A defect in the mitochondrial Complex III, but not Complex IV, triggers early ROS-dependent damage in defined brain regions

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Abstract

We have created two neuron specific mouse models of mitochondrial electron transport chain deficiencies involving defects in Complex III (CIII) or Complex IV (CIV). These conditional knockouts (cKO) were created by ablation of the genes coding for the Rieske iron sulfur protein (RISP) and COX10 respectively. RISP is one of the catalytic subunits of CIII and COX10 is an assembly factor indispensable for the maturation of Cox1, one of the catalytic subunits of CIV. Although the rate of gene deletion, protein loss and complex dysfunction were similar, the RISP cKO survived 3.5 months of age, whereas the COX10 cKO survived for 10-12 months. The RISP cKO had a sudden death, with minimal behavioral changes. In contrast, the COX10 KO showed a distinctive behavioral phenotype with onset at 4 months of age followed by a slower but progressive neurodegeneration. Curiously, the piriform and somatosensory cortices were more vulnerable to the CIII defect whereas cingulate cortex and to a less extent piriform cortex were affected preferentially by the CIV defect. In addition, the CIII model showed severe and early ROS damage, a feature not observed until very late in the pathology of the CIV model. These findings illustrate how specific respiratory chain defects have distinct molecular mechanisms, leading to distinct pathologies, akin to the clinical heterogeneity observed in patients with mitochondrial diseases.
Introduction

Genetic defects affecting the function of the electron transport chain and the oxidative phosphorylation (OXPHOS) system are known as mitochondrial disorders. This group of diseases involves defects in either the nuclear or the mitochondrial DNA (mtDNA) and is heterogeneous in nature. Mitochondrial diseases can affect single or multiple organs. Tissues with higher energetic demands such as brain and muscle are most commonly affected (1).

In the last few years, effort has been concentrated in understanding the molecular bases of the phenotypic variability of mitochondrial disorders. The heterogeneous nature of mitochondrial diseases poses a challenge for the development of effective treatments. Advances in this area have been hampered by the lack of appropriate animal models with a single respiratory defect. In the last few years, mouse models of mitochondrial diseases have started to emerge (2), allowing the testing of therapeutic approaches (3, 4). Here we characterized two animal models of mitochondrial encephalopathy caused by complex III (CIII) or complex IV (CIV) deficiency in neurons. Surprisingly, we found significant differences in their phenotypes.

Mammalian CIII is comprised of 11 subunits with one of them, cytochrome \( b \), being encoded by the mitochondrial genome. In the case of mammalian CIV, there are 13 subunits of which 3, that comprise the catalytic core, are encoded by the mtDNA. The rest of the subunits are encoded in the nucleus and imported into the mitochondria.

CIII deficiencies are relatively rare among the respiratory chain defects. They have been associated with mutations in catalytic and structural subunits of the complex and with assembly factors. CIII deficiencies comprise a broad spectrum of symptoms and exhibit tissue specificity (review in (5)). Among the clinical presentations that are associated with mutations in the
catalytic subunit cytochrome b are: encephalopathy, Leber’s hereditary optic neuropathy (LHON), cardiomyopathy and myopathy (6, 7). Mutations in UQCRB and UQCRQ, structural subunits of CIII, cause hypoglycemia, lactic acidosis and psychomotor retardation respectively (8). Mutations in the assembly factors (BCS1L and TTC19) also show various clinical presentations. BCS1L is a molecular chaperone that assists in the incorporation of the Rieske iron sulfur protein (RISP, one of the catalytic subunits) and UQCR10 into the complex. Defects in BCS1L can cause Björnstad syndrome affecting multiple organs (muscle weakness, optic atrophy, encephalopathy, liver failure and tubulopathy) or GRACILE syndrome (growth restriction, aminoaciduria, cholestasis, iron overload, lactic acidosis and early death) affecting the liver. Defects in TTC19 cause the accumulation of CIII-assembly intermediates and leads to neurological abnormalities (reviewed in (9)). The specific function of TTC19 remains unknown.

CIV deficiencies are more common defects of the electron transport chain. Mutations in COX subunits encoded by the mtDNA have been associated with encephalopathy, sideroblastic anemia, myopathy, myoglobinuria, Leigh-like syndrome, multi systemic disease and metabolic acidosis among other pathologies. In the case of mutations in structural subunits only two cases have been reported with defective COX6b1 supporting the idea that perhaps mutations in the structural components are not compatible with life. The majority of the cases of CIV deficiency correspond to defects in the auxiliary proteins. In yeast, over 40 assembly factors for CIV have been identified (10). CIV ancillary factors associated with disease are SURF1, SCO1, SCO2, LRPPRC, COX10, COX15, TACO1 and FASTKD2 and their clinical characteristics include Leigh syndrome, metabolic acidosis, hypertrophic cardiomyopathy, French-Canadian Leigh syndrome and encephalopathy [reviewed in (9)].
In addition to specific mitochondrial disorders, impairment of mitochondrial function has been linked also to many neurodegenerative diseases and aging, possibly because impairment of the electron transport chain can produce excess free radicals leading to oxidative stress/damage (11). The role of oxidative damage in mitochondrial diseases has not been extensively documented \textit{in vivo} and most of the studies refer to increased ROS production in cultured cells derived from patients with mitochondrial disorders.

To gain a better understanding on the pathophysiological mechanisms of mitochondrial diseases, we created two conditional knockout (cKO) models with either CIII or CIV defect in the same subgroup of neurons. The CIII deficiency was achieved by ablating the Rieske iron sulfur protein (RISP), one of the catalytic subunits of the complex and the CIV deficiency by ablating the assembly factor COX10. COX10 encodes a heme \textit{a} farnesyl transferase required for heme \textit{a} biosynthesis. Heme \textit{a} is an indispensable cofactor of Cox1, a catalytic subunit of CIV. Although the two mitochondrial defective mice recapitulate mitochondrial disease phenotypes, there were marked differences between them.

\textbf{Results}

\textbf{Neuron specific CIII and CIV deficient mice}

We created two mouse models of mitochondrial CIII and CIV deficiency in neurons using the Cre-loxP system. To produce the CIII deficient mouse, a knock-in mouse homozygous for the floxed UQCRFS1 gene (12) was crossed with a transgenic mouse expressing the Cre recombinase under the control of the CaMKII\textit{a} (calcium-calmodulin kinase II alpha) promoter (13). The UQCRFS1 gene encodes the Rieske iron sulfur protein (RISP), one of the catalytic subunits of CIII. To create the CIV deficient mouse we crossed a knock-in mouse homozygous
for the floxed COX10 gene with the same Cre transgenic mouse mentioned above (14). Cox10 is indispensable for Cox1 maturation and stability.

Both RISP and COX10 cKO mice had shortened life spans albeit the RISP cKO was markedly shorter than the COX10 cKO. RISP cKO died between 3-3.5 months of age whereas the COX10 cKO mice died between 8-12 months (Figure 1A). RISP cKO weighted less than their control littermates, particularly RISP cKO females (Figure 1B). In the case of COX10 cKO mice, a significance weight difference was observed only later in life (after 4 and 5 months of age for females and males respectively, Figure 1B).

There were no overt functional phenotypes in the first 3 months of age for either model. The RISP cKO displayed lower nocturnal ambulatory movement than their control littermates (supplementary figure S1). In the case of the COX10 cKO mice, a clear and reproducible behavioral difference was only detected at 4 months of age, including alternate cycles of hyperactivity. Analysis of nocturnal ambulatory movement at 2 months of age showed indistinguishable behavior between COX10 cKO and their control counterparts. At 3 months of age the COX10 cKO started to decrease their nocturnal activity and by 4 months, cycles of hyperactivity were obvious (Supplementary Figure S1A). This behavioral abnormality continued for their life span and only when terminal the animals became markedly quiet.

Despite of the absence of overt phenotypes at early age, a motor coordination test in the rotarod revealed that the RISP cKO had decreased performance at 2 months of age with a rapid decline, being unable to perform the test 15 days later (mice were unable to stay in the rotating rod for more than few seconds without falling). In the case of the COX10 cKO, decrease in the rotarod performance had a later onset than the RISP cKO with differences between COX10 cKO
and control mice first observed at 3 and 4 months of age for males and females respectively (Supplementary Figure S1B).

**Effect of ablation of RISP and COX10 on OXPHOS complexes**

We estimated the extent of gene ablation over time by amplification of the remaining floxed alleles in the two models and found them to be similar (Supplementary Figure S2). Assuming that both alleles were recombined, we calculated that the percentage of recombination in cortex was $50 \pm 9.8$ and $52 \pm 3.2$ % (mean ± SD) for RISP and COX10 respectively at 1 month of age (supplementary Figure S2A). Similar values were obtained for hippocampus ($54 \pm 8$ and $54 \pm 3$ respectively). The levels of floxed alleles remaining at 4 months were comparable to the levels observed at 1 month of age indicating that most of the deletion of both RISP and COX10 had already occurred at an early age (supplementary figure S2B). However, lower % of deletion values were obtained for RISP cKO at 3 months and there was a significant difference between RISP and COX10 deletion at 3-4 months of age in cortex but not in hippocampus (supplementary figure S2A). Since the determination of the deletion was performed in whole tissue samples that include other cell types besides CamKIIα-neurons, we assessed the specific effect of the gene ablation in neurons by immunohistochemistry using RISP and Cox1 specific antibodies. Supplementary Figure S3 shows confocal microscopy images of the immunostaining of brain sections of control and cKO mice with RISP (green) and Cox1 (red) antibodies. In merged images of control mice, neurons stained in yellow indicating the presence of both RISP and Cox1 proteins, however in the RISP cKO, neurons only stained in red or positive for Cox1 (arrowheads) indicating the absence of RISP protein. Similarly, in the COX10 cKO the merged
image shows neurons stained in green or positive for RISP (arrowheads) indicating the absence of Cox1 protein.

Western blot analysis of steady state levels of respiratory complex subunits showed decrease levels of RISP at 1 month of age in hippocampus homogenates of RISP KO and the levels progressively decreased with age in both cortex and hippocampus reaching about 50% and 3% of control levels respectively at 3 months (figure 2 and supplementary S4). In cortex homogenates, the levels of UQCRC2 (another CIII subunit), NDUFA9 (CI subunits) and SDHA (CII subunit) normalized to actin remained comparable to control values whereas there was a slight increase in the levels of Cox1, Cox4 (CIV subunits), VDAC1 and Tim23 (1.4, 3.6, 1.7 and 1.3 fold respectively) at 3 months of age (figure 2, top panel). In the case of hippocampus homogenates, an increase in all OXPHOS proteins ranging from 1.3 to 1.9 fold was observed with the exception of NDUFA9 which levels remained unchanged (supplementary figure S4). In the case of piriform cortex, increases of 2.3 to 3 fold in all mitochondrial proteins relative to actin were observed at 79 days (Figure 2, bottom panel).

Ablation of COX10 affected the steady-state levels of Cox1 as well as Cox4 in a progressive manner and these markers were barely detectable by 4 months of age in both cortex and hippocampus homogenates (Figure 2 and S4). The levels of other OXPHOS subunits and mitochondrial membrane proteins were slightly increased in cortex homogenates (ranging from 1.2 to 2.2 fold from control when normalized to actin). In the case of hippocampus, there was a slight increase in the levels of OXPHOS complexes at 3 months of age (ranging from 1.2 to 1.7 fold of control normalized to actin) but by 4 months of age there was a slight decrease (0.84-0.98 fold, supplementary figure S4).
We also determined the effect of the deletion of RISP and COX10 on the enzymatic activity of different complexes of the electron transport chain. Figure 3 shows that as expected, deletion of RISP had a severe impact on CIII activity. At 1 month of age RISP KO mice displayed about 43% ± 8.8% (mean ± SD) of CIII activity of control levels in cortex homogenates. The enzymatic defect was progressive and reached about 25% ± 12% of control values at 110 days of age. Interestingly, increases in the enzymatic activity of CIV and citrate synthase (CS) of about 114% ± 6% and 115% ± 17% respectively were observed in the RISP cKO at about 2.5 months of age (Figure 3). Similar results were obtained in hippocampus homogenates where CIII was about 40% ± 11% of control values at 1 month of age and progressed to 14% ± 6% of controls by 110 days and CIV and CS were elevated after 70 days of age to 120% ± 17% and 121% ± 16% of controls respectively (supplementary figure S5A).

Likewise, the ablation of COX10 produced a progressive CIV deficiency. At 1 month of age cortex homogenates of COX10 cKO showed about 80% ± 10% of control levels of CIV activity and by 4 months of age the values decreased to about 33% ± 11% of controls (figure 3). Similar results were observed in hippocampus homogenates although the differences with control values were more pronounced (supplementary figure S5A). The CIV deficiency in single neurons was also confirmed immunohistochemically using the double activity stain of CII/CIV (SDH/COX activity, Figure 4D). Regarding the enzymatic activity of other respiratory complexes, the COX10 cKO did not show a significant increase in CII+CIII or CS activities in contrast to the results observed for the RISP cKO (Figure 3).

Increases in the levels of other respiratory complexes and CS in RISP cKO are suggestive of mitochondrial proliferation. Therefore, we examined the levels of mtDNA/nDNA by quantitative PCR. Supplementary figure S5B shows that indeed, the ratio of ND1 to actin was significantly
higher in the RISP cKO than control mice at 3 months of age whereas this was not the case for the COX10 cKO mice. As mentioned above, this was reflected by a slight increase of mitochondrial proteins (figure 2). There was no clear evidence of mitochondrial proliferation (mtDNA/nDNA) at earlier ages (1-2 months) in either cKO mice (not shown).

**Pathological features of ablation of RISP and COX10 in brain**

Gross examination of the RISP and COX10 cKO brains at 3 and 4 months of age, respectively, did not show obvious alterations in structure (figure 4A), however, the brain of the RISP KO weighted less than the control counterpart (Figure 4B). COX10 cKO brain weight did not show any changes at 1-4 months of age. However, by 8 months of age severe cortical atrophy was observed and brains weighted about half of controls (Figures 4A and 4B). To assess the extent of neuronal cell death we performed tunel staining in brain sections at different ages and found that the RISP cKO mice consistently had tunel positive cells in the somatosensory cortex, piriform cortex and hippocampus at 3 months (Figure 4E), right before their death but not at earlier ages (not shown). In the case of the COX10 cKO, the tunel positive cells were consistently found in the cingulate cortex, piriform cortex and hippocampus/dentate gyrus at 4 months (Figure 4E), long before their death but not at earlier time points (not shown). Immunohistochemical staining with the neuronal marker, NeuN, revealed no obvious differences between control and RISP cKO mice at 3 months in hippocampus although tunel positive cells were present, indicating that the cell death was a recent event (Figure 4C). In contrast, the hippocampus CA1 region of the COX10 cKO showed evident neuronal loss at 4 months (Figure 4C).
The contribution of glia to the neurodegenerative process was examined by both immunohistochemistry and western blot (Figure 5). Both RISP and COX10 cKO mice showed increase immunoreactivity to GFAP in both cortex and hippocampus when compared to control mice (Figure 5A). Analysis of the steady-state levels of GFAP showed that RISP cKO mice had a slight increase in GFAP levels (normalized to Tuj1) in cortex, hippocampus and piriform cortex ranging from 1.6 to 2.7 fold of controls at 3-3.5 months of age (Figure 5B). In the case of the COX10 cKO, a dramatic increase in GFAP levels (16.9-18.4 fold) was observed in 4-5 months old mice cortex homogenates. Although increased GFAP was observed in hippocampus and piriform cortex (4.9-6.8 fold), the levels were not as high as the ones observed in cortex (Figure 5B).

**Progressive neurodegenerative process in RISP and COX10 cKO mice**

To follow the progression of the neurodegenerative process we performed MRI in the same animals at different ages. Figure 6 shows different brain regions of control, RISP cKO (66 and 84 days old) and COX10 cKO (120 and 240 days old). The RISP cKO mice showed lesions in the piriform area at 66 days of age. Eighteen days later, these lesions worsened and extended to posterior parts of the brain. In the case of the COX10 cKO, small lesions were observed in the piriform cortex at 4 months of age (Figure 6). By 6 months, lesions in the striatum, outer cortical layers and hippocampus were evident (not shown) and by 8 months there was massive degeneration of the cortex (Figure 6). These results are consistent with cell death observed at 3 and 4 months in the RISP and COX10 cKOs respectively (Figure 4E).

*In vivo* proton magnetic resonance spectroscopy (MRS) of the mice brain permitted the detection of choline (Cho), total creatine (tCr), glutamate (Glu), n-acetyl aspartate (NAA) and
lactate (Figure 7). The COX10 KO showed metabolic changes with elevated levels of lactate (plotted in the inverse phase to distinguish it from the lipids resonance). However, we were unable to detect brain lactate in control or in RISP cKO mice by this method (Figure 7). MRS also revealed a decrease in NAA in the COX10 cKO. NAA is synthesized in the mitochondria and it is considered to be only present in neurons and dendrites and thus a marker for the neuronal population. NAA reduction has been associated with energetic impairments [reviewed in (15)].

**Oxidative Damage in RISP and COX10 KO mice**

To gain a better understanding of the pathological differences of the CIII and CIV deficient mice we examined the extent of oxidative damage in both mice models. Immunohistochemistry of brain sections with 8-hydroxyguanosine, a marker of nucleic acid oxidation, showed strong staining in the piriform cortex of the 3 month old RISP cKO mice. COX10 cKO brain was only weakly stained in this region (Figure 4F arrowheads). Examination of other oxidative stress markers such as protein adducts of nitrosylation (n-tyrosine) and lipid peroxidation (4-hydroxynonenal, 4-HNE) by western blot showed that at 1 month of age the RISP cKO mice already have higher levels of nitrosylated proteins and 4-HNE protein adducts than control mice in the piriform cortex. The levels of nitrosylated proteins normalized to actin ranged from 2.8 to 5.0 fold higher and 4-HNE adducts where 1.7 to 14.8 times higher than control values in the RISP KO at different ages (Figure 8). In the case of the COX10 mice, an increase in protein oxidation (8.1 and 12.6 fold increase in N-Tyrosine and 4-HNE respectively) was only observed later, at 4 months, and not at earlier ages. In addition, the RISP cKO mice also had a dramatic increase in the levels of mitochondrial superoxide dismutase 2 (SOD2) ranging from 3 to 31 fold
increase at different ages in piriform cortex when compared to control mice. In contrast, the levels of SOD1 were only slightly increased (1.7 fold) in RISP cKO mice (Figure 8). Likewise, the levels of SOD2 were also increased in the COX10 cKO although the changes were modest (2.6 to 3.6 fold higher than controls) when compared to the levels observed in the RISP cKO. SOD1 levels were slightly lower in COX10 KO compared to control mice (Figure 8).

We compared the steady state level of mitochondrial proteins of control and cKO mice in piriform cortex, cingulate cortex and hippocampus in both RISP and COX10 cKO mice. From the molecular markers analyzed, there were no obvious differences that could explain the increased susceptibility of piriform cortex in the cKO mice (supplementary figure S6).

**Discussion**

In the present study we compared two animal models of mitochondrial encephalopathy caused by defects in mitochondrial respiratory complexes III or IV produced by the ablation of UQCRFS1 or COX10, respectively. To date there are no reports of any patient with mutations in UQCRFS1 gene (encoding for Rieske iron sulfur protein RISP) perhaps accounting for its incompatibility with life. Mutations in the COX10 gene in humans result in a fatal outcome early in life (2-24 months of age) and have been associated with leukodystrophy, tubolopathy, hypertrophic cardiomyopathy and Leigh-like syndrome (16-18).

To gain insight into the heterogeneous nature of mitochondrial diseases and pathophysiological mechanisms underlying the phenotype of a single mitochondrial respiratory complex defect, we crossed our knock-in floxed UQCRFS1 (12) and COX10 (19) mice with the same CamKIIα-Cre expressing transgenic line to be able to establish a direct comparison
between the two mitochondrial deficiencies (13). We used the CamKII\(\alpha\)-Cre to drive the postnatal deletion of the floxed alleles in forebrain neurons affecting mainly hippocampus and cortex, however in humans, mitochondrial disorders of the nervous system affect other structures like striatum, basal ganglia, brain stem and cerebellum. For this reason, our mouse models might display limited recapitulation of the human disorders, however they prove useful to study the molecular mechanisms leading to the heterogeneous nature of mitochondrial encephalopathies.

In addition, although our studies compare the deletion of a structural subunit (RISP) with the deletion of an assembly factor (COX10), we do not think that the differences in phenotypes are due to this fact. We have confirmed previous observations that COX10 is indispensable for stability of the structural subunit Cox1. Therefore, both models have defects in a structural/catalytic subunit and their corresponding steady-state levels (Figure 2 and supplementary figure S4) where affected in a similar way.

Our results showed that the pathologic mechanisms in mitochondrial diseases are not solely caused by the depletion of ATP due to a dysfunctional OXPHOS, but also by differential regional susceptibility to oxidative stress. Our results are supported by several in vitro studies involving deficiencies in other respiratory complexes or electron carriers (CI, CV or CoQ deficiencies) were the partial defect in the different cell lines generated an increased in free radical leading to oxidative stress and cell death (20-22). Mitochondria are regarded as the main generators of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the cell. In particular, the established site for ROS production are respiratory complexes I, II and III although other mitochondrial enzymes such as \(\alpha\)-ketoglutarate dehydrogenase, electron transfer flavoprotein and dihydro-orotate dehydrogenase are able to produce superoxide when donating electrons to CoQ (23, 24). CI and CII produce superoxide radicals in the mitochondrial matrix.
and CIII produces superoxide in both the matrix and intermembrane space. In addition, CIV also contributes to the mitochondrial free radical species pool by catalyzing the production of nitric oxide (NO). Superoxide radicals can react with NO and produce peroxynitrate (24, 25).

Free radicals participate in intracellular signaling in a variety of metabolic processes but during pathological conditions when they are produced in excess, ROS and RNS are the cause of oxidative stress. Since free radicals can cause extensive damage in the structure/function of proteins, nucleic acids and membrane lipids they are considered the culprit of degenerative conditions, although more concrete evidence of their effects \textit{in vivo} and their role in mitochondrial diseases is needed.

Our RISP cKO mice showed extensive oxidative stress already detectable from early age preceding neuronal death. In contrast, in the COX10 cKO mice, detection of oxidative damage coincided with neuronal death. In particular, the piriform cortex was the main region affected in the RISP cKO and to a less extent in the COX10 cKO. The oxidative stress detected included damage to nucleic acids, lipids and proteins as shown by 8-hydroxyguanosine staining and nitrotyrosine and HNE-adducts western blots (Figures 4F and 8).

RISP is thought to be required for the production of reactive oxygen species as RISP knockdown experiments showed the inability of cells to produce the ROS required for hypoxic signaling (26, 27). However, RISP KO mouse lung fibroblast showed increased levels of superoxide, presumably originating from sources other than CIII (28).

In the COX10 cKO mice, we observed a delayed onset of oxidative stress correlating with the first signs of neurodegeneration when compared to the RISP cKO. However, our previous studies showed that the neuronal ablation of COX10 reduced the oxidative stress and concomitantly the number of plaques caused by the expression of mutant β-amyliod precursor
protein and presenilin-1 in an Alzheimer’s mouse model (14). A similar phenotype was observed in the neuron-specific Tfam (mitochondrial transcription factor) cKO mouse. Neuronal Tfam cKO developed a late onset encephalopathy, however there was no evidence of oxidative stress even at the latest stages of disease (29). Other mitochondrial encephalopathy mouse model involving a defect in complex I by disruption of NDUFS4 gene in both neurons and glia (nestin promoter driven deletion) was associated with increased protein oxidation in olfactory bulb of the KO mice (30).

We also observed a specific vulnerability of different brain regions to specific defects in the respiratory chain. The piriform cortex was the main region affected in the CIII defect whereas retrosplineal/cingulate cortex and to a less extent piriform cortex was more affected by the CIV deficiency. This also has been observed in other models of OXPHOS deficiency. For example, in the Tfam cKO (deletion also driven by the CamKII-α promoter) a massive cell loss was found in cingulated cortex and hippocampus (29). In the NDUFS4 KO, spongiform vacuolization was observed in brainstem (vestibular nuclei), inferior olive, cerebellar vermis and olfactory bulb (30). In a mtDNA depletion model caused by expression of mitochondrial targeted endonucleases in CamKII-α neurons, the striatum was severely affected when compared to cortex and hippocampus (31).

Such differences in regional vulnerability are common in neurodegenerative diseases associated with defects in the mitochondrial electron transport chain. Interestingly, as observed in the COX10 cKO mice, Alzheimer’s disease (AD) patients and AD mice models show metabolic abnormalities in the posterior cingulated cortex. These metabolic abnormalities in AD patients as well as in young adults at high risk of developing late-onset AD (apolipoprotein E ε4 carriers) are related to decreased glucose metabolism and decreased complex IV activity in this
brain region (32, 33). In the case of Huntington’s disease (HD), striatal GABAergic neurons are particularly affected as disease progresses. The vulnerability of this neuronal population in HD has been attributed to alterations in the mitochondrial calcium homeostasis and more recently to impairments on mitochondrial trafficking (34). Regarding preferential vulnerability of the piriform cortex, as observed in the RISP cKO mouse, has been described in animal models of temporal lobe epilepsy (35, 36). Interestingly, the structural damage to piriform cortex and other limbic structures (hippocampus, amgdaloid nuclei and entorhinal cortex) during drug-induced status epilepticus is preceded by metabolic activation (37). Moreover, the excitotoxicity of kainic acid (epileptogenic drug) is associated to increased oxidative damage and does not correlated with decreased antioxidant defenses in affected brain regions (38, 39). It is thought that the increase oxidative damage is caused by marked increase in the levels of intracellular calcium during overstimulation of receptors and perhaps reduced mitochondrial calcium buffering capacity (40).

Another interesting aspect concerning mitochondrial diseases is a phenomenon observed in many mitochondrial myopathies, where increased mitochondrial biogenesis takes place to compensate for the respiratory defect. No clear documentation of this phenomenon has been reported in mitochondrial encephalopathies. However, brain tissue appears to have the capability to increase mitochondrial biogenesis as it has been recently shown in a Huntington’s mouse model by inducing the expression of PGC-1α, the master regulator of mitochondrial biogenesis, with bezafibrate treatment (41). Our previous studies showed mitochondrial proliferation as a compensatory mechanism in our muscle and liver COX10 cKO models; however this did not occur in the neuron specific COX10 cKO, at least at the ages tested (1-4 months of age). In contrast, increase in the mtDNA/nDNA ratio, citrate synthase and complex IV activities and
slight increase in steady-state levels of mitochondrial proteins were observed in the RISP cKO mouse, suggesting that increased mitochondrial biogenesis can take place in neurons, and could be a potential compensatory mechanism.

The RISP and the COX10 KO mice characterized in this study illustrate how different OXPHOS defects can have different pathogenic mechanisms. These well characterized models will be instrumental in the development of new therapeutic approaches.

Materials and Methods

Generation of RISP and COX10 conditional knockout mice

Conditional RISP KO mice were generated by crossing heterozygous floxed UQCRFS1 (12) with transgenic mouse expressing Cre recombinase under the CamKIIα promoter kindly supplied by Dr. Zeitling (University of Virginia) (13). The COX10 conditional KO was generated by crossing the homozygous floxed mice (19) with the CamKIIα transgenic mouse.

All experiments were conducted according to protocols approved by the University of Miami Institutional Animal Care and Use Committee. The mice colony was maintained in a virus antigen free facility at the Division of Veterinary Resources at the University of Miami in controlled 12 h light/dark cycle at room temperature. Food (standard rodent diet) and water were administered ad libitum.

RISP KO mice had to be maintained in a mixed genetic background (129svj and C57Bl6) since mice backcrossed to pure C57Bl6 genetic background were sterile. Homozygous floxed UQCRFS1 were incapable of producing offspring. In the case of COX10 KO the colony is maintained in C57Bl6 background. We did not observed obvious phenotypic differences in COX10 animals of mixed compared to pure background. Crosses to obtain KO mice were
performed using female animals expressing Cre and not males, since there is a CamKIIα driven Cre expression in testis/sperm tissue. Both cKO mice were born in the expected Mendelian ratios. As control animals for RISP and COX10 cKO, we used littermate mice (same genetic background as the KO) with RISP$^{\text{flox/flox}}$ or COX10$^{\text{flox/flox}}$ genotype. When the previous genotypes were not obtained, we also used RISP$^{\text{flox/wt;Cre+}}$ or COX10$^{\text{flox/wt;Cre+}}$ respectively as controls since there was no difference in the values of the enzymatic activity of either CIII or CIV when compared to the values obtained for the respective homozygous floxed mice.

**Determination of Enzyme Activities**

Homogenates of different brain regions (cortex and hippocampus) were prepared in PBS containing complete protease inhibitors cocktail (Roche Diagnostics) in a volume of approximately 10x the weight. Homogenates were prepared in microcentrifuge tubes using a motor driven pestle and tissue was disrupted by 10-15 strokes. Homogenates were centrifuged at 100 x g for 5 minutes and supernatants were used for enzymatic assays. Complex III or ubiquinol cytochrome c reductase, Complex IV or cytochrome c oxidase and citrate synthase activities were measured spectrophotometrically as described previously (42). Protein concentrations were determined using the Bio-Rad Bradford Assay Kit and BSA as standard. Specific activity was determined and values represented as percentage of control values performed simultaneously.

**Western Blot Analysis**

Proteins were separated by SDS-PAGE in 4-20% acrylamide gels and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in 0.1% Tween 20 in PBS and subsequently incubated with specific antibodies. Horseradish peroxidase-conjugated secondary
antibodies were used and signal was developed by chemiluminescence using the Superwest signal reagent (Pierce) or RapidStep (EDM). Antibodies against RISP, UQCRC2, Cox1, Cox4, NDUFA9, SDHA, VDAC1 were obtained from Mitosciences; Actin from Sigma; Tim23 from BD Biosciences; GFAP from Cell Signaling; Tuj1, N-Tyrosine and 4-HNE from Abcam; SOD1 from Research Diagnostics and SOD2 from Upstate. All antibodies were used in a 1/1000 dilution (in 0.15% blocking) except for N-tyrosine used at 1/400 and 4-HNE at 1/80 in (blocking buffer-5% milk).

**Rotarod Analysis**

Mice motor coordination was tested at different ages using a Rotarod (IITC 755, IITC Inc, California) set at a ramp speed of 6 to 20 rpm over 180 sec. The test consisted of three trials performed for each animal at the corresponding age and the latency to fall was recorded. Animals were trained in the rotarod two times of three trials each about 2 weeks prior to the first test.

**Activity Cage Analysis**

Spontaneous ambulatory movement of mice was recorded using the Opto-M3 activity meter (Columbus Instruments) equipped with infrared beams along the cage. Movement was counted as the number of times the infrared beams were disrupted. Mice were housed individually in a new cage 30 minutes prior to their daily dark cycle and ambulatory counts were recorded for a period of 12 hr (7 pm to 7 am).
Quantitative PCR of genomic DNA

Genomic DNA was extracted from cortex and hippocampus using standard proteinase K, phenol, chloroform extraction and isopropyl alcohol precipitation. The ratio of mitochondrial to nuclear DNA was determined by quantitative real-time PCR using 10 ng of genomic DNA in a 20 µl reaction mixture using SsoFast EvaGreen Supermix (Biorad) following PCR conditions stipulated by the manufacturer in a CFX96 Real Time PCR system (Biorad). Primers for the mtDNA were ND1-F: 5’-CAG CCT GAC CCA TAG CCA TA-3’ and ND1-B: 5’-ATT CTC CTT CTG TCA GGT CGA A-3’ and for the genomic DNA β-actin-F: 5’-GCG CAA GTA CTC TGT GTG GA-3’ and β-actin-B: 5’-CAT CGT ACT CCT GCT TGC TG-3’. DNA amounts were quantified using the delta delta Ct method and expressed as a ratio of ND1/Actin.

To quantify the deletion of the floxed RISP and COX10 alleles in knockout animals, 20 ng of genomic DNA were amplified using corresponding primers and PCR conditions mentioned above. Standard curves were obtained using genomic DNA from RISP\textsuperscript{flox/flox}, RISP\textsuperscript{flox/wt}, RISP\textsuperscript{flox/wt;Cre+}, RISP\textsuperscript{wt/wt} and COX10\textsuperscript{flox/flox}, COX10\textsuperscript{flox/wt}, COX10\textsuperscript{flox/wt;Cre+}, COX10\textsuperscript{wt/wt} respectively. Values were normalized to an unrelated gene (EGF) as described previously (43).

The forward primer used to amplify the RISP and COX10 floxed alleles was common to both genes since its target sequence comprises part of the loxP site. The common forward primer sequence is 5’-CGG GGA TCA ATT CGA GCT CGG CC-3’. The reverse primers were specific for each gene. The sequence for the reverse primers is RISP-B: 5’-AAC TTC CTA CAT GGT TGT TTC AAT T-3’ and COX10-B: 5’-CAC TGA CGC AGC AGC GCC AGC ATC TT-3’ respectively. Primer sets produced an amplicon of 191 bp and 167 bp for RISP and COX10 respectively. The percentage of deletion was calculated by assuming that recombination occurs in both floxed alleles in cre-expressing neurons(43).
Immunohistochemistry

Frozen brains sections were obtained in a cryostat and processed for immunohistochemistry using NeuN (Chemicon), 8-hydroxy guanosine (QED Bioscience), RISP (Mitosciences) and Cox1 (Alexa-conjugated, Molecular Probes) antibodies. Either fluorescent labeled or HRP labeled secondary antibodies were used for immunodetection using standard immunohistochemical techniques. To detect cell death, the “in situ cell death detection kit-TMR red” from Roche was used following manufacturer instructions.

Cytochrome c oxidase or COX activity stain was performed in cryostat sections (~20 µm) as described in (14). Briefly sections were incubated in 100 mM sodium phosphate buffer pH 7.4 containing 0.5 mg/ml diaminobenzidine, 0.2 mg/ml cytochrome c and 40 mg/ml sucrose at 37ºC for 50 min. Succinate dehydrogenase or SDH activity stain was performed as described in (19) by incubating sections in 10 mM sodium phosphate buffer pH 7.5 containing 1.6 mg/ml EDTA, 0.65 mg/ml KCN, 0.06 mg/ml phenazine methosulfate, 1.3 mg/ml succinic acid and 1.2 mg/ml nitroblue tetrazolium at 37ºC for 20 min. For double COX/SDH stain, sections were first stained with COX reagents, washed 3 times in PBS and then incubated with SDH reagents.

In vivo MRI and Proton Magnetic Resonance Spectroscopy

MRI and MRS studies were conducted in the University of Miami High Field MRI (magnetic resonance imaging) Biomedical Research Core Facility equipped with a 4.7 Tesla horizontal bore Bruker magnet designed for small animal experimentation. Mice were anesthetized by spontaneous breathing of 1.3-1.6% isofluorane and oxygen mixture through a nose cone using an Ohmeda veterinary anesthesia unit. The mice were placed in a MRI compatible cradle, their body
temperature was maintained at 37°C by a water bath circulation system and their respiratory rate was monitored by a pneumatic pillow and an accompanying pressure transducer. Both temperature and breathing were recorded by a small animal monitoring system (SA instruments Inc). Anesthesia was adjusted to obtain a respiratory rate of about 90 breaths per minute. Following localizer scans high resolution axial and coronal T2 weighted datasets were acquired in order to visualize any cerebral or structural atrophy and to investigate for potential focal pathologies (44). High resolution T2 axial datasets were manually segmented for whole brain volume for comparisons between groups and time points. Water suppressed point resolved spectroscopy, PRESS, was acquired in order to glean the metabolic status from the mouse brain (45). The MRI dataset was collected with the following settings: a voxel size of 4x4x2 mm, a bandwidth of 3 kHz, 4096 acquisition points, a TR period 2.5 s, a TE period of 144 ms and 256 averages. To optimize the quality of the MRS data, the magnetic field was shimmed to a line width of less than 20 Hz measured at full width at half maximum on the unsuppressed water signal. The voxel was centered in the cortex and hippocampus regions. Spectral data was processed using software developed by JMRUI (Java MR User Interface: http://www.mrui.uab.es/mrui/mrui_Overview.shtml). To obtain estimates of the lactate levels in vivo, a long TE time of 144ms was employed to differentiate the lactate from lipid signal contamination and is shown as an inverted peak in the spectra (46).
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Conflict of Interest statement

The authors declare no conflict of interest.
References


Figure Legends

Figure 1. Survival and growth curves of RISP and COX10 cKO mice.

A) Comparison of the survival curve of the RISP and COX10 cKO mice. RISP cKO [closed diamonds, control male and female mice (n=20 ea); open squares, KO male mice (n=7); open triangles, KO female (n=11)]. COX10 cKO [closed diamond, control male and female mice (n=35 ea); open squares, KO male mice (n=35); open triangle, KO female (n=25). There is a significant difference in the survival of RISP compared to the COX10 cKO. The premature death of the RISP cKO occurs much earlier than the COX10 cKO. No difference between male and female survival was observed in both cKO models. B) Growth curve of RISP and COX10 cKO. RISP cKO mice weight less than control counterparts from an early age, particularly female mice [open diamonds, control male (n=7); closed diamonds, KO males (n=5); open circles, control females (n=15); closed circles, KO females (n=7)]. COX10 cKO mice start to lose weight at about 4-5 months of age [open diamonds, control male (n=9); closed diamonds, KO males (n=8); open circles, control females (n=13); closed circles, KO females (n=8)]. Values represent mean ± standard deviation.

Figure 2. Steady-state levels of mitochondrial oxidative phosphorylation complex subunits in RISP and COX10 cKO cortex.

Immuno blots of cingulate and piriform cortex homogenates (20 µg protein) of RISP and COX10 cKO at different ages using antibodies against different subunits of mitochondrial respiratory complexes. RISP and UQCRC2 are subunits of complex III; Cox1 and Cox4 are subunits of complex IV; NDUFA9 is a complex I subunit; SDHA is a complex II subunit. VDAC1 (voltage
dependent anion channel-1), Tim 23 (subunit of inner membrane import translocator) and actin were used as loading controls.

**Figure 3. Mitochondria respiratory complex activities in RISP and COX10 cKO cortex.**

Enzymatic activity of complexes III and IV and citrate synthase (CS) were measured spectrophotometrically in cortex homogenates of RISP and COX10 cKO mice at different ages. Mean values of specific activity of the KO mice were compared to mean values of age matched control mice and represented as percentage of control. Each symbol corresponds to one KO mouse (some symbols overlap, n=6 per group). A progressive decline of CIII and CIV activity is observed in RISP and COX10 cKO respectively.

**Figure 4. Pathological features of RISP and COX10 cKO brains.**

A) Gross anatomy of RISP and COX10 cKO brain. No overt differences were observed in RISP or COX10 cKO brain at 3 and 4 months respectively. However at 8 months severe cortical atrophy occurred in the COX10 cKO. B) Weight of RISP and COX10 cKO brains were compared to control brains at different ages. RISP cKO brain weighted significantly less than controls at 3 months of age although no differences were apparent in gross anatomy. No changes were observed up to 4 months of age in COX10 cKO brain. RISP control (n=5) and KO (n=5); 3-4 months COX10 control (n=6) and cKO (n=5); 8-12 months (n=3). C) Frozen brain sections were immunohistochemically stained with NeuN antibody. Images showed hippocampal CA1 region of RISP and COX10 KO at 3 and 4 months of age. D) Brain sections of control and COX10 cKO mice (hippocampus) were stained for complex IV (COX), complex II (SDH) or combined activity stain. Insert shows magnification of CA3 region with COX negative neurons.
stained in blue/purple by the SDH stain. E) Tunel staining of control and KO mice in different brain regions (cingulate, somatosensory and piriform cortex and hippocampus/dentate gyrus). Insert in bottom panel shows NeuN stain (green) in control mice. No signs of tunel positive cells were detected in earlier ages in both RISP and COX10 cKO mice (not shown). F) Immunohistochemical stain of the 8-hydroxy guanosine (nucleic acid oxidative damage marker) in RISP and COX10 cKO mice at 3 and 4 months of age respectively.

**Figure 5. Reactive glia in RISP and COX10 cKO mice.**

A) Immunohistochemical stain of frozen brain sections of control, RISP and COX10 cKO mice at 3 and 4 months respectively with GFAP antibody showing reactive glia. B) Steady-state levels of GFAP and Tuj1 (astrocytic and neuronal markers respectively) in RISP and COX10 cKO mice at different ages in homogenates of different brain regions.

**Figure 6. In vivo Nuclear Magnetic Resonance Imaging of RISP and COX10 cKO brain.**

Progression of encephalopathy was followed in the same animals at different ages using T2 weighted MRI datasets. Representative images of different brain regions are shown. RISP cKO at 66 days of age shows hyper-intense areas in the piriform cortex that extend to different areas and posterior regions of the brain 18 days later. COX10 cKO mice also demonstrate lesions in piriform cortex at 120 days of age and by 240 days severe striatum and cortical damage were observed.
Figure 7. *In vivo* Proton Nuclear Magnetic Resonance Spectroscopy of control, RISP and COX10 cKO mice.

Representative proton spectra of control, RISP and COX10 cKO mice where the detection of some brain metabolites by MRS is demonstrated (n=3-4 for each group). Peaks corresponding to Choline (Cho), total creatine (tCr), Glutamate (Glu), n-acetyl aspartate (NAA) and lactate (represented as the inverted peak to differentiate it from lipid signal resonance) are shown. COX10 cKO mice have increased lactate levels. No peak corresponding to lactate was detected in either control or the RISP cKO mice.

Figure 8. Oxidative stress markers in RISP and COX10 cKO mice.

Steady-state levels of different oxidative markers were analyzed by western blot in piriform cortex and hippocampus homogenates (20 µg of protein) of control, RISP and COX10 cKO at different ages. Increased levels of N-tyrosine and 4-hydroxy nonenal (4-HNE) protein adducts indicate reactive nitrogen species and lipid peroxidation in RISP cKO mice from an early age. SOD1 and SOD2: superoxide dismutase 1 and 2. Actin was used as loading control.
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**PIRIFORM CORTEX**
- N-tyrosine
- 4-HNE
- Actin
- SOD2
- SOD1

**HIPPOCAMPUS**
- N-tyrosine
- 4-HNE
- Actin
- SOD2
- SOD1