxide ion
$HO^{\bullet}$: Hydroxyl radical
$HO_2^{\bullet}$: Hydroperoxyl radical
NOS: Nitrogen monoxide synthase
NADPH: Nicotinamide adenine dinucleotide phosphate (reduced)
$NADP^+$: Nicotinamide adenine dinucleotide phosphate (oxidized)
$NO_2^-$: Nitrite ion
$^{\bullet}NO$: Nitrogen monoxide
$NO^+$: Nitrosonium ion
$^{\bullet}O_2NO$: Nitrite radical
$^{\bullet}NO_2$: Nitrogen dioxide radical
$N_2O_3$: Dinitrogen trioxide
$ONOO^-$: Peroxynitrite
ONOOH: Peroxynitrous acid
$ONOOH^*$: Peroxynitrous acid (metastable)
$NO_3^-$: Nitrate
RSH: Thiol (reduced)
ROH: Alcohol
RNHR: Secondary amine
RH: Alkane
Tyr: Tyrosine
$^{\bullet}$Tyr: Tyrosyl radical
RSNO: S-Nitrosothiol
RNRNO: Nitrosamine
RONO: Nitrosyl alcohol
RNO: Nitrosoalkane
$TyrNO_2$: 3-Nitro-L-tyrosine
$PheNO_2$: 3-Nitro-L-phenylalanine
$TrpNO_2$: 6-Nitro-L-tryptophan
$TyrNO^{\bullet}$: 3-Nitrosotyrosine radical
TyrNO: 3-Nitrosotyrosine
Phe: L-Phenylalanine
Trp: L-Tryptophan
$NO_2^+$: Nitronium ion
$Me^nX$: Metal complexes
$O{=}Me^nX$: Oxo-metal complexes
$ONOOCO_2^-$: Nitrosoperoxycarbonate
$ONOO\text{-}Me^nX$: Metal-peroxynitrite complexes
$RS^{\bullet}$: Thiyl radical
RSSR: Thiol (oxidized)
$RSOO^{\bullet}$: Thiol peroxyl radical
$RSSR^{\bullet-}$: Disulphide radical anion
$RS^-$: Thiolate anion
LH: Lipid
$L^{\bullet}$: Lipid radical
LOOH: Lipid peroxide
$LOO^{\bullet}$: Lipid peroxyl radical
$R^{\bullet}$: Alkyl radical
LOH: Lipid hydroperoxide
$LO^{\bullet}$: Lipid alkoxy radical
Gred: Glutathione reductase
PHGPX: Phospholipid glutathione peroxidase
TOH: Tocopherol (reduced)
$TO^{\bullet}$: Tocopherol (semiquinone)
$AscH^-$: Ascorbate (reduced)
$Asc^{\bullet-}$: Ascorbate (oxidized)
$2'dG$: $2'$-Deoxyguanosine
$8\text{-}OH\text{-}2'dG^{\bullet}$: 8-Hydroxy-$2'$-deoxyguanosine.

## Conflict of Interests

The authors report no competing interests.

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*Research Article*

# Dietary Tocotrienol/$\gamma$-Cyclodextrin Complex Increases Mitochondrial Membrane Potential and ATP Concentrations in the Brains of Aged Mice

**Anke Schloesser,[1] Tuba Esatbeyoglu,[1] Stefanie Piegholdt,[1] Janina Dose,[1] Naoko Ikuta,[2] Hinako Okamoto,[3] Yoshiyuki Ishida,[3] Keiji Terao,[2,3] Seiichi Matsugo,[4] and Gerald Rimbach[1]**

[1]*Institute of Human Nutrition and Food Science, University of Kiel, Hermann-Rodewald-Straße 6, 24118 Kiel, Germany*
[2]*Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan*
[3]*CycloChem Bio Co., Ltd., KIBC, 5-5-2 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan*
[4]*School of Natural System, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan*

Correspondence should be addressed to Gerald Rimbach; rimbach@foodsci.uni-kiel.de

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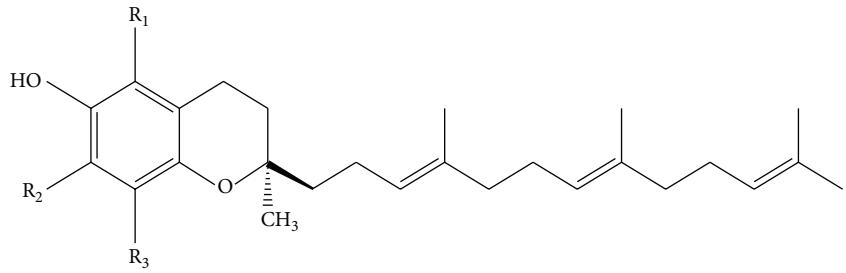
Brain aging is accompanied by a decrease in mitochondrial function. In vitro studies suggest that tocotrienols, including $\gamma$- and $\delta$-tocotrienol (T3), may exhibit neuroprotective properties. However, little is known about the effect of dietary T3 on mitochondrial function in vivo. In this study, we monitored the effect of a dietary T3/$\gamma$-cyclodextrin complex (T3CD) on mitochondrial membrane potential and ATP levels in the brain of 21-month-old mice. Mice were fed either a control diet or a diet enriched with T3CD providing 100 mg T3 per kg diet for 6 months. Dietary T3CD significantly increased mitochondrial membrane potential and ATP levels compared to those of controls. The increase in MMP and ATP due to dietary T3CD was accompanied by an increase in the protein levels of the mitochondrial transcription factor A (TFAM). Furthermore, dietary T3CD slightly increased the mRNA levels of superoxide dismutase, $\gamma$-glutamyl cysteinyl synthetase, and heme oxygenase 1 in the brain. Overall, the present data suggest that T3CD increases TFAM, mitochondrial membrane potential, and ATP synthesis in the brains of aged mice.

## 1. Introduction

Tocotrienols are plant derived bioactives. Tocotrienols occur in nature in four different isoforms: $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-tocotrienol (T3). The $\alpha$-form of tocotrienol contains 3 methyl groups, the $\beta$- and $\gamma$-forms contain two groups, and the $\delta$-form contains only one methyl group at the chromanol ring [1]. Important sources of $\alpha$-tocotrienol are palm oil, rice bran, and barley, whereas annatto contains significant amounts of $\gamma$- (10%) and $\delta$-tocotrienol (90%). The chemical structure of the tocotrienol isoforms is illustrated in Figure 1.

Cyclodextrins are widely used to improve the solubility and stability of lipophilic molecules including tocotrienols [2]. It has been recently shown that complexation of annatto-derived tocotrienols with $\gamma$-cyclodextrin (CD) significantly improved tocotrienol bioavailability in rats [3] and mice [2] by enhancing intestinal absorption. Furthermore, complexation of T3 with CD significantly prolonged the life span of the model organism, *Caenorhabditis elegans* [4].

Brain aging is accompanied by decreased mitochondrial function, increased oxidative stress, decreased proteasomal activity, and the accumulation of cytotoxic amyloid beta peptides. TFAM and PGC1$\alpha$ are key transcription factors that control mitochondrial biogenesis. TFAM activates the duplication of mitochondrial DNA molecules, thereby orchestrating mitochondrial bioenergetics and ATP production [5]. Mitochondrial dysfunction may result in elevated production of reactive oxygen species. In addition, an age-related loss of endogenous antioxidant defense mechanisms such as superoxide dismutase (Sod), heme oxygenase 1 (Hmox1), and




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| $\alpha$ | $CH_3$ | $CH_3$ | $CH_3$ |
| $\beta$ | $CH_3$ | H | $CH_3$ |
| $\gamma$ | H | $CH_3$ | $CH_3$ |
| $\delta$ | H | H | $CH_3$ |

Figure 1: The chemical structure of tocotrienol isoforms.

$\gamma$-glutamyl cysteinyl synthetase (Gclm) accelerates oxidative stress in the brain. Similarly, during the aging process, amyloid beta peptides may enter mitochondria and disrupt mitochondrial function [6]. It has also been demonstrated that proteasomal activity decreases in the aging brain; thus, the cellular ability to degrade oxidized proteins may be impaired [7]. Collectively, all of these processes are interrelated and contribute to an accelerated brain aging phenotype.

There is increasing experimental evidence that T3 prevents cellular senescence [8]. In fact, the age-dependent decrease in the number of brain cells may be slowed down or partly counteracted by T3-rich nutraceuticals [9]. It has been suggested that dietary T3 reaches the brain [10] and exhibits neuroprotective properties [11–14]. However, data on neuroprotection, in the context of mitochondrial function, due to tocotrienols, are scarce. Therefore, in this study, we investigated the effect of annatto-derived dietary tocotrienols complexed with $\gamma$-cyclodextrin on mitochondrial membrane potential and ATP concentrations in the brains of aged mice.

## 2. Materials and Methods

*2.1. Mice and Diet.* Animal studies were performed according to German regulations of animal welfare and with the permission of the appropriate local authorities.

Male C57BL/6J mice were purchased from Taconic Europe A/S (Ry, Denmark) at the age of 15 months. The mice were housed individually in Makrolon cages under controlled environmental conditions (22–24°C, 45–55% relative humidity, and 12 h light/dark cycle) with free access to diet and tap water. The experimental diet (Table 1) was a purified semisynthetic energy dense high fat and high sugar Western-type diet purchased from Ssniff (TD 88137 modified, Soest, Germany). For the first 2 weeks, all mice were fed the Western-type diet ad libitum without tocotrienol/$\gamma$-cyclodextrin. Subsequently, mice were randomly assigned to body weight-matched diet groups, namely, the control ($n = 6$) and the tocotrienol/$\gamma$-cyclodextrin (T3CD, $n = 8$) supplemented group. Annatto-derived tocotrienol/$\gamma$-cyclodextrin (Cyclochem Bio Co., Ltd., Kobe, Japan) was supplemented at 369 mg/kg (providing 100 mg T3/kg diet) in the diet for 24 weeks. Diets were supplemented with 20 mg/kg diet $\alpha$-tocopherol.

Feed intake and body weight were determined on a weekly basis. At week 20, the energy expenditure was measured using a TSE PhenoMaster (TSE Systems GmbH, Bad Homburg, Germany) as previously described [13]. We lost one mouse in the control group during the supplementation period. At the end of the trial, the mice were killed by cervical dislocation, and the brains were immediately removed and dissected into hemispheres. One entire hemisphere was used for the preparation of dissociated brain cells, and the other one was snap-frozen in liquid nitrogen and stored at −80°C until further analysis (except for samples used for RNA isolation, which were placed in RNAlater (Qiagen, Hilden, Germany) and stored at −20°C).

Table 1: Composition of the experimental diets.

| | Control | T3CD |
|---|---|---|
| Tocotrienol/$\gamma$-cyclodextrin | — | 369 mg/kg* |
| Crude protein | 17.1% | 17.1% |
| Crude fat | 21.2% | 21.2% |
| Crude fiber | 5.0% | 5.0% |
| Crude ash | 4.5% | 4.5% |
| Nitrogen free extracts | 49.3% | 49.3% |
| Starch | 14.5% | 14.5% |
| Sugar | 32.8% | 32.8% |
| Cholesterol | 0.2% | 0.2% |
| Vitamin E | 20 mg/kg | 20 mg/kg |
| Metabolizable energy | 19.1 MJ/kg | 19.1 MJ/kg |

*Providing 100 mg T3 per kg diet.

*2.2. Preparation of Dissociated Brain Cells.* The preparation of dissociated brain cells was performed according to [15] with some modifications. The cerebellum was excluded, and the remaining tissue was quickly dissected on ice, washed, and then minced in 2 mL of medium I (138 mM NaCl, 5.4 mM KCl, 0.17 mM $Na_2HPO_4$, 0.22 mM $KH_2PO_4$, 5.5 mM glucose, and 58.4 mM sucrose; pH 7.35) with a scalpel. The brain cells were further dissociated first by filtration through a crude filter (200 $\mu$m) using a Pasteur pipette and later through a fine filter (100 $\mu$m). The cell suspension was then washed twice in medium II (110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, 70 mM sucrose, and 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES); pH 7.4). After a final centrifugation step (1000 ×g, 5 min, 4°C), the cell pellet was resuspended in 5 mL Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, Germany) and distributed 50 $\mu$L/well in white 96-well plates for adenosine triphosphate (ATP) measurements and

Table 2: Primer sequences and annealing temperatures used for qRT-PCR analyses in murine brain tissue.

| Gene symbol | Gene name | Forward 5′-3′ | Reverse 3′-5′ | Annealing temperature |
|---|---|---|---|---|
| Actb | Actin, beta | GACAGGATGCAGAAGAGATTACT | TGATCCACATCTGCTGGAAGGT | 55°C |
| Cat | Catalase | GGAGCAGGTGCTTTTGGATA | CTGACTCTCCAGCGACTGTG | 55°C |
| Gclm | Glutamate-cysteine ligase, modifier subunit | TCCCATGCAGTGGAGAAGAT | AGCTGTGCAACTCCAAGGAC | 57°C |
| Gpx4 | Glutathione peroxidase 4 | ATGAAAGTCCAGCCCAAGG | CGGCAGGTCCTTCTCTATCA | 59°C |
| Hmox1 | Heme oxygenase 1 | GAGCCTGAATCGAGCAGAAC | AGCCTTCTCTGGACACCTGA | 59°C |
| Sod2 | Superoxide dismutase 2, mitochondrial | GCCTGCTCTAATCAGGACCC | TAGTAAGCGTGCTCCCACAC | 59°C |

250 $\mu$L/well in 24-well plates for mitochondrial membrane potential (MMP) measurements. The plated dissociated brain cells were incubated at 37°C in 5% $CO_2$ for 3 h prior to measurements. ATP concentrations and MMP are expressed as fluorescence per milligram protein, and the protein content was determined using the BioRad DC Protein Assay (BioRad, Munich, Germany).

*2.3. Measurement of Mitochondrial Membrane Potential (MMP) and Adenosine Triphosphate (ATP) Concentrations.* Basal MMP was determined using the Rhodamine 123 (Sigma-Aldrich, Steinheim, Germany) fluorescence dye as previously described [9]. Basal ATP concentrations were measured using the ViaLight Plus Bioluminescence Kit (Lonza, Walkersville, USA) according to [15].

*2.4. Cerebral Proteasome Activity.* Proteasomal activity in the brain tissue was measured according to [16] with some modifications. Up to 10 mg brain tissue was homogenized 1 : 10 in ice-cold lysis buffer (20 mM Tris-HCl, 10% glycerol, 0.5 mM EDTA, 0.5% Nonidet P-40, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM adenosine triphosphate; pH 7.8) for 2 × 2 min at 25 Hz with the TissueLyser II (Qiagen, Hilden, Germany), incubated on ice for 30 min, and centrifuged (15,700 ×g; 10 min; 4°C) to maintain the supernatant for further analysis. In addition, 20 $\mu$g protein of each sample was incubated with 185 $\mu$L reaction buffer (20 mM Tric-HCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM adenosine triphosphate; pH 7.8) and 5 $\mu$L of the fluorescent substrate N-succinyl-leucineleucine-valine-tyrosine-7-amino-4-methylcoumarin (Suc-LLVY-AMC, Enzo Life Sciences, Lörrach, Germany). The initially quenched fluorescence signal was measured on a Tecan infinite F200 plate reader (Tecan, Grödig, Austria) at 360 and 465 nm excitation and emission wavelengths, respectively. The calculation of the proteasomal activity was based on an external standard curve.

*2.5. Gene Expression Using qRT-PCR.* RNA isolation and qRT-PCR were conducted as previously described [15]. Primer sequences are given in Table 2. Relative mRNA levels were calculated with an external standard curve and were related to housekeeping gene expression (Actb). The mean value of expression in the control group was set to an arbitrary unit of 1.

Table 3: Primary antibodies used for Western blot analyses in murine brain tissue.

| Name | Manufacturer information | Dilution |
|---|---|---|
| ADAM10 | AB-19026, Merck Millipore | 1 : 500 |
| BACE1 | AB-5832, Merck Millipore | 1 : 400 |
| PGC1 | sc-5816, Santa Cruz Biotechnology | 1 : 200 |
| TFAM | sc-166965, Santa Cruz Biotechnology | 1 : 200 |

*2.6. Western Blotting.* Protein expression was determined in cytosolic lysates prepared from brain tissue except for BACE1, which was detected in whole cell lysates. Samples (40–60 $\mu$g protein) were mixed with loading buffer (0.5 M Tris-HCl, 8% glycerol, 1.6% sodium dodecyl sulfate, 0.001% brome phenol blue, and 5% $\beta$-mercaptoethanol), denatured at 95°C for 5 min, and loaded on Criterion TGX Stain-Free Precast gels (BioRad, Munich, Germany) for separation by SDS–PAGE. Protein fluorescence was activated by UV-exposition for 5 min before transfer onto a PVDF membrane using the Trans-Blot Turbo System (BioRad, Munich, Germany). The target proteins were identified using respective primary (Table 3) and secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) as previously described [16].

*2.7. Statistical Analysis.* All presented data are expressed as the means ± SEM. Statistical analysis was based on Mann-Whitney $U$ test. Values of $p < 0.05$ were considered statistically significant and are indicated with asterisks (∗). The statistical analysis was performed using PASW Statistics 18 (IBM, Chicago, IL, USA).

## 3. Results

There were no significant differences in feed intake, final body weight, or energy expenditure between the control mice and the mice receiving the T3CD enriched diet as summarized in Table 4.

To assess brain bioenergetics, we determined the ATP concentrations and mitochondrial membrane potential in the mice. The ATP concentrations in dissociated brain cells were significantly higher in the T3CD-fed mice than in those of the controls (Figure 2(a)). Consistent with higher brain ATP levels, we observed a significantly higher membrane

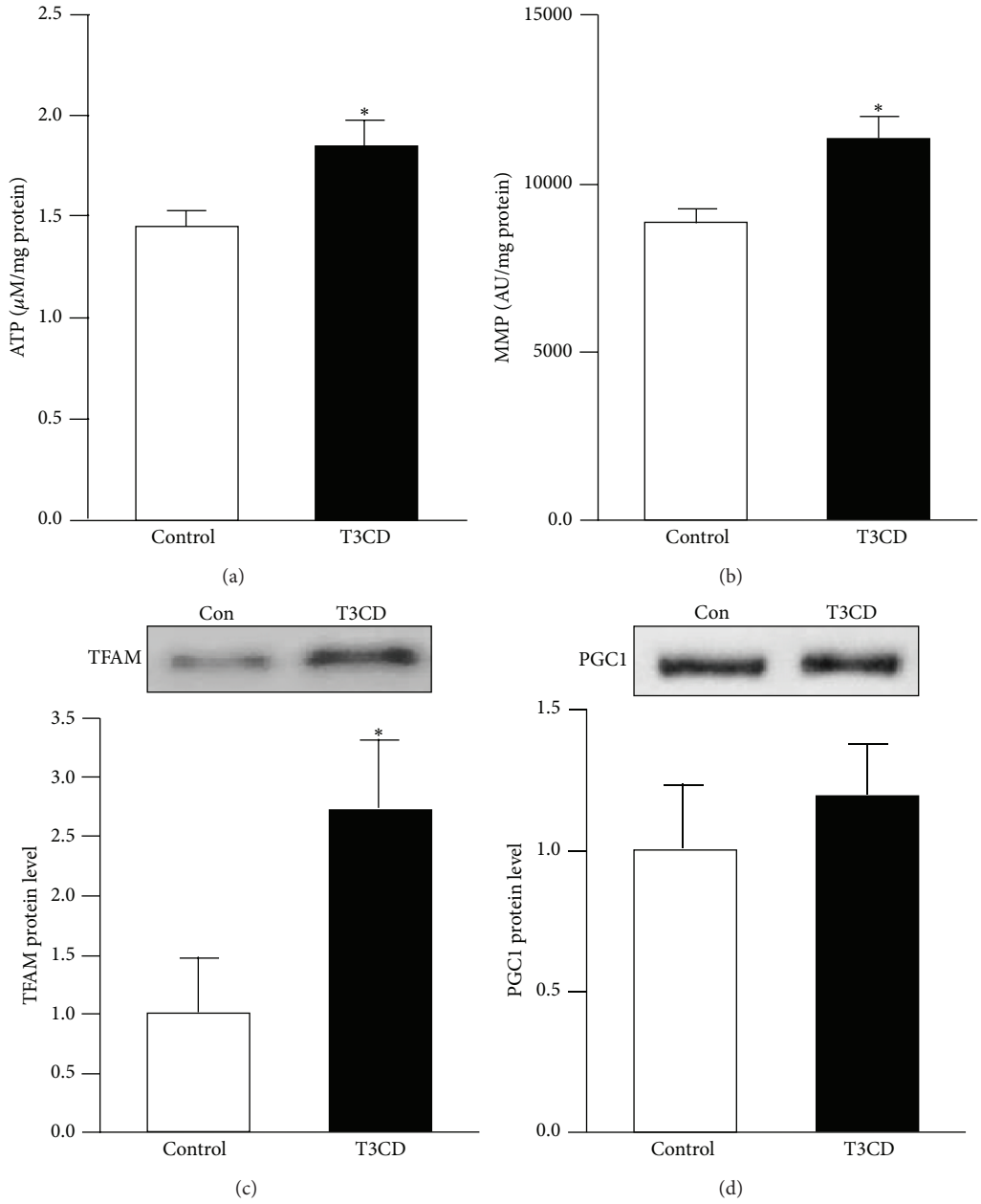



Figure 2: Basal adenosine triphosphate (ATP) concentration, mitochondrial membrane potential (MMP), and TFAM protein levels in mouse brain were elevated by tocotrienol/$\gamma$-cyclodextrin supplementation. (a) Basal ATP [$\mu$M/mg protein] and (b) MMP [AU/mg protein] levels were measured in dissociated brain cells that were freshly isolated from the mice fed either a control diet or a diet supplemented with tocotrienol/$\gamma$-cyclodextrin (T3CD) complex. (c) TFAM and (d) PGC1 protein levels were determined by Western blotting and subsequent densitometric analysis of target bands. Target protein expression was related to the total protein fluorescence transferred to the PVDF membrane. Representative blots from one of 5–8 animals per groups are shown. Values are the means + SEM from 5 to 8 animals per group. The asterisks indicate a significant difference ($p < 0.05$) between the groups.

potential in the brains of the mice fed the T3CD enriched diet (Figure 2(b)). Under the conditions investigated, T3CD versus control mice exhibited significantly higher TFAM protein levels (Figure 2(c)). However, the protein levels of PGC1$\alpha$ were similar between the groups as shown in Figure 2(d).

Dietary T3CD slightly increased (44% increase) Sod2 (Figure 3(a)) compared to that of controls. We also observed slightly higher Hmox1 (22% increase) and Gclm (14% increase) levels as well as higher proteasomal activity (28% increase) in the brains of mice fed the T3CD enriched diet; however, the differences did not reach statistical significance (Figures 3(b), 3(c), and 3(f)). Brain Gpx4 and Cat mRNA concentrations (Figures 3(d) and 3(e)) remained unchanged by the different dietary treatments.

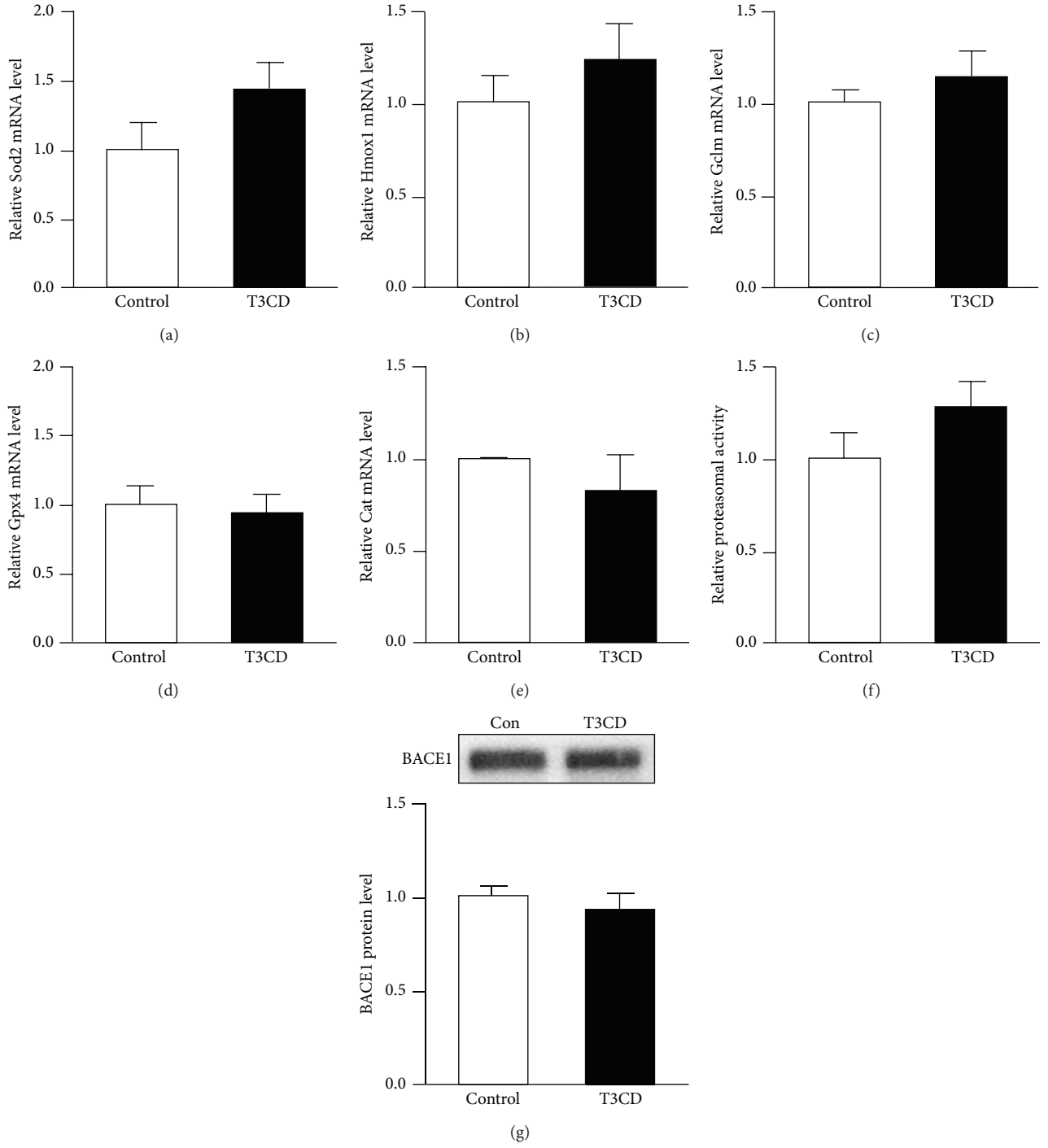



Figure 3: Effect of tocotrienol/$\gamma$-cyclodextrin supplementation on the mRNA levels of Sod2, Hmox1, Gclm, Gpx4, and Cat, proteasomal activity, and the BACE1 protein levels in mouse brain. Relative mRNA levels of (a) Sod2, (b) Hmox1, (c) Gclm, (d) Gpx4, and (e) Cat were determined by qRT-PCR and were related to the mean of the housekeeping gene expression. (f) Proteasomal activity was measured in the brain tissue by releasing the initially quenched fluorescence signal of the substrate through cleavage by the specific proteasome site. (g) BACE1 protein levels were determined by Western blotting and subsequent densitometric analysis of target bands. Target protein expression was related to the total protein fluorescence transferred to the PVDF membrane. Representative blots from one of 5–8 animals per group are shown. Values are the means + SEM from 5 to 8 animals per group.

Table 4: Feed intake [g], final body weight [g], and energy expenditure [kJ/(h∗kg$^{0.75}$)] in mice fed a control diet or a diet supplemented with tocotrienol/$\gamma$-cyclodextrin (T3CD).

| | Control | T3CD |
|---|---|---|
| Feed intake [g/d] | 2.91 ± 0.04 | 2.94 ± 0.03 |
| Final body weight [g] | 41.2 ± 2.11 | 44.5 ± 1.65 |
| Energy expenditure [kJ/(h∗kg$^{0.75}$)] | 22.9 ± 0.46 | 22.3 ± 0.47 |

Values are means ± SEM.

Furthermore, the BACE1 protein concentrations were measured in this study in response to dietary T3CD. However, there were no significant differences in the BACE1 protein concentrations between the T3CD and control mice (Figure 3(g)). In addition, gamma secretase protein concentrations (data not shown) remained unchanged by the different dietary treatments.

## 4. Discussion

It is well documented in the literature that mitochondrial biogenesis is impaired in aging [5]. Therefore, in this study, we included middle-aged mice (21 months old by the end of the study) in our feeding trial.

Differences in feed intake may affect mitochondrial function. In fact, caloric restriction has been reported to affect mitochondrial biogenesis and specific TFAM binding to mitochondrial DNA in laboratory rodents [17]. Therefore, over the 6-month experiment, we continuously monitored feed intake, which was not significantly different between the controls and the T3CD-fed mice. Accordingly, no differences in body weight or energy expenditure were evident between the groups.

In the present study, we found higher ATP concentrations and higher mitochondrial membrane potential in the murine brain due to dietary T3CD. Thus T3CD may partly counteract an aging brain phenotype in terms of mitochondrial function. We suggest that higher ATP concentrations and MMP may be related to higher TFAM concentrations. Studies in transgenic mice indicate that TFAM is a direct regulator of mtDNA copy number [18], thereby driving mitochondrial biogenesis. Our in vivo data in mice are consistent with in vitro studies demonstrating that T3 improves mitochondrial respiration, coupling, and membrane potential and maintains oxidative phosphorylation and ATP levels in cultured cells [19].

PGC1$\alpha$ is a master switch in energy metabolism. A decrease in PGC1$\alpha$ may impair mitochondrial respiratory capacity, thereby increasing the production of reactive oxygen species [20]. Unlike the reported effects of coenzyme Q10, lipoic acid [20], and tocopherol [21], we did not observe an increase in PGC1$\alpha$ in T3CD treated animals. Thus, the increase in mitochondrial membrane potential and ATP concentrations in the brain of our aged mice due to T3CD was possibly not directly mediated via a PGC1$\alpha$-dependent signal transduction pathway. Possibly, other key regulators of mitochondrial biogenesis (e.g., sirtuins, endothelial nitric oxide synthase, and mitofusin) may be involved in the tocotrienol-mediated increase in mitochondrial membrane potential and ATP concentration, which warrants further investigation.

Superoxide dismutase, $\gamma$-glutamyl cysteinyl synthetase, and heme oxygenase 1 are Nrf2 target genes. Studies in laboratory mice have revealed that the induction of the Nrf2-driven antioxidant response confers neuroprotection during mitochondrial stress in vivo [22]. Furthermore, it has been shown in cultured cells that $\gamma$- and $\delta$-tocotrienol may induce Nrf2-dependent signal transduction pathways [23, 24]. In addition, there is crosstalk between Nrf2 and the proteasome [25]. Therefore, we measured Sod2, Hmox1, and Gclm gene expression as well as proteasomal activity in mice brain in response to dietary T3CD. Under the conditions investigated, only a moderate increase in Sod2, Hmox1, Gclm, and proteasomal activity due dietary T3CD was observed. Further studies should address the question of whether higher dietary T3CD concentrations lead to a more pronounced induction of Nrf2 and its target genes.

We observed no changes in BACE1 in response to dietary T3CD. Similar results were observed in our previous studies in mice [26] and rats [27] that were fed diets containing different concentrations of $\alpha$-tocopherol; thus tocopherols and tocotrienols seem to have little to no effect on BACE1 expression in laboratory rodents.

The present study has several limitations. First, we did not measure T3 transfer into the brain because of the limited availability of brain tissue, all of which was used for ATP, MMP, Western blot, and RT-PCR analyses. Brain T3 concentrations have been reported elsewhere [28]. However, we measured the transfer of $\delta$-T3 into white adipose tissue. In the T3CD-fed mice, we analyzed 16.0 ± 1.90 nmol T3 per g fresh matter indicating sufficient absorption and tissue distribution of tocotrienols provided as dietary T3CD. Furthermore, we did not conduct brain imaging [29], behavioral and cognitive testing in response to dietary T3 as previously described [28]. Only few rodent species spontaneously develop the cognitive, behavioral, and neuropathological symptoms of age-related diseases [30]. Thus, such tests should be conducted in mice exhibiting a pronounced brain aging phenotype (e.g., transgenic mouse models of Alzheimer's disease).

## 5. Conclusions

In conclusion, our results suggest that complexation of annatto-derived tocotrienols with $\gamma$-cyclodextrin significantly enhances mitochondrial membrane potential and ATP concentrations in the brains of aged mice. Further in vivo studies are required to elucidate whether dietary T3CD affects behavior and cognitive function in animal models of age-related brain disorders.

## Abbreviations

ADAM: A disintegrin and metalloproteinase domain-containing protein 10
ATP: Adenosine triphosphate
BACE1: Beta secretase 1
Cat: Catalase
CD: Cyclodextrin

Gclm: Glutamate-cysteine ligase, modifier subunit
Gpx4: Glutathione peroxidase 4
Hmox1: Heme oxygenase 1
MMP: Mitochondrial membrane potential
mtDNA: Mitochondrial DNA
Nrf2: Nuclear factor erythroid-like 2 derived factor 2
PGC1: Peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$)
Sod2: Superoxide dismutase 2
T3: Tocotrienol
T3CD: Tocotrienol/$\gamma$-cyclodextrin
TFAM: Transcription factor A, mitochondrial.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# The Role of Oxidative Damage in the Pathogenesis and Progression of Alzheimer's Disease and Vascular Dementia

**Maria Luca,[1] Antonina Luca,[2] and Carmela Calandra[1]**

[1]*Psychiatry Unit, Department of Medical and Surgical Sciences and Advanced Technologies, University Hospital Policlinico-Vittorio Emanuele, Santa Sofia Street 78, Catania, 95100 Sicily, Italy*
[2]*Department of "G.F. Ingrassia", University Hospital Policlinico-Vittorio Emanuele, Santa Sofia Street 78, Catania, 95100 Sicily, Italy*

Correspondence should be addressed to Carmela Calandra; c.calandra@unict.it

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Oxidative stress (OS) has been demonstrated to be involved in the pathogenesis of the two major types of dementia: Alzheimer's disease (AD) and vascular dementia (VaD). Evidence of OS and OS-related damage in AD is largely reported in the literature. Moreover, OS is not only linked to VaD, but also to all its risk factors. Several researches have been conducted in order to investigate whether antioxidant therapy exerts a role in the prevention and treatment of AD and VaD. Another research field is that pertaining to the heat shock proteins ($Hsp_s$), that has provided promising findings. However, the role of OS antioxidant defence system and more generally stress responses is very complex. Hence, research on this topic should be improved in order to reach further knowledge and discover new therapeutic strategies to face a disorder with such a high burden which is dementia.

## 1. Oxidative Stress and Brain Aging

Redox homeostasis is a complex mechanism that can be resumed as the maintenance of the balance between reactive oxygen species (ROS) production and elimination [1]. Largely generated from mitochondria, ROS are by-products of cellular metabolism [2]. Among them, we include free radicals (superoxide), hydroxyl radicals (the most reactive species), and nonradicals (hydrogen peroxide). Even though ROS exert a role in crucial physiological processes, such as signaling and apoptosis [3, 4], they are highly reactive species; as a result, they can damage proteins, lipids, deoxyribonucleic acid (DNA), and sugars with remarkable negative consequences on the cellular functioning [5]. The antioxidant defence system, composed by nonenzymatic and enzymatic antioxidants (e.g., glutathione, flavonoids, superoxide dismutase (SOD), catalases, and glutathione peroxidase (GPx)), protects the cells from the ROS-related injuries [6]. If the redox homeostasis fails, this system is not sufficient to counteract the high amount of ROS and the so-called "oxidative stress" (OS) occurs [7]. The balance between oxidants and antioxidants is not a static condition and a great number of stimuli can interfere with the redox status. Hence, OS has been recently redefined as a "disruption of redox signaling and control" [8]. OS and its detrimental effects on the cellular functioning have been demonstrated to be involved in aging [9], as well as in a variety of illnesses, particularly age-related ones, among which are diabetes [10], atherosclerosis [11], mild cognitive impairment (MCI) [12], Parkinson's disease [13], and other neurodegenerative disorders, such as Huntington's disease [14] and amyotrophic lateral sclerosis [15]. In addition, OS seems to be involved in the pathogenesis of the two major types of dementia: Alzheimer's disease (AD) and vascular dementia (VaD) [16]. The importance of OS in so many neurodegenerative disorders is not surprising, since the brain is highly susceptible to ROS, because

(1) it is rich in fatty acids, which are sensible to peroxidation;

(2) it has not a powerful antioxidant activity;

(3) it consumes a lot of oxygen; therefore, it is exposed to free-radicals accumulation [17].

Previous researches highlighted the importance of OS in both normal brain aging and pathological brain aging [18], sharing the same altered biochemical and anatomopathological pattern: neural loss and altered mitochondrial activity and accumulation of degradated mitochondria and tangles [19–21]. However, AD brains show substantial qualitative and quantitative differences when compared to controls. More specifically, the redox homeostasis is different: the activity of mitochondrial pyruvate dehydrogenase, ketoglutarate dehydrogenase, and cytochrome oxidase is more severely affected; moreover, the antioxidant defence system is critically impaired [20, 22]. In addition, even though normal brain aging is related to the accumulation of degradated mitochondria and tangles, both conditions are more represented in AD [20, 21]. The amount of tangles certainly increases with age, particularly in the hippocampus. However, it is remarkably higher in demented brains and the more the dementia is severe, the more the tangles affect the neocortex, which is usually spared in normal brain aging [21].

High levels of peripheral markers of oxidative stress and low antioxidant power have been reported in patients with MCI, late onset AD, and VaD. Even though the three disorders seem to share a common oxidative-related pathogenesis, they maintain distinctive features, since other variables (e.g., homocysteine levels) allow for their differentiation [16]. An interesting study published in 2013 reported how young healthy individuals at risk of developing AD (as determined through genetic analysis) presented an apparently paradoxical condition: high levels of antioxidants and *reductive*, rather than oxidative, stress. On the contrary, in case of overt AD, the opposite situation was noticed: low antioxidants and high indicators of oxidative stress. The hypothesis is that the individuals at risk of developing AD, presenting an increased generation of ROS, respond with an overexpression of antioxidants, thus suffering from reductive stress. Later on, the antioxidant defence system collapses and the OS becomes evident, along with the symptoms of dementia [23]. The role of OS in the physiopathology of dementia is very complex and the knowledge pertaining to this topic needs to be enhanced. In the present review, data on the role of OS in AD and VaD, as well as a discussion on the therapeutic implications of such a role, are reported.

## 2. OS and AD

In the last decades, several researches investigating the role of OS in neurodegenerative disorders have been conducted. Despite the fact that the knowledge on this topic is certainly larger than before, it is still unclear whether OS is the cause or the consequence of the neurodegenerative processes. Notwithstanding, it is almost certain that OS is involved in the crucial events leading to the neural death and in the propagation of such events. Hence, if the complex and various neurodegenerative phenomena are intended as a cycle for more than a cascade, it is clear that, one way or another, OS is the "main actor" [24]. AD, the most common cause of dementia in the elderly, is an age-related neurodegenerative disorder causing the progressive loss of the higher cerebral functions, such as memory, language, and cognitive thinking, with huge consequences on mood, behaviour, and self-sufficiency [25]. This type of dementia is characterized by the accumulation of misfolded beta-amyloid (A$\beta$), a protein produced from the cleavage of the amyloid precursor protein (APP), and neurofibrillary tangles (aggregates of hyperphosphorylated tau protein) in the brain [26, 27]. Evidence of OS and OS-related damage in AD is largely reported in the literature [27–30]. More specifically, markers of lipid peroxidation have been found in plasma, urine, and cerebral tissue of transgenic mice models of AD amyloidosis [31]; OS-related DNA damage has been demonstrated in AD patients, so that some authors suggested that urinary oxidized nucleosides could be used as biomarkers [32]; high levels of carbonyls, markers of protein oxidation, have been found in AD brains [33]. Also tau phosphorylation has been related to OS and it is known that hyperphosphorylation is responsible for its misfolding [34, 35]. It is known that the ROS production is crucially involved in the physiological mechanisms regulating folding, misfolding, and the elimination of unfolded proteins [36–38]. The endoplasmic reticulum (ER) plays a fundamental role in the regulation of protein folding. In case of abundance of misfolded proteins, ER stress occurs, thus determining an enhanced production of ROS during the oxidative folding process (formation of disulfide bonds) and the uncontrolled accumulation of unfolded/misfolded proteins [36, 39]. The consequential depletion of the antioxidant glutathione and the ROS-related damage of the mitochondrial electron transfer system amplify the production of ROS and lead to cell death. In fact, the ER alteration is one of the common features linking neurodegenerative disorders, mostly characterized by protein misfolding, to each other [36]. In addition, a sustained OS alters the functions of the ubiquitin-proteasome pathway, which is responsible for the degradation of the damaged proteins [38]. As far as AD is concerned, there are several conditions causing the excessive production of ROS: mitochondrial dysfunctions, A$\beta$-related microglial activation, inflammation, and binding of redox active metals to deposits [40]. A deficiency in cytochrome c oxidase has been reported in platelets and in postmortem brain tissue of AD patients. As a result, mitochondrial degradation is stimulated and neurons are damaged by the mitochondrial debris and the excessive production of ROS [41]. As if that were not enough, the altered mitochondria generate high levels of ROS, exposing themselves to the OS-related injuries to which they are so sensitive [42]. Another important issue in AD is inflammation. In fact, this neurodegenerative disorder is characterized by an uncontrolled inflammatory activation of microglial cells [43]. The peroxisome proliferator-activated receptor gamma (PPAR-$\gamma$) is a regulator of the inflammatory processes which exerts anti-inflammatory properties [44]. OS, leading to the phosphorylation of PPAR-$\gamma$, is responsible for the functional alteration of this important transcription factor [45]. In addition, it has been reported that mild OS could trigger the amyloid cascade being, therefore, involved in the very early stages of AD: in fact, it causes an alteration of the subcellular compartmentalization of BACE1 (beta-site APP cleaving enzyme 1), an enzyme involved in the $\beta$-secretase cleavage of the APP; as a result, the amyloidogenic processing of APP is favoured [46]. As in a vicious cycle,

A$\beta$ produces ROS through a metal-catalyzed reaction [47]. The lesions observed in brains suffering from AD are those typical of OS (e.g., damage to DNA, protein oxidation, and lipid peroxidation) [18] and contain metals (e.g., zinc, iron) exerting catalytic activity and causing ROS production [48]; these metals have been demonstrated to be highly represented in AD brains [49]. From what has been discussed above, it is apparent that OS is involved in the occurrence of the core aspects of AD, that is, phosphorylation and misfolding [46, 50]. Obviously, the exposure of OS stimulates the activation of compensatory responses [51]. Unfortunately, both enzymatic and nonenzymatic antioxidant defences seem to be impaired in AD patients [52, 53]. Even though literature data are not exempted from inconsistencies [22], it is plausible that SOD could be induced by OS in the early stages of AD and, then, consumed in the advanced stages [22]. GPx and glutathione reductase (GR) seem to be, respectively, higher and lower in AD patients versus controls. Hence, the GR/GPx activity ratio turns out to be higher in healthy subjects, intermediate in MCI, and lower in AD patients. In addition, the ratio has been found to be positively correlated with the scores at the Mini Mental Sate Examination (MMSE), a tool used to assess the cognitive performances [12]. It is easy to imagine that since GR (which regenerates reduced glutathione from oxidized glutathione (GSSG)) is low, the levels of GSSG should be high. In fact, the levels of GSSG are higher in AD patients and relate to the severity of the dementia [22]. As far as nonenzymatic defences are concerned, even in this case literature data show some inconsistencies, but various studies report vitamin deficiency in patients versus controls [22]. Since the redox homeostasis is so deeply altered, the neurons are dangerously exposed to the detrimental effects of OS and to the fearsome mechanism of neurodegeneration.

## 3. OS and VaD

VaD is the second cause of dementia in the elderly. Executive functions, more than memory, are severely impaired in this type of dementia [54]. Even if hypoxia and haemorrhagic stroke (e.g., subdural haematoma) can cause VaD, the latter is mostly related to ischaemic stroke [55]. In particular, one of the most common forms of VaD, the subcortical one, is caused by multiple subcortical ischaemic lesions. Hypertension, diabetes mellitus, hypercholesterolemia, and hyperhomocysteinemia favour the occurrence of atherosclerosis, cardiovascular diseases, and stroke and represent important risk factors for VaD [54]. As far as oxidative stress is concerned, it is linked not only to VaD, but also to all its risk factors; in fact, OS has been demonstrated to play a role in the pathogenesis of diabetes [10] and to be involved in the tissue toxicity determined by hypercholesterolemia [56] and hyperhomocysteinemia [57]. Moreover, it has been reported that the dysfunction of mitochondrial proteins, leading to OS, is involved in the hypertension-related target organ damage affecting vasculature, heart, kidneys, and brain [58]. Mitochondrial dysfunction is considered to be an important step in the pathogenesis of atherosclerosis, also because it subtends the previously mentioned risk factors [59]. The OS-related oxidation of low-density lipoproteins (LDL) is crucial in the atherosclerotic process [60, 61] and high levels of lipid hydroperoxides have been reported in patients with ischemic stroke [62]. As a matter of fact, patients with VaD have been found to show high levels of malondialdehyde (a marker of lipid peroxidation) and these levels were higher than those reported in AD patients [63]. The association between folate deficiency and OS-related LDL dysfunction seems to be typical of VaD and could help to differentiate it from other types of dementia [64]. In addition, OS is certainly a mediator of the stroke-related neuronal damage and cognitive dysfunctions, as demonstrated by the high levels of plasmatic ROS in patients with ischaemic stroke as well as the finding of oxidative DNA damage within the peri-infarct brain regions in rats [65–67]. Moreover, OS is indirectly and directly involved in the deep alterations of the blood-brain barrier (BBB) occurring after an ischaemic brain injury. More specifically, the activation of metalloproteinases and the proteases involved in the proteolytic disruption of the BBB and in the white matter lesions typical of VaD is strictly linked to OS [68, 69]. In addition, ROS are directly responsible for the alterations in the cerebral perfusion and permeability, thus contributing to the cerebrovascular disease [70]. The OS-induced dysfunction of the previously cited anti-inflammatory agent PPAR-$\gamma$ is involved in vascular aging [71]. In practice, OS and inflammation "cooperate" in determining the endothelial damage and the BBB failure occurring in VaD [72]. As in AD, even in VaD the antioxidant defence system seems to be insufficient. Apart from the previously reported folate deficiency [64], also vitamin E has been found to be lower in VaD versus controls and also versus AD [73]. In addition, SOD and GR are reduced in VaD too [74]. However, it is worth pondering over that, from a clinical point of view, the difference between AD and VaD is not so strict. In fact, microvascular degeneration and atherosclerotic processes are often documented in AD, in which an ROS-related amyloid cerebral angiopathy occurs [75–77]. In addition, the LDL oxidation, involved in the atherosclerotic process as previously mentioned [60, 61], seems to be a common feature shared by AD and VaD [78]. An important antioxidant enzymatic system, influenced by the redox status [79], protecting LDL from oxidation and exerting anti-inflammatory properties, is represented by the serum paraoxonases [78, 80]. Paraoxonase activity (as a protective factor) and macrophage OS (as a deleterious condition) take part in atherogenesis [81, 82]. Additionally, macrophage OS has been related to the paraoxonase 1 deficiency [83]. Considering dementia, both AD and VaD patients have been found to show a lower paraoxonase activity when compared to controls [78]. In the light of what has been discussed, OS represents a common important contributor in the pathogenesis of these two forms of dementia, whether alone or in comorbidity (mixed AD and VaD) [76]. Moreover, not only GR/GPx ratio, but also other markers of OS have been related to cognitive performances in dementia. High levels of 4-hydroxynonenal and malonaldehyde relate to worse scores at the MMSE [12, 84]. Notwithstanding, the use of biomarkers as predictors of

severity or outcome of dementia seems to be not strongly enough supported, at least for now [22].

## 4. Antioxidants in AD and VaD: Future Therapeutic Perspectives

Several researches have been conducted in order to investigate whether antioxidant therapy exerts a role in the prevention and treatment of AD and VaD. The findings arisen from a cohort study considering more than 1300 subjects indicate that the intake of flavonoids is inversely related to the risk of incident dementia [82]. Hence, vitamin supplementation could play a positive effect in both AD and VaD [85, 86]. It has been demonstrated that curcumin reduces the levels of A$\beta$ in cell lines and mouse primary cortical neurons and exerts a neuroprotective effect in vascular dementia enhancing the expression of antioxidants in rats and ischemic cells [87, 88]. In fact, the enhancement of the enzymatic defences is the key of the efficacy of treatments such as the EUK-207 (SOD/catalase mimetic) in mice with AD and resveratrol in rats with VaD [89, 90]. It has been reported that resveratrol increases SOD activity and glutathione levels in the cerebral cortex and hippocampus of rats with VaD [90]. Another research field is pertaining to the heat shock proteins ($Hsp_s$), which has provided interesting findings. It is well known that OS is involved in the activation of such important chaperons, which regulate the aggregation of misfolded proteins and apoptosis [91]. However, in case of neurodegeneration, the chaperones could paradoxically facilitate the aggregation of disease-related proteins, attempting to repair them and trying to avoid the formation of toxic aggregates [92]. This data explains why Hsp90 inhibitors are one of the promising therapeutic tools for the treatment of AD [93, 94]. It has been affirmed that Hsp90 "may play a role in maintaining pathogenic changes that lead to neurodegenerative diseases" [95]. This concept is easier to understand if it is considered that the tau protein is a client protein for Hsp90 [96] and that the latter is a repressor of the heat shock factor-1 (HSF-1), which regulates the heat shock response through the expression of $Hsp_s$ [93, 97]. Hsp90 inhibitors, through the activation of HSF-1, exert neuroprotective effects favouring the induction of $Hsp_s$, such as Hsp70 [93, 98], that has been found to exert therapeutic properties in mice with AD [99]. $Hsp_s$ are also expressed in response to ischemic brain lesions [100, 101] and transgenic mice expressing human inducible Hsp70 have shown to have ischemia-resistant hippocampal neurons [102]. Since inflammation plays an important role in the pathogenesis of AD and VaD [43, 72], the enhancement of the anti-inflammatory defence, through PPAR-$\gamma$ agonists, could represent another potential target for the treatment of these severe dementias. In mice models of AD, PPAR-$\gamma$ agonism resulted in the reduction of parenchymal A$\beta$, microglial activation, and neural loss [43]. In addition, it showed efficacy in improving reversal learning [103]. The oral antidiabetic drugs pioglitazone and rosiglitazone, exerting agonistic properties on PPAR-$\gamma$, were found to reverse some clinical (memory, learning) and biochemical (OS, endothelial dysfunction) features of diabetes-induced VaD dementia [104]. Moreover, their antiatherogenic effect is not only linked to their insulin sensitizing properties, but also to the modulation of endothelial activation markers, platelet activity, and vasodilatation. Hence, their therapeutic effects could be useful also for patients without diabetes mellitus affected by dementia [105]. However, the antioxidant therapy has not reached the aimed results. Among the more credible causes of this failure, (a) the activity of many nutritional antioxidants is strictly linked to that of other antioxidants; hence, monotherapy could not be sufficient; (b) therapy is often administered in too advanced stages of dementia; (c) since the brain is separated from the periphery trough the BBB, the peripheral redox status may not reflect the cerebral homeostasis; (d) the researches available used different methods to analyze the antioxidant levels in blood in order to evaluate the outcome after therapy [106]. Therefore, there are many fascinating plausible therapeutic targets that need to be further investigated to add new and more efficient therapeutic tools to the nowadays available disappointing options.

## 5. Concluding Remarks

In the light of what has been discussed, OS seems to be a crucial contributor in the pathogenesis of AD and VaD, directly or indirectly affecting the steps leading to neurodegeneration. AD and VaD are linked by many anatomical features, as well as by OS. However, the role of OS, antioxidant defence system and, more generally, stress responses, is very complex. Hence, research pertaining to this topic should be improved in order to reach further knowledge and discover new therapeutic strategies to face a disorder with such a high burden which is dementia.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Maria Luca and Antonina Luca contributed equally to this work.

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*Research Article*

# Epicatechin Reduces Striatal $MPP^+$-Induced Damage in Rats through Slight Increases in SOD-Cu,Zn Activity

**M. Rubio-Osornio,[1] E. Gorostieta-Salas,[1] S. Montes,[2] F. Pérez-Severiano,[2] C. Rubio,[1] C. Gómez,[3] C. Ríos,[2] and J. Guevara[4]**

[1]*Laboratorio Experimental de Enfermedades Neurodegenerativas, Instituto Nacional de Neurología y Neurocirugía, Manuel Velasco Suárez, 14269 Mexico City, Mexico*
[2]*Departamento de Neuroquímica, Instituto Nacional de Neurología y Neurocirugía, Manuel Velasco Suárez, 14269 Mexico City, Mexico*
[3]*Departamento de Farmacología, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 Mexico City, Mexico*
[4]*Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 Mexico City, Mexico*

Correspondence should be addressed to J. Guevara; jorge.guevara@comunidad.unam.mx

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Parkinson's disease is a neurodegenerative disorder characterized by movement alterations caused by reduced dopaminergic neurotransmission in the nigrostriatal pathway, presumably by oxidative stress (OS). $MPP^+$ intrastriatal injection leads to the overproduction of free radicals (FR). The increasing formation of FR produces OS, a decline in dopamine (DA) content, and behavioral disorders. Epicatechin (EC) has shown the ability to be FR scavenger, an antioxidant enzyme inductor, a redox state modulator, and transition metal chelator. Acute administration of 100 mg/kg of EC significantly prevented ($P < 0.05$) the circling $MPP^+$-induced behavior (10 $\mu$g/8 $\mu$L). Likewise, EC significantly ($P < 0.05$) reduced the formation of fluorescent lipid products caused by $MPP^+$. $MPP^+$ injection produced ($P < 0.05$) increased enzymatic activity of the constitutive nitric oxide synthase (cNOS). This effect was blocked with acute EC pretreatment. Cu/Zn-dependent superoxide dismutase (Cu/Zn-SOD) activity was significantly ($P < 0.05$) reduced as a consequence of $MPP^+$ damage. EC produced a slight increase (≈20%) in Cu/Zn-SOD activity in the control group. Such effects persisted in animals injured with $MPP^+$. The results show that EC is effective against $MPP^+$-induced biochemical and behavioral damage, which is possible by an increase in Cu/Zn-SOD activity.

## 1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by deficiencies in dopaminergic neurotransmission of the ascending nigrostriatal pathway. PD is the second most common neurodegenerative disorder after Alzheimer's disease, reaching millions around the world, prevalently in people over 60 years of age [1]. The main clinical features in patients with PD are resting tremors, rigidity, bradykinesia, and loss of postural stability [2]. Several theories propose that dopaminergic neuronal death is the result of oxidative stress (OS), mitochondrial dysfunction, and iron deposits (Fe) in the *Substantia nigra pars compacta* (*SNpc*) [1]. Oxidative stress is associated with mitochondrial dysfunction and the concomitant overproduction of radical anion superoxides ($^{\bullet}O_2^-$), along with decreased levels of reduced glutathione (GHS) in the SNpc [3]. Accumulation of Fe in the same region contributes to the overproduction of free radicals (FR), since the reaction between ferrous iron ($Fe^{2+}$) and hydrogen peroxide ($H_2O_2$) leads to the formation of hydroxyl radicals ($^{\bullet}OH$) via Fenton's reaction [4]. This generates lipid peroxidation and neuron death [5]. Intrastriatal injection of 1-methyl-4-phenylpyridinium ($MPP^+$) in rats reproduces the main biochemical characteristics of PD. $MPP^+$ is incorporated into dopaminergic terminals via the dopamine reuptake transporter. $MPP^+$ neurotoxicity is based on the complex I inhibition of the mitochondrial electron transport chain, leading to FR overproduction, lower oxidative phosphorylation, and ATP levels [6]. The entry of $Ca^{2+}$ through the NMDA receptor channel stimulates

$NO^{\bullet}$ overproduction by nNOS [7]. The $NO^{\bullet}$ readily reacts with $^{\bullet}O_2^-$ to generate ONOO-, thus promoting tyrosine residue nitration [8] in proteins such as tyrosine hydroxylase (TH), which results in diminished enzymatic activity of TH, which reduces striatal DA content and generates behavioral alterations [9, 10].

Polyphenols are present in high concentrations in fruits, vegetables, and beverages such as green tea and red wine, contributing to their beneficial effects [11]. Epicatechin (EC) is a polyphenol, a secondary metabolite produced in plants from the biosynthetic pathway of flavonoids [12]. It has been suggested that the flavonoids' neuroprotective effect is due mainly to its ability to trap FR [13] and to chelate transition metals [14]. Moreover, experimental evidence indicates that the flavonoid-metal complex mimics superoxide dismutase [15].

The aim of this study was to determine the effect of EC on both behavioral and oxidative damage induced by $MPP^+$. We hypothesize that the administration of EC in rats is able to decrease the effect of free radicals, in response to oxidative stress associated with the microinjection of $MPP^+$ in the rat striatum.

## 2. Materials and Methods

### 2.1. Experimental Procedures

*2.1.1. Animals, Epicatechin, and $MPP^+$ Injection.* The use and care of animals were done according to official regulatory guidelines (NOM-62-ZOO-1999). Male Wistar rats (280–300 g) were used throughout the study. The animals were housed in acrylic box cages and placed under constant conditions of temperature, humidity, and lighting (12 h light/dark cycles) and provided with a standard commercial rat chow diet and water *ad libitum*. Rats were administered with a 100 mg/kg oral dose (o.p.) of epicatechin (EC) (Sigma-RBI, St. Louis, MO, USA, Cat. E1753) in 10% DMSO. Five hours after the administration of EC, the animals were infused in the right striatum with 10 $\mu$g of $MPP^+$ iodide (Sigma-RBI, St. Louis, MO, USA, Cat. D048) in 8 $\mu$L of sterile saline (s.s.), under sodium pentobarbital anesthesia (40 mg/kg i.p.). The stereotaxic coordinates were 0.5 mm anterior to bregma, −3.0 mm lateral to bregma, and −4.5 mm ventral to the dura, according to the stereotaxic atlas of Paxinos and Watson [16].

*2.1.2. Circling Behavior.* Apomorphine-induced circling behavior was assessed in rats as previously described [10]. Six days after $MPP^+$ intrastriatal injection, animals were treated with apomorphine (1 mg/kg, subcutaneous) and then placed into individual box cages. Five minutes later, the number of ipsilateral rotations at the lesioned striatum was recorded for 60 min. Rotations were considered as 360° turns and results were expressed as the total number of ipsilateral turns in a one-hour period (turns/h).

*2.1.3. Striatal Dopamine Levels Measurement.* Animals were sacrificed by decapitation and the striatum dissected twenty-four hours after the circling behavioral test. HPLC with electrochemical detection was used to measure striatal levels of DA. Samples were sonicated in 10 volumes of a perchloric acid-sodium metabisulfite solution (1 M 0.1% w/v) and centrifuged at 10,000 ×g for 10 min, and the supernatant was analyzed. Data were collected and processed by interpolation in a standard curve, as previously described [9]. Results are expressed as $\mu$mol of DA per mg of wet tissue.

*2.1.4. Fluorescent Lipid Products.* The effect of epicatechin on fluorescent striatal lipid product (FLP) formation was evaluated six h after $MPP^+$ injection. The striata were homogenized in 2.2 mL of sterile saline. One mL of the homogenate was then added to 4 mL of chloroform-methanol mixture (2 : 1, v/v). Tubes were then capped and vortexed for 10 s and the mixture was ice-cooled for 30 min to allow phase separation. The aqueous phase was discarded and a 1 mL of chloroformic layer was transferred to a quartz cuvette, to which 100 $\mu$L of methanol was added. Fluorescence was measured in a Perkin-Elmer LS50B luminescence spectrometer at 370 nm of excitation and 430 nm of emission. The protein content was measured according to the method described by Lowry and colleagues [17]. Results are expressed as arbitrary fluorescence units/$\mu$g of protein.

*2.1.5. Nitric Oxide Synthase (NOS) Activity.* NOS activity was measured based on the stoichiometric conversion of L-arginine to NO and L-citrulline [7], with slight modifications as previously described [18]. A volume of homogenized tissue containing 500 mg of protein was incubated for 30 minutes at 37°C in the presence of 10 mM of L-arginine HCl, 0.2 Ci of [3H]-L-arginine, 1 mM of NADPH, 100 nM of calmodulin, 2.5 mM of $CaCl_2$, and 30 mM of tetrahydrobiopterin. To quantify the activity of inducible $Ca^{2+}$-independent NOS, the incubation was performed in the presence of 0.1 mM of EGTA and 0.1 mM of EDTA, without adding $CaCl_2$. Reactions were stopped by adding a buffer containing 2 mM of EGTA, 2 mM of EDTA, and 20 mM of HEPES, pH 5.5. The reaction mixture was applied onto a 1 mL column of cationic interchange resin (Dowex-50W), which had been previously equilibrated with the stop buffer. This column retained labeled arginine and allowed [3H]-L-citrulline to elute through the column. [3H]-L-citrulline was quantified using a Beckman LS6500 scintillation counter. Results were expressed as ng [3H]-L-citrulline/500 mg of protein per 30 min.

*2.1.6. Superoxide Dismutase (SOD) Activity.* The superoxide dismutase activity (SOD) was measured using the xanthine/xanthine oxidase method described in [19]. The striatum was carefully weighed and homogenized (1 : 10 w/v) in a buffer consisting of 20 mM sodium bicarbonate, 0.02% Triton X-100, pH 10.2. After homogenization, 50 $\mu$L of clarified supernatant was added to 950 $\mu$L of reaction mixture, consisting of 10 $\mu$M of sodium azide, 100 $\mu$M of xanthine, 10 $\mu$M of reduced cytochrome c, and 1 mM of EDTA in 20 mM sodium bicarbonate, 0.02% Triton-X100, pH 10.2. The reaction was initiated by adding xanthine oxidase enzyme and monitored by measuring the changes in absorbance at 560 nm in a Lambda-20 Perkin Elmer UV/Vis spectrophotometer.

The samples were carried out in duplicate. Firstly, we analyzed the total SOD activity. Later, the Mn-SOD activity was evaluated by the addition of 5 mM of sodium cyanide to the reaction mixture, to selectively inhibit the Cu/Zn-SOD activity. The difference between total SOD and Mn-SOD reflected Cu/Zn-SOD activity. Results were expressed as the percentage variation compared with the respective control values (international units of SOD activity/g of wet tissue).

*2.1.7. Serum Alanine Aminotransferase, Alkaline Phosphatase, and γ-Glutamyl Transpeptidase Activity Determination.* Serum was obtained by cardiac puncture. Determination of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma glutamyl transpeptidase ($\gamma$-GT) activity was determined as reported elsewhere [20]. All results are in units of $\mu$mol/L/min.

*2.1.8. Statistics.* Results from the evaluation of circling behavior were analyzed by Kruskal-Wallis' test followed by Mann-Whitney's test. Results from dopamine quantification, lipid peroxidation, nitric oxide synthase activity, superoxide dismutase activity, and hepatic damage markers assays were analyzed by ANOVA followed by Tukey's test.

## 3. Results

*3.1. Circling Behavior.* The evaluation of the apomorphine-induced behavioral test was carried out 6 days after damage induced by $MPP^+$. In a pilot experiment to select the dose and the time to inject the EC, we observed that a subchronic oral dose of 40, 60, and 80 mg/kg of EC for 5 days was not able to reduce the turning behavior induced by $MPP^+$ (308 ± 28 ipsilateral turns) (data not shown). The subchronic dose of 100 mg/kg for five days reduced the turning behavior after $MPP^+$ to 204.1 ± 20.7, ipsilateral turns/60 min. This effect was not different from a similar, previous dose of EC given 5 h before the $MPP^+$ injury (data not shown). We therefore evaluated the effect of an acute dose of 100 mg/kg of EC administrated 5 h before the damage was induced by $MPP^+$ and then 6 days after the circling behavior was induced by apomorphine. The results indicated that a single acute oral EC dose of 100 mg/kg significantly reduced ($P < 0.05$) the circling behavior (213.3 ± 24.94, ipsilateral turns/60 min) related to the damage induced by $MPP^+$ (314.4 ± 29.87, ipsilateral turns/60 min). Single EC oral dose administration partially prevented the unbalanced turning behavior in $MPP^+$-treated rats (Figure 1(a)) in the same manner as the chronic oral administration.

*3.2. Striatal DA Content.* Twenty-four hours after the turning experiment, the animals were sacrificed. Striatal tissue was dissected and the DA content was measured. The group treated with the vehicle and saline solutions was considered as the control (51.72 ± 4.92 $\mu$g DA/mg wet tissue). EC treatment alone plus saline did not show differences from the control group (58.41 ± 7.4 $\mu$g DA/mg wet tissue). In the animals group treated with $MPP^+$, a significant ($P < 0.01$) decrease in dopamine content (2.26 ± 0.43 $\mu$g DA/mg wet tissue) was found. Whereas the experimental group treated with EC 100 mg/kg plus $MPP^+$ prevented the dopamine decay (32.42 ± 6.84 $\mu$g DA/mg wet tissue), this result was consistent with the behavioral assay (Figure 1(b)).

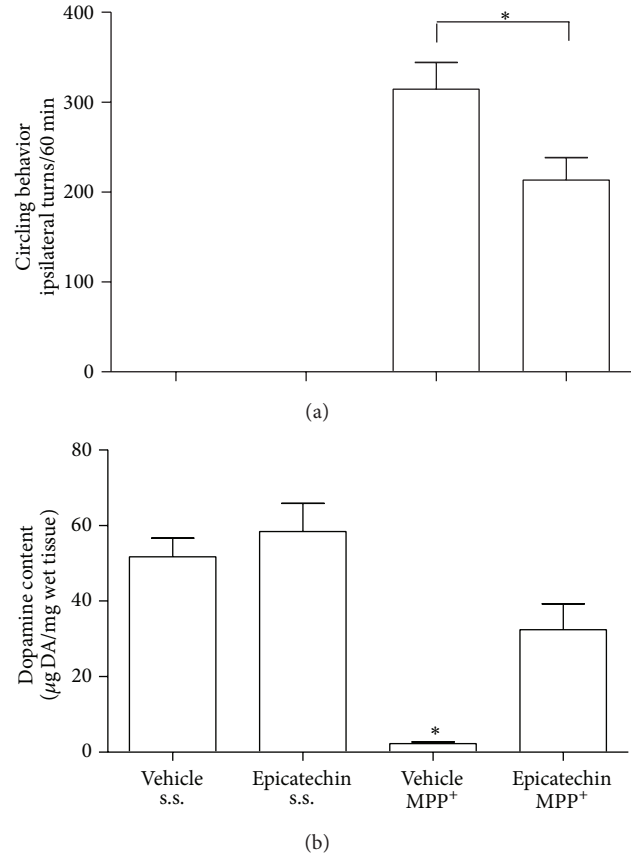


Figure 1: Effect of oral 100 mg/kg epicatechin (EC) on circling behavior and striatal dopamine content in $MPP^+$ model. (a) Six days after $MPP^+$-induced damage, the EC effect on apomorphine-induced circling behavior was evaluated. The results are expressed as mean ± S.M.E. of 6–8 animals per group. $^*P < 0.05$; data were analyzed by Kruskal-Wallis' test followed by U Mann-Whitney's test. (b) Twenty-four hours after the behavioral test evaluation, dopamine was determined by HPLC coupled to an electrochemical detector. Results are expressed as mean ± S.E.M. of 6–8 animals per group. $^*P < 0.01$, two-way ANOVA followed by Tukey's test.

*3.3. Lipid Peroxidation.* The results obtained from EC administration against $MPP^+$-induced oxidative damage are shown in Figure 2. The formation of fluorescent lipid products was measured 6 h after $MPP^+$ infusion as a short-term damage marker. Animals treated with the vehicle solution (oral pathway) and sterile saline (intrastriatal) were considered as the control group (1.315 ± 0.073, fluorescent units). We did not find any changes with respect to the control group with EC administration (1.347 ± 0.082) more than with the saline solution. However, $MPP^+$ infusion statistically increased ($P < 0.05$) the formation of lipid fluorescent products (1.969 ± 0.179). EC pretreatment prevented the formation of oxidized lipids induced by $MPP^+$ to levels shown in the control group (1.455 ± 0.087).

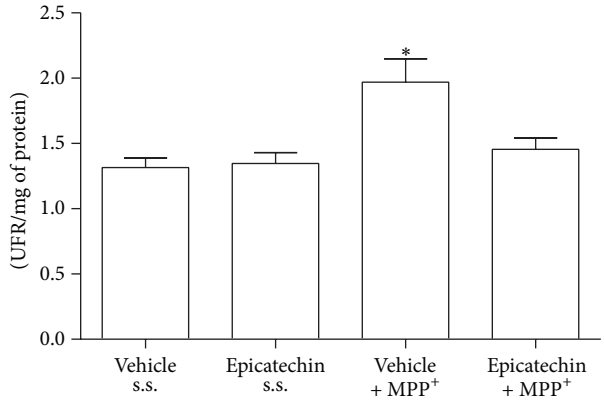


Figure 2: EC reduces $MPP^+$-induced striatal lipid peroxidation in rat. Six hours after $MPP^+$-induced damage, the formation of lipid fluorescent products was measured as an index of lipid peroxidation. The results are expressed as fluorescence arbitrary units. Each bar represents the mean ± S.E.M. of 8 animals per group. $^*P < 0.05$, two-way ANOVA followed by Tukey's test.

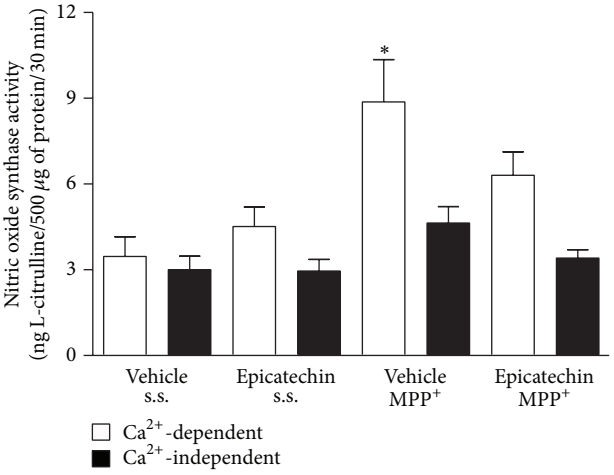


Figure 3: EC effect on striatal $Ca^{2+}$-dependent and $Ca^{2+}$-independent nitric oxide synthase activity. The $Ca^{2+}$-dependent NOS activity was significantly increased after $MPP^+$ treatment. The results are expressed as mean ± S.E.M. of 5–7 animals per group in ng L-citrulline/500 mg of protein/30 min. $^*P < 0.05$, two-way ANOVA followed by Tukey's test.

*3.4. Striatal Nitric Oxide Synthase Activity.* NOS striatal enzymatic activity was also assayed 6 hours after $MPP^+$ infusion. The control group showed levels of $Ca^{2+}$-dependent NOS activity (constitutive nitric oxide synthase (cNOS)) (3.46 ± 0.68, ng L-citrulline/500 mg protein/30 min), similar to those of the EC pretreated group (4.51 ± 0.69). However, NOS activity was statistically found to increase ($P < 0.05$) (8.86 ± 1.48) in the $MPP^+$-treated group. Acute oral, 100 mg/kg, administration of EC attenuated the effect of $MPP^+$ on $Ca^{2+}$-dependent NOS activity (6.30±0.82). $Ca^{2+}$-independent NOS activity remained unchanged in the striatal tissue from all groups studied in this time and no statistical differences were found among groups (Figure 3).

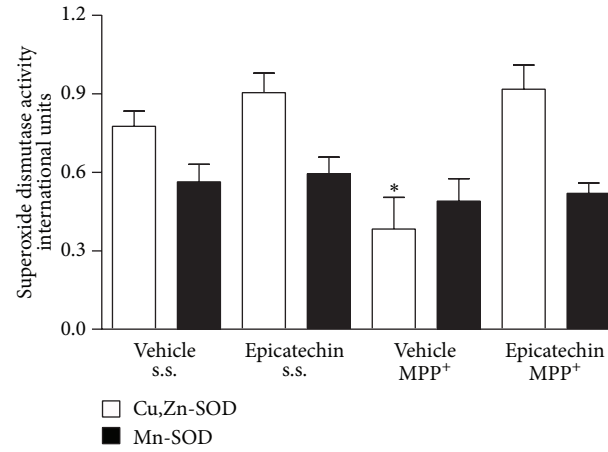


Figure 4: Effect of EC administration on the reduction in Cu/Zn-SOD activity $MPP^+$-induced. The infusion of $MPP^+$ statistically reduced Cu/Zn-SOD activity (white bars). The Mn-SOD activity did not change among groups (black bars). The results are expressed as mean ± S.E.M. of 6–8 animals per group. $^*P < 0.05$, two-way ANOVA followed by Tukey's test.

*3.5. Striatal Superoxide Dismutase Activity.* Figure 4 shows the results obtained from superoxide dismutase activity within the present paradigm. The white bars represent the results of Cu,Zn-dependent superoxide dismutase (SOD-Cu,Zn) activity. The animals treated with $MPP^+$ showed a significant ($P < 0.05$) decrease in superoxide dismutase activity (0.361 ± 0.14), international units, while the rest of the groups remained unchanged (0.774 ± 0.05, vehicle + s.s.; 0.909 ± 0.07, EC + s.s.; and 0.916 ± 0.09, EC + $MPP^+$). In this case, it is possible to observe a slight increase of 17.4% in the group treated with EC, more than in the saline solution group, while it is preserved in the experimental group treated with EC + $MPP^+$ (18.3%) and Mn-dependent superoxide dismutase (SOD-Mn) activity remained unchanged among groups (black bars).

*3.6. Liver Damage Indicators.* None of the hepatic damage markers analyzed in this study showed a toxic effect after EC administration. The group of animals treated with a single oral dose of 100 mg/kg EC had no effect on ALT, $\gamma$-GT, and ALP activity when compared with the control group. Likewise, differences were found between the control group treated with vehicle solution and $MPP^+$ and the groups treated with EC and $MPP^+$ (Table 1).

## 4. Discussion

Parkinson's disease (PD) is a neurodegenerative disorder with increasing incidence as life expectancy increases. There is currently no effective drug therapy to counteract the overproduction of free radicals (FR) and oxidative stress (OS) of the nigrostriatal pathway of patients with this disease. In this study, we initially compared the effect of the oral subchronic administration, of increasing doses (40, 60, 80, and 100 mg/kg), and of epicatechin (EC) with the circling

Table 1: Serum alanine aminotransferase, $\gamma$-glutamyl transpeptidase, and alkaline phosphatase as hepatic damage markers. No statistical differences among groups were found.

| Treatment | Liver damage indicators | | |
|---|---|---|---|
| | ALT | $\gamma$-GT | ALP |
| Vehicle + s.s. | 20.10 ± 1.90 | 56.20 ± 4.26 | 15.54 ± 2.45 |
| EC 100 mg/kg + s.s. | 21.28 ± 1.82 | 66.52 ± 6.21 | 14.52 ± 1.22 |
| Vehicle + $MPP^+$ | 17.50 ± 1.76 | 61.27 ± 9.32 | 13.80 ± 1.09 |
| EC 100 mg/kg + $MPP^+$ | 18.92 ± 2.14 | 52.53 ± 2.91 | 12.53 ± 1.20 |

behavior of rats treated with the dopaminergic toxin $MPP^+$, in order to determine an adequate EC dose. The results showed that the subchronic administration of 100 mg/kg of EC was able to decrease the ipsilateral turns induced by apomorphine administration in $MPP^+$-injured rats. We subsequently evaluated the effect of a single, 100 mg/kg, dose of EC. This dose decreased the circling behavior to the same level that we obtained by the same dose in a subchronic paradigm. This result of the circling behavior was corroborated by quantifying the striatal dopamine content and we found that the effect of a single oral administration of EC was consistent at both levels. The effect of polyphenols extracted from green tea against neurotoxicity induced by $MPP^+$ has been related to the blocking of the presynaptic DA transporter (DAT) [6, 21]. However, in this work, the EC exerted partial protection, both behaviorally and in DA content. This evidence suggests that the dose of EC used in this study did not block the DA transporter. Therefore, the effects observed might be the result of some other mechanism.

$MPP^+$-induced fluorescent lipid products formation was prevented by EC pretreatment, which presumably counteracted oxidative damage produced by $MPP^+$. EC ability to scavenge free radicals appears to be related to the chemical structure of the catechin molecule. A catechol functional group and a hydroxyl group activated a double bond C5 ring neutralizing free radical [22, 23]. Possibly the antioxidant action of EC resembles the action of (−)-epigallocatechin-3-gallate by trapping iron [22], thereby reducing the free iron intraneuronal pool, thus decreasing the overproduction hydroxyl radical ($^\bullet OH$) by the entry of the metal by Fenton's reaction [10]. Given the consistency of our results with the findings reported in the literature, our next goal was to identify the EC action mechanism in the striatum of the rat. We found that EC pretreatment was able to reduce the calcium-dependent nitric oxide synthase activity (cNOS) in $MPP^+$-injured rats, indicating that EC has a regulatory effect on the overproduction of $^\bullet ON$, probably through the ROS-$^\bullet ON$ pathway [24]. Interestingly, we observed that EC administration produces a slight increase in Cu,Zn superoxide dismutase (Cu,Zn-SOD) activity, which can be closely linked to its neuroprotective effect. Experimental evidence shows that the formation of flavonoid-metal complexes mimics SOD activity [15]. The overexpression of Cu,Zn-SOD in mice reduces striatal tyrosine hydroxylase nitration and inactivation induced by MPTP administration [25]. This supports the idea of a critical role for superoxide anion in $MPP^+$-induced neurotoxicity.

The effect of the acute administration of 100 mg/kg of EC on liver damage was evaluated for the classic markers. No adverse effects were found (Table 1). These results are consistent with the subchronic oral administration of green tea extracts, containing approximately 120 mg/kg of EC, in Wistar rats and showed no adverse effects on the liver [26]. Some reports indicate that the administration of 100 mg/kg of epigallocatechin-3-gallate in mice increases the plasma concentration of alanine transaminase (ALT), while intraperitoneal administration of 150 mg/kg is lethal [27]. Consequently, in the present study we show evidence of the neuroprotective effect of EC on the behavior and oxidative damage of $MPP^+$-induced in the rat striatum and on its circling behavior. These changes might be through the induction of an increase of Cu,Zn-SOD activity.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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