INVESTIGATION OF BRAIN BIOENERGETICS IN DIFFERENT MITOCHONDRIAL MODELS

PhD thesis

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THE LIST OF ABBREVIATIONS

AD:	Alzheimer's disease
ADP:	adenosine diphosphate
ATP:	adenosine triphosphate
ANT:	adenine nucleotide translocase
α-GP:	α-glycerophosphate
α-GPDH:	α-glycerophosphate dehydrogenase
α-KG:	α-ketoglutarate
BSA:	bovine serum albumin
CAT:	carboxyatractyloside
CoQ:	coenzyme Q, coenzyme Q10, ubiquinone, ubidecarenone
FAD:	flavin adenine dinucleotide
DLD:	dihydrolipoamide dehydrogenase, E2 subunit of KGDHC
DLST:	dihydrolipoamide S-succinyltransferase, E3 subunit of KGDHC
EGTA:	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ETS:	electron transport chain
FCCP:	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
GPX:	glutathione peroxidase
GR:	glutathione reductase
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2:	hydrogen-peroxide
KGDH:	α –ketoglutarate dehydrogenase. E1 subunit of KGDHC
KGDHC:	α-ketoglutarate-dehydrogenase complex
MB:	methylene blue
NAD^+:	nicotinamide adenine dinucleotide (oxidized form)
$NADP^+$:	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADH:	nicotinamide adenine dinucleotide (reduced form)
NADPH:	nicotinamide adenine dinucleotide phosphate (reduced form)
OGDH:	2-oxoglutarate dehydrogenase, E1 subunit of KGDHC
RET:	reverse electron transport
RCR:	Respiratory control ratio
ROS:	reactive oxygen species

Succ:	succinate
SDH:	succinate dehydrogenase, respiratory complex II
SOD:	superoxide dismutase
$\Delta\Psi m$	transmembrane potential

1. INTRODUCTION

1.1 Mitochondria

Mitochondrion is an important cell organelle, which has a major role in the energy homeostasis of the cells. Mitochondria are also called the primary power source (in other terms "the powerhouse") of cells. Their primary role is to provide ATP for cell functions; but they also take part in several other cellular processes, e.g., intracellular Calcium, ROS homeostasis, regulation of cell death and thermogenesis. Several important biochemical pathways are also located in the mitochondria (oxidative phosphorylation in the mitochondrial inner membrane, citric acid cycle (except the inner membrane associated part of succinate dehydrogenase), beta oxidation, part of heme synthesis in the mitochondrial matrix, etc.) [1]. Furthermore results of the last decades highlighted the association between the impaired mitochondrial functions and several pathological conditions, such as excitotoxicity, [2], ischemia/reperfusion-, neurodegenerative diseases [3], and oxidative stress [4].

As mentioned above, the citric acid cycle is localized in the mitochondrial matrix. The reactions of the citric acid cycle are carried out by eight enzymes participating in the consumption of acetyl-CoA and water, reducing NAD^+ to $NADH+H^+$, FAD to $FADH_2$, producing GTP and/or ATP, releasing carbon dioxide. The generated $NADH+H^+$ and $FADH_2$ are used by the oxidative phosphorylation pathway to generate ATP from ADP and Pi. The fourth enzyme of the citric acid cycle is the α-ketoglutarate dehydrogenase complex (KDGHC) a large multienzyme complex in the mitochondrial matrix [5].

1.2. α-Ketoglutarate-dehydrogenase complex

The KGDHC catalyzes the rate-limiting step in the tricarboxylic acid (TCA) cycle converting α-ketoglutarate (α-KG) to succinyl-CoA in an oxidative decarboxylation reaction reducing NAD^+ to $NADH+H^+$, releasing CO_2 and utilizing HS-CoA [6;6;7]. The KGDHC consists of three components: α-ketoglutarate/2-oxoglutarate dehydrogenase (succinyl-transferring KGDH/OGDH, E1; EC 1.2.4.2), dihydrolipoyl succinyltransferase (DLST; E2; EC 2.3.1.61), and dihydrolipoyl dehydrogenase (DLD, E3; EC 1.8.1.4) [8-10]. The three subunits form an octahedral cube where multiple E2 proteins form the inner core surrounded by the E1 and E3 subunits. The E1 and E2 subunits are strongly connected; in contrast to that E3 binds relatively weakly to the E2

subunits [11-13]. In E. Coli, the subunit composition of the native KGDHC is about 12 E1 chains, 24 E2 chains, and 12 E3 chains [14]. Different chain ratios (E1:E2:E3) have been reported in the literature for mammalian KGDHC including pig heart 1:1:1.5[15], 1:1.2:1.4 [16], and 1:1:0.5 [17;18]. The variations of the composition have been reviewed by our colleagues, binding stoichiometries for the highest catalytic efficiency were determined from the rate of NADH generation of the complexes at physiological pH [19;20]. In contrary with the isoform in the heart, KGDHC in the brain is heavily regulated and plays a role in energy coupling [17;18;21;22]. α-KG can be a major input of the TCA cycle because α-KG can be produced in the matrix (mouse and human) in 30 reactions see https://metabolicatlas.org/explore/Mouse-GEM/gem-browser/metabolite/MAM01306m ; (accessed on 21 September 2023). Besides producing NADH (substrate for respiratory Complex I in the electron transfer system; ETS) for the terminal oxidation, the KGDHC reaction is also a major source of succinyl-CoA, a substrate for succinyl-CoA ligase (SUCL). The SUCL reaction leads to the formation of ATP or GTP via substrate-level phosphorylation (SLP) which is a major source of mitochondrial ATP in the absence of oxidative phosphorylation [23;24]. In energetically impaired mitochondria, ATP generated by SLP can prevent hydrolysis of the glycolytic ATP via avoiding the reversal of the ADP-ATP transporter, the adenine nucleotide translocase (ANT) [24-26].

New isoforms of KGHDC were discovered in brain tissues, where oxoglutarate dehydrogenase L (KGDHL) substitutes for E1, which may be particularly critical in neurodegeneration [27]. Sequence analysis indicated that KGDHL represents a previously unidentified isoform of KGDH.[27]. KGDHL expressed only in brain tissue, but not in the heart [16]. KGDHL activity also declined in mouse models of Alzheimer's disease. Li and colleges confirmed a decrease of KGDHL in triple transgenic Alzheimer's mice and showed by overexpression of KGDHL reduced amyloid plaque load [28] Brain samples from Alzheimer's disease patients showed a low expression of KGDHL, and investigation on Alzheimer mice models demonstrates the importance of these changes. Mass spectrometry analysis and two-dimensional gel electrophoresis indicate the reduced KGDHL expression in the triple transgenic Alzheimer's mice model [28;29]. The increased KGDHL expression in cancer cells is coupled with an increased ROS production which can lead to apoptosis of cancerous

cells. Furthermore, the overexpression of KGDHL restricts cancer cell migration and invasion [30].

1.3. Deficiencies of α-Ketoglutarate dehydrogenase complex

The human (dihydro)lipoamide/lipoyl dehydrogenase enzyme h(DLD) is a pyridine nucleotide disulfide oxidoreductase, whose insufficiency/deficiency is a rare genetic disease with autosomal recessive inheritance [29;31;32]. The majority of carriers of this disorder are in the Ashkenazi Jewish population (1:94-1:110, for the G194C-hE3 pathogenic variant; with a disease frequency of 1:35000-1:48000) [33;34].

Pathogenic hDLD variants show a wide range of DNA sequence alterations leading to incorporation of different amino acids (missense mutation) or generation of early stop codon (nonsense), splice site perturbation, and (small) deletions/insertions [35;36]. Although excision (loss) or duplication of the entire gene has not yet been described as a cause of hDLD deficiency [32] (skipping an exon combined with a reading frame shift resulting in a shorter protein product, however occurred in one documented case [37]. Regarding the mature hDLD enzyme, 14 disease-causing amino acid changes (or deletions) have been described so far in the clinical literature [38]. Previously described hDLD X-ray crystallographic structural analysis showed that the pathogenic amino acid substitutions are concentrated in three regions of the protein structure: the cofactor-binding region (e.g. I12T, K37E), to the active center (e.g. P453L), and to the dimerization surface (e.g. E340K, D444V) [39]. The resulted protein structural changes (such as low protein expression, an unfavorable protein folding/stability and faster degradation, unfavorable conformational changes, the (partial) loss of the FAD cofactor (since it is strongly, but not covalently bound to the enzyme), termination of critical amino acid interactions, or the breakdown of the enzyme are responsible for the detectable -either in vitro or in vivo -– decrease of hDLD activity.[39;40].

The disease primarily affects tissues with intensive metabolism and high oxygen consumption, and for this reason the relevant clinical symptoms are primarily neurological and cardiological in nature, although functional disorders affecting the liver are also quite common [32;35;36]. Characteristic clinical phenotypes of hDLD deficiency: growth failure, hypertrophic cardiomyopathy, encephalopathy, hypotonic muscles, lactic acidosis, hypoglycemia, Leigh syndrome, ataxia, developmental disorders, visual impairment, microcephaly, and liver failure.

In the early-onset form of the disease, the clinician often encounters a hypotensive infant and detects lactic acidosis. The patient usually dies during the first or repeated metabolic decompensation, while if he survives these, he will be developmentally retarded, and will have permanent neurological damage (e.g. intellectual deficit, ataxia).
Clinical biochemical diagnostic may detect reduced functioning (reduced activity) of hDLD or the mitochondrial α-ketoacid dehydrogenase enzyme complexes that contain hDLD (α-ketoglutarate/2-oxoglutarate dehydrogenase (KGDH/OGDH), pyruvate dehydrogenase (PDH), branched chain α-ketoacid dehydrogenase (BCKDH) complexes). hDLD is the common third subunit/component of these complexes (hE3). It is an interesting phenomenon that 2-Oxoadipate Dehydrogenase enzyme uses the E2 subunit of hKGDHC for its function [41;42].

1.4. Effect of hDLD deficiency in the of α-ketoacid dehydrogenase enzyme complexes

Due to its role in BCKDH, the hDLD/hE3-deficiency is called maple syrup urine disease type 3 (MSUD-3, maple syrup urine disease type 3) or branched-chain ketoaciduria. Since the hypofunction of the hDLD enzyme simultaneously affects all of the above metabolic complexes, hDLD deficiency is usually associated with much more severe symptoms than isolated enzyme complex deficiencies. The hDLD is also part of the the glycine cleavage system as L-Protein. Interestingly this function is generally not affected by hDLD deficiencies [32;38]. In addition to its metabolic role, hKGDHC has also been associated with HIF1-α turnover [43], reprogramming of metabolism, epigenetic changes [44], with cell proliferation, histone succinylation [45], or MYC-mediated in cancer with leukemogenesis [46], which functions can also be affected by the hDLD pathogen mutations.
In this connection the clinical literature also clearly shows that the activity of the above mentioned enzyme complexes decrease significantly more than the activity of the hDLD itself [35;36]. The fact that the activities of the various enzyme complexes are reduced to different extents at the same time, is likely dependent on the specific pathogenic hDLD mutants in question - as well as the wild-type hDLD itself [47] – which binds with different affinities to complexes [35;48;49].
Decrease in the relevant enzyme complex activities cause either continuous or intermittent lactic acidemia, increased α-ketoglutarate (KG) level (in urine), increased

branched-chain amino acid (Leu, Ile, Val) levels in plasma. Often elevated transaminase, citrulline, and creatine kinase levels are also detectable during the course of the disease [32]. Defects of lipoic acid metabolism often have a similar clinical course to E3-deficiency; however, differential diagnosis achieved by detection of elevated concentration of Gly in different body fluids, characteristic of the E3-deficiency [50]. For diagnostic purposes reduced hDLD activity is usually measured from fibroblasts, lymphocytes, from liver or muscle tissue samples [32;35;36].

Between acute attacks, patients are treated on an empirical basis, which takes into account the involvement of the relevant enzyme complexes [BCKDH:] reducing the intake of amino acids and proteins, but avoiding starvation; KGDHC: none accepted recommendation; PDHC: ketogenic/fat diet, thiamine intake, administration of dichloroacetate (to inhibit PDHkinase)]. In general, patients also receive vitamins (mainly B2, B7), antioxidants, Coenzyme Q10, lipoic acid and intravenous glucose during acute episodes [32]. In an acute episode metabolic acidosis must be controlled and a normoglycemia must be maintained, and stable metabolic status in general is attempted to be achieved (for detailed specific clinical recommendations, see [32]). Currently no accepted clinical protocol exist to prevent acute episodes, however, it was observed that the frequency of acidic phases usually decreases with age [32].

1.5. Regulation of α-Ketoglutarate dehydrogenase complex

KGDHC is a highly regulated enzyme, which has a major role in the flux control of the citrate cycle. Regulation of KGDHC is complex involving ATP/ADP, NADH/NAD^+ ratio, Ca^{2+} and the availability of metabolic substrates in mitochondria. In details: KGDHC is activated by calcium and ADP and inhibited by NADH and ATP[51]. DLST is inhibited by Succinyl-CoA and ATP [52]. DLD inhibited by NADH and ATP[51;53;54]. Calcium has an essential role in activating three mitochondrial dehydrogenases, pyruvate dehydrogenase (PDH), isocitrate dehydrogenase and KGDHC in the 0.1–10 μM range [55;56] rat heart mitochondria [57].

Calcium, in high concentrations (higher or equal to 100 μM), however, inhibits KGDHC [58], isocitrate dehydrogenase [59] and pyruvate dehydrogenase [60]. This effect could significantly contribute to the detrimental effects of high calcium concentration in neurons under pathological conditions. KGDHC is sensitive to ROS and inhibition of

this enzyme could be critical in the metabolic deficiency induced by oxidative stress [61].

On the other hand, it has been revealed that the enzyme itself is able to generate ROS, therefore could contribute to the induction of oxidative stress [17;62-64]. This thesis will focus on the relevance of KGDHC in ROS generation, concentrating mainly on the brain enzyme and discussing its relation to ischemia-reperfusion injury and neurodegeneration

1.6. Reactive oxygen species generation from α-ketoglutarate dehydrogenase complex

Mitochondria are the powerhouses of the cell, but like all powerhouses, they also have some less desirable by-products such as reactive oxygen species (ROS) [65;66]. Although, traditionally ROS production is mainly attributed to the ETS [67] many observations showed that soluble enzymes in mitochondria can also play a role in the ROS formation [17;62;68].

Superoxide is a primary reactive oxygen species (ROS) which, with spontaneous dismutation/ can be converted into H_2O_2 [69;70]. In the case of flavins and flavoenzymes, it has already been described that they are able to (partially) reduce O_2 [71-73]. Zn^{2+} can also stimulate ROS formation by DLD, which can occur in ischemia-reperfusion and Alzheimer's disease, since the concentration of Zn^{2+} increases in these conditions [74-76]. DLD can exist independently of the multienzyme complexes [77;78], and it was also established that the flavoprotein was present in the highest concentration is muscle and brain in mitochondria [79]. The free form of DLD binds much significantly weaker to KGDHC and BCKDH than to PDH [12;71-73;80-82] and certain pathogenic mutations can weaken the binding even further). DLD is a ROS generator which property is also planned to be used in cancer therapy [83]. Increased mitochondrial ROS production was described in acute ischemia-reperfusion syndrome as well as in chronic neurodegenerative diseases [84;85]. It is interesting to note that, although KGDHC is very sensitive to oxidative stress agents [18;61;86]. ROS production by hKGHDC is more significant when it is physiological electron acceptor, NAD^+, is not available in sufficient quantity (the forward reaction), or if the $NADH/NAD^+$ ratio increases (which supports the ROS-forming E3 reaction, see above)

[63;64]. Increased NADH/NAD^+ ratio was described, in ischemia, in case of increased caloric intake or in complex I insufficiency [87].

The self-regulation of the KGDHC was demonstrated by the inactivation of E1 subunit, in which the lipoic acid cofactor has the primary role (through the formation of a thiyl radical and interaction with the E1 catalytic intermediate; this mechanism is suspended by thioredoxin) [88;89].

H_2O_2-mediated inactivation of KGDHC through the lipoic acid cofactor is associated with a decline of NADH production and consequently the oxidative phosphorylation. However this inactivation is reversible by glutathionylation. Therefore KGDHC is proved to be a target of antioxidant response [90;91].

It is also an interesting observation that the activity of KGDHC enzyme in the case of acidosis (pH 6,4-6,8) is the highest [92], although this isn't play an important role in brain tissue (here optimum of pH is between 7.2-7.4) [58]. Even a small decrease in the activity of KGDHC leads to energy deficit and oxidative stress, which starts a vicious cycle that will be stimulated by acidosis, resulting in further oxidative stress, metabolic deregulation, and finally clinical symptoms [87;93]. Typical clinical symptoms of isolated KGDHC deficiency are: muscle hypotonia, impairment of motor skills, progressive hypertension disease, dystonic movement, encephalopathy, seizures, liver enlargement, cognitive disorders, metabolic decompensation and sudden death in young age [32].

1.7 Disrupted function of α-ketoglutarate dehydrogenase in pathological conditions

Insufficient functioning of hKGDHC and the formation of ROS plays a role in many diseases and pathological processes, such as: hypoxia- or glutamate-induced brain damage, Wernicke-Korsakoff's syndrome, neurodegenerative diseases (Alzheimer's (AD), Parkinson's, Huntington's diseases), Friedreich's ataxia, ischemia-reperfusion syndrome, progressive supranuclear paresis, (cellular) aging, lactic acidosis in infancy, various neoplastic changes, hDLD deficiency, etc. [87]. Some strategies proposed to increase hKGDHC activity (such as for example, inhibition of the tissue transglutaminase enzyme), or the pharmacological down regulation of ROS formation by hKGDHC may play a role in the therapy in the future [93-96].

Disruption of E2 subunit function is associated with psychomotor retardation in the childhood[98]. Mutation of the E3 subunit of KGDHC in infantile lactic acidosis is

linked to severe psychomotor retardation. [97]. The G229C mutations in the DLD gene cause intermittent neuropsychiatric diseases with ataxia and in children with attention deficit disorders and also coordination deficits [99]. Friedreich's ataxia, which is the most common congenital ataxia, caused by a GAA repeat in the FRDA gene resulting the progressive degeneration of the long tracts of the spinal cord, furthermore coupled to a deficiency of the E3 subunit of KGDHC [100]. For instance, the DLD-deficient mice exhibited an increased vulnerability to mitochondrial toxins and diminished neurogenesis [101]. The mitochondrial DLST deficiency in a transgenic mouse model accelerates amyloid plaques and memory deficiency [102]. The succinylation of subunit is directly linked KGDHC to amyloid plaque and neurofibrillary tangle formation [103]. The protein levels of KGDHL declined in Alzheimer's mouse models. Genetic reduction of the KGDHL increases amyloid plaques formation and overexpression of KGDHL diminishes it [104]. Accordingly to the multiple animal models of Alzheimer's disease support the key role of KGDHC in pathogenesis of the neurodegenerative disease.

The KGDHC activity is also reduced in the brain of Wernicke–Korsakoff syndrome patients.[105]

The decreased activity of the KGDHC activity was a consistent finding in the brain of Alzheimer's disease patients, covering the regions affected by the disease as well as regions that remained normal [106-108]. The decline in the KGDHC activity was as consistent and characteristic as the abnormal amyloid plaque formation. The KGDHC activity was also reduced in fibroblasts from patients with presenilin-1 mutation [109] or had increased vulnerability to oxidative stress [110]. The reduced enzyme activity may not be a cause in the pathophysiology of Alzheimer's disease but the mitochondrial dysfunction due to the inhibition of KGDHC could be a critical factor in the degeneration process [107]. The role of KGDHC in the pathogenesis of neurodegenerative diseases is underlined by the observation that in rare cases of genetic defects of KGDHC [107] progressive neural degeneration was observed in patients [111].

The key role of a decreased KGDHC activity in the events leading to neuronal death in the central nervous system is very clearly indicated in animal models of thiamine deficiency, in which the activity of KGDHC is reduced in brain regions where the

neurons die, whereas in regions that survive the activity of the enzyme is spared [61;112;113]).

1.8. Properties and history of methylene blue

Methylene blue (MB, 3,7-bis-(dimethylamino)- phenazathioniumchlorid, methylenthioniniumchlorid) was synthetized at the end of the 19th century, initially it was used as a textile dye, and later was used for killing microorganisms [114]. Paul Ehrlich and Guttman first used it in 1891 for treatment of malaria, [115]. Since then it has been recognized that MB selectively inhibits the glutathione reductase of Plasmodium falciparum, which protozoan species is responsible for the spread of malaria, thus reducing the protection against oxidative stress, could result in parasite cell death [116;117]. MB was considered the primary antimalarial agent until the end of World War II [117]. However, as a common side effect for its use, turns the urine blue or green. Therefore the medical use as an antimalarial drug has been declined. Meanwhile, in the 1920s, it was recognized that the injury caused by cyanide poisoning can be prevented with MB [118]. In the second half of the 1900s, the first antidepressant compound named chlorpromazine was designed by introducing the aliphatic side chain to the nitrogen atom of the phenothiazine skeleton [119;120]. Besides successfully used in methemoglobinemia [121], ifosfamide (Holoxan)-induced toxicity, and also in the treatment of encephalopathy [122] and septic shock [123]. In the last decade a greater role is attributed to its neuroprotective effect in Alzheimer's and Parkinson's disease in animal models [124-126], as well as its memory-stimulating and anti-aging properties [125]. Today, MB is successfully used worldwide to treat methemoglobinemia [127]. During methemoglobinemia, Fe^{2+} ions of hemoglobin are oxidized to Fe^{3+} ions resulting in methemoglobin, which is accompanied by a decrease in oxygen carrying capacity. In the presence of MB, the NADPH-dependent methemoglobin reductase reduces MB to MBH_2 (leuco-methylene blue) and the produced MBH_2 donates electrons to methemoglobin to regenerate hemoglobin [128].

MB has a redox potential of 11 mV (39). Due to this redox potential, MB is very efficient in cycling between reversible forms an oxido-reduction by receiving and gaining electrons to various molecules. MB is also efficiently reduced by NAD(P)H-dependent dehydrogenases to form the colorless MBH_2. MB loses the blue color on reduction due to the disappearance of the absorbance bands at 600 and 666 nm [129].

MBH_2 can readily reoxidize to MB by O_2 if suitable electron acceptor such as cytochrome c is present [130].

MB has a heterocyclic structure, for this reason it is a highly lipophilic compound that easily passes through biological membranes [131]. Thus, MBH_2 and MB can enter the mitochondria and other intracellular compartments such as lysosomes [132].

The photosensitizing effect of MB is used in therapy of skin cancer and under light radiation the antifungal and antibacterial effects also increase [133]. MB is capable of absorbing energy from various light sources and transferring this energy to molecular O_2, creating the singlet oxygen ($^1O_2$). Singlet oxygen is an electrophilic molecule that oxidizes various macromolecules (nucleotides, lipids) and biological membranes. Consequently the increased lipid peroxidation and reduced membrane integrity leads to the loss of the fluidity of the membrane and disrupted functioning of various ion channels and transporters [134].

1.9. Mitochondrial effects of methylene blue

The beneficial effects of MB exerted on respiratory chain-inhibited mitochondria are summarized in the "alternative electron transport" theory [126]. According to that, MB is able to receive electrons both from NADH or $FADH_2$ and can transport them to cytochrome c [135] transport transports it to cytochrome c [136] in case of inhibition of the mitochondrial respiratory chain [137]. The redox potential of NAD^+/NADH is -320 mV, while $FAD^+/FADH_2$ is -220 mV, so they can donate electrons to MB, because of their more negative standard redox potential. During inhibition of the respiratory chain complexes CI, CII and CIII, electrons cannot continue to flow in the respiratory chain and NADH cannot be oxidized by Complex I, and similarly $FADH_2$ cannot be oxidized by Complex II, so the reducing equivalents accumulate. ATP production and mitochondrial oxygen consumption will be decreased. The accumulated NADH inhibits the rate-determining enzyme of the citrate cycle enzyme the KGDHC, which leads to the slowing down of the citrate cycle. However, if MB is present it may oxidize NADH and $FADH_2$. The electrons can be transported as MBH_2 to cytochrome c. Then electrons are transferred to molecular oxygen via CIV and reduce it.

MB increases the activity of CIV or cytochrome c oxidase [138]. MB also increases mitochondrial heme synthesis by promoting the uptake of iron, which is the precursor

for heme groups. The CIV is a heme A-containing enzyme complex, so MB can promote heme synthesis to enhance the activity of CIV [125].

1.10. The Significance of Methylene blue in Alzheimer's diseases

Alzheimer's disease (AD) is late-onset progressive neurodegenerative disease, its main symptom is the cognitive decline and is responsible for 60–70% of dementia cases. The cause of Alzheimer's disease is not fully understood. The disease process is largely associated with amyloid plaques, neurofibrillary tangles, and loss of neuronal connections in the brain. In Alzheimer's disease the amyloid ß-protein aggregates bind to the heme group of CIV [139]. In addition, amyloid ß-proteins bind to citrate cycle enzymes [140], inhibiting the production of succinyl-CoA for the heme synthesis. During the process the activity of CIV decreases, which leads to mitochondrial dysfunction with decreased energy production and increased mitochondria-associated free radical production [141].

The effects of MB on complex IV and mitochondrial metabolic pathways may provide protection against age-related mitochondrial associated neurodegeneration. Complex IV decay is a key mitochondrial dysfunction in Alzheimers-disease [130].

Additionally, MB at a concentration higher than 15 μM has been shown to inhibit amyloid β oligomerization by promoting fibrillization [142]. Aggregation of phospho-tau has been prevented at ≈3 μM MB *in vitro* [124]. These effects of MB on cell metabolism and mitochondrial complex IV in particular suggest that MB may delay the onset of AD. If the concentration of MB used are very high about 10 μM. It also may lead to neurotoxicity [143].

MB showed also an improve of spatial memory retention in behavioral studies on rats and psychological tests, and all were manifested in other biochemical effects [144;145].

Thus, MB is a drug with an extended medical and safety record in humans, and FDA approval for its testing in clinical trials in connotation to aging and age-related disorders may not be denied on safety grounds.

2. OBJECTIVES

The aim of our study was to investigate the role of KGDHC in brain mitochondrial bioenergetics, oxygen consumption, ROS production and antioxidant expression. Various genetically modified mouse strains have been used having a heterozygous mutation either in the dihydrolipoyl succinyltransferase ($DLST^{+/-}$) or in dihydrolipoyl dehydrogenase ($DLD^{+/-}$) leading to reduced protein expression.

Advising the following questions:

•How important are the DLST and DLD subunits of the KGDHC in mitochondrial ROS production?

•How does the reduced expression of DLD subunit affects the ROS generation during reverse electron transfer (RET) induced by either succinate, or α-glycerophosphate?

•How does the expression of mitochondrial glutathione peroxidase change in transgenic mice?

On the other hand, we investigated the mitochondrial effect of methylene blue (MB), which cloud determinate its beneficial effects found in several pathological conditions:

• How does MB influence the mitochondrial oxygen consumption by the presence of glutamate-malate respiratory substrates?

• How does MB affect ATP production in respiration-impaired mitochondria?

• What is the effect of MB on the mitochondrial membrane potential in resting and respiration-compromised mitochondria?

• What causes the elevated ROS production by methylene blue in both energized and respiration-impaired mitochondria in the present of different respiratory substrates?

3. METHODS

3.1. Animals

Heterozygous DLD+/- (DLD+/-, C57BL/6) mice and wild type (WT) littermates were obtained from Jackson Laboratory (JAX mice; https://www.jax.org/strain/008333 Jackson Laboratory Repository, Bar Harbor, ME, USA). Mice deficient in the DLST subunit (DLST+/-; C57BL/6 and 129SV/EV hybrid) and WT littermates were obtained from Lexicon Pharmaceuticals (https://www.informatics.jax.org/allele/MGI:4367457, The Woodlands, TX, USA). WT mice were reproduced by crossing DLD+/- and DLST+/-; thus, all WT mice have DLD+/- or DLST+/- progeny. The animals used in our study were of either sex and between 3 and 6 months of age. Mice and guinea pigs were housed in a room maintained at 20–22 °C on a 12-h light-dark cycle with food and water available ad libitum. Animals were decapitated by a process in accordance with the International Guiding Principles for Biomedical Research Involving Animals and Guidelines for Animal Experiments at Semmelweis University according to the EU Directive "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes".

3.2. Mitochondria isolation

Synaptic and non-synaptic mitochondria were isolated from adult mice brain using a discontinuous Percoll gradient, as detailed earlier [146;147]. Brains were immediately removed and homogenized in ice-cold buffer A (in mM: 225 mannitol, 75 sucrose, 5 HEPES, 1 EGTA; pH 7.4) and then centrifuged for 3 min at 1300 g. The supernatant was centrifuged for 10 min at 20 000 g. The pellet was then suspended in 15 % Percoll and layered on a discontinuous gradient consisting of 40 % and 23 % Percoll layers, which was then centrifuged for 8 min at 30 700 g. After resuspension of the lowermost fraction in buffer A, it was centrifuged at 16 600 g for 10 min, and then the pellet was resuspended in buffer A and centrifuged again at 6 300 g for 10 min. After the supernatant was discharged, the pellet was resuspended in buffer B (in mM: 225 mannitol, 75 sucrose, 5 HEPES, pH 7.4) yielding ~30 mg/mL mitochondrial protein concentration. Mitochondrial protein concentration was determined by a modified Biuret assay [148]. Mitochondria were prepared and used within 4 hours in each

experiment and added with a 10 uL pipette to the cuvettes or the O2k-chamber after careful resuspension. Unless otherwise indicated, 0.1 mg/mL mitochondrial protein was applied in the experiments.

3.3. Mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured using high-resolution respirometry (Oroboros O2k; Oroboros Instruments, Innsbruck, Austria) at 37 °C in 2-mL chambers under continuous stirring [146]. Data were digitally recorded and analyzed. Oxygen concentration was monitored by the polarographic oxygen sensor (POS) and the oxygen flux was calculated as the negative time derivative of the oxygen concentration [149]. POS was calibrated routinely at air saturation and in oxygen-depleted medium. Oxygen consumption was measured in the following standard medium (in mM): 125 KCl, 20 HEPES, 2 KH_2PO_4, 0.1 EGTA, 1 MgCl2 and 0.025 % bovine serum albumine (BSA), pH 7.4. Mitochondria were energized with α-KG (5 mM), α-glycerophosphate (α–GP; 20 mM), or succinate (5 mM).

3.4. Mitochondrial H_2O_2 formation

The rate of H_2O_2 generation was determined using the Amplex UltraRed assay [150]. Horseradish peroxidase (5 U per 2 mL), Amplex UltraRed (2 μM), and mitochondria were added to the standard medium. Fluorescence was recorded at 37 ºC at 550 nm excitation and 585 nm emission wavelengths in a PTI Deltascan fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ, USA). The fluorescence signal was calibrated with known quantities of H_2O_2 at the end of each experiment..

3.5. Measurement of NAD(P)H steady state

The matrix NAD(P)H autofluorescence was measured in parallel with the Amplexn assay using the double excitation and double emission mode of PTI Deltascan fluorescence spectrophotometer. Mitochondria were incubated at 37°C as described above and the fluorescence was measured using 344 nm excitation and 460 nm emission wavelengths. Changes in the NAD(P)H level were expressed in photon count × 10^3.

3.6. Measurement of mitochondrial ATP synthesis

Synthesis of ATP was measured by a coupled enzymatic assay [150;151]. Standard assay medium was supplemented with $NADP^+$ (1.5 mM), hexokinase (2U/ml), glucose 6-phosphate dehydrogenase (3.84 U/ml), 2.5 mM glucose, 50 μM P^1,P^5-Di(adenosine-5') pentaphosphate (inhibitor of adenylate kinase). In the medium ATP phosphorylated glucose to glucose 6-phosphate in the presence of hexokinase, then glucose 6-phosphate was converted by glucose 6- phosphate dehydrogenase to 6-phosphogluconate with the concomitant reduction of $NADP^+$ to NADPH. Absorbance of NADPH was measured at 340 nm using GBC-UV double beam spectrophotometer. Measurements were calibrated with known amounts of ATP.

3.7. Measurement of membrane potential

ΔΨm was determined using the cationic dye safranine O, which is accumulated and quenched in energized mitochondria [152]. The dye concentration was 2 μM. The excitation and emission wavelengths were 495 and 586 nm respectively as described previously [153]. Measurements were performed at 37 °C with 0.1 mg/ml mitochondrial protein using a Hitachi F-4500 spectrofluorimeter (Hitachi High Technologies, Maidenhead, UK).

3.8. Measurement of Ca^{2+} uptake

Mitochondria (0.1 mg/ml) were added to the incubation medium in the presence of ADP and glutamate plus malate (5 mM each) then Ca^{2+} pulses were given in 100 sec intervals. The free Ca^{2+} concentration at each added concentration of Ca^{2+} was calculated and measured. Ca^{2+} uptake by mitochondria was followed by measuring Calcium Green-5N (100 nM) fluorescence at 505 nm excitation and 535 nm emission wavelengths at 37°C using a Hitachi F-4500 spectrofluorimeter.

3.9. Western blotting

Frozen-thawed mitochondrial pellets were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to a methanol-activated polyvinylidene difluoride membrane. Immunoblotting was performed as recommended by the manufacturers of the antibodies. Mouse monoclonal anti-cyclophilin D (cypD; Mitosciences, Eugene, OR, USA), rabbit polyclonals anti-

OGDH, anti-DLST, anti-DLD, anti-VDAC1, anti-CypD, anti-GR, anti-GPX1, anti-TRX and anti-PRX3 (Abcam, Cambridge, UK) primary antibodies were used at concentrations of 1 microg/mL, while rabbit polyclonal anti-manganese superoxide dismutase (MnSOD; Abcam) at 0.2 microg/mL. Immunoreactivity was detected using the appropriate peroxidase-linked secondary antibody (in 1:4,000 dilution, donkey anti-mouse or donkey anti-rabbit; Jackson Immunochemicals Europe Ltd., Cambridgeshire, UK) and enhanced chemiluminescence detection reagent (ECL system; Amersham Biosciences GE Healthcare Europe GmbH, Vienna, Austria).

3.10. Materials

All laboratory chemicals were obtained from Sigma Aldrich (St. Louis, MO, US) except for ADP (Merck Group, Darmstadt, Germany), α-GP (Santa Cruz Biotechnology, Dallas, TX, US), and Amplex UltraRed (ThermoFisher Scientific, Waltham, MA, US).

3.11. Statistics

Data in general are presented as the means ± S.E.M. Normal distribution was tested by the F-probe. Statistical differences were evaluated with ANOVA (SIGMASTAT; Systat Software Inc., San Jose, CA, USA) followed by the Bonferroni's test for multiple comparison; $p<0.05$ represents significant difference.

4. RESULTS

4.1 Effect of the KGDHC subunit deficiencies

In order to examine the mitochondrial bioenergetics of the KGDHC subunit deficient mice, oxygen consumption, ROS production and protein expression were monitored in normal versus genetically modified mitochondria.

4.1.1. Oxygen consumption of mitochondria using various respiratory substrates

Mitochondria energized with α-KG isolated from control and transgenic KGDHC heterozygote animals exhibited acceptable coupling (see Table 1). The respiratory control ratio (the rate of respiration in the presence of respiratory substrate and ADP/the rate of respiration in the absence of ADP) is a widely used parameter for the quality control of mitochondrial preparations. Importantly, there were no significant differences between the control and transgenic groups in terms of mitochondrial quality and coupling regardless of the respiratory substrates used.

Table 1. Respiratory control ratio (RCR) in mitochondria isolated from wild-type (WT), dihydrolipoyl succinyltransferase $DLST^{+/-}$ and dihydrolipoyl dehydrogenase $DLD^{+/-}$ transgenic mice. α-ketoglutarate (α-KG; 5 mM), succinate (5 mM), or α-glycerophospate (α-GP; 20 mM) were used as respiratory fuel substrates. RCR was calculated as the ratio of the O_2 consumption rates measured in the presence and absence of ADP (2 mM). Original traces and data are shown in Figures 1-3. The results are expressed as mean±S.E.M. (N=4-16).

	WT	$DLST^{+/-}$	$DLD^{+/-}$
α-KG	5.99±0.50	4.48±0.60	4.26±0.65
succinate	2.97±0.07	2.87±0.08	2.92±0.07
α-GP	2.08±0.07	1.99±0.09	1.97±0.10

Table 2. P-L control efficiency (OXPHOS coupling efficiency) in mitochondria isolated from wild-type (WT), dihydrolipoyl succinyltransferase $DLST^{+/-}$ and dihydrolipoyl dehydrogenase $DLD^{+/-}$. α-ketoglutarate (α-KG; 5 mM), succinate (5 mM), or α -glycerophospate (α-GP; 20 mM) were used as respiratory fuel substrates. P-L (OXPHOS) control efficiency was calculated as [1-(O_2 consumption measured without ADP/O_2 consumption measured with ADP)]. 1 refers to fully coupled mitochondria, 0 refers to zero respiratory phosphorylation capacity. Original traces and data are shown in Figures 1-3. The results are expressed as mean±S.E.M. (N=4-16).

	WT	$DLST^{+/-}$	$DLD^{+/-}$
α-KG	0.81±0.02	0.75±0.03	0.75±0.04
succinate	0.66±0.01	0.65±0.01	0.66±0.01
α-GP	0.51±0.01	0.49±0.02	0.49±0.03

4.1.1.1. Respiration of α-ketoglutarate-supported mitochondria

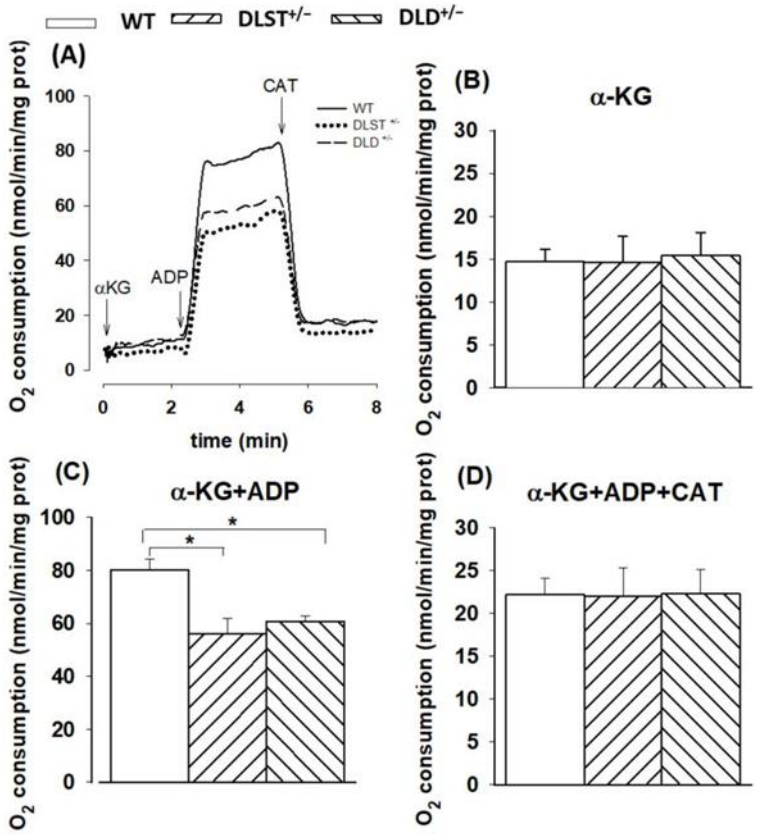

Figure 1. Oxygen consumption of mitochondria isolated from wild-type and KGDHC subunit heterozygote KO mice respiring on α-ketoglutarate (α-KG) in the absence (B) or presence (C,D) of ADP, and after addition of carboxyatractilozide (CAT), (D). (A) Traces are representatives of experiments with wild-type mice (WT; solid line), dihydrolipoyl succinyltransferase transgenic mice ($DLST^{+/-}$; dotted line), and dihydrolipoyl dehydrogenase transgenic mice ($DLD^{+/-}$; dashed line). Oxygen consumption was monitored by high-resolution respirometry. Mitochondria (0.1 mg/mL) were incubated in a standard medium, as described under Materials and Methods. Afterwards, α-KG (5 mM), ADP (2 mM); (B,C), and carboxyatractyloside (CAT; 2 M); (D) were given. White bars: WT; bars with left diagonal stripes: $DLST^{+/-}$ mutation; bars with right diagonal stripes: $DLD^{+/-}$ mutation. The results are expressed as the means of oxygen consumption in nmol/min/mg protein SEM (N = 4–16). Statistically significant differences are indicated by asterisks; * $p < 0.05$.

α-KG is the substrate specific for the KGDHC in mitochondria. In the presence of respiratory substrate (α-KG) but in the absence of ADP, there was no significant changes between the isolated mitochondria observed. (Figure 1B). Upon addition of ADP the rate of respiration was elevated, however, in all the transgenic groups a decrease of oxygen consumption was detected (Figure 1C). After inhibition of the ANT

by CAT (Figure 1D), the rate of respiration was decreased in all types of mitochondria. However, the rates of CAT-inhibited respiration were not significantly different between the groups.

4.1.1.2. Respiration of succinate-supported mitochondria

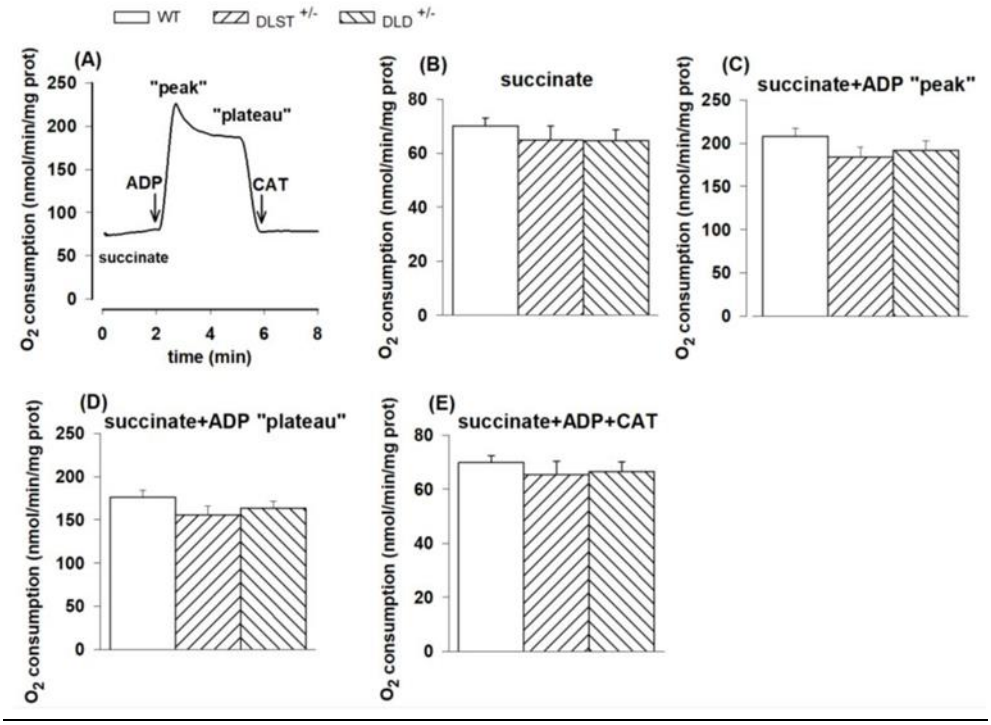

Figure 2. Oxygen consumption of mitochondria isolated from wild-type and KGDHC subunit heterozygote KO mice respiring on succinate in the absence (B) or presence (C,D) of ADP, and after addition of carboxyatractyloside (CAT), (E). (A) Trace is representative of a single experiment. Succinate (5 mM), ADP (2 mM); (C–E) and CAT (2 M); (E) were given as indicated. All other conditions and representations are as in Figure 1.

In order to assess whether mutations in various KGDHC subunits affect oxidation of other substrates independent of the activities of the KGDHC and respiratory Complex I, succinate was also utilized as a respiratory substrate; which is oxidized by Complex II.

In succinate-energized mitochondria, none of the KGDHC transgenic conditions led to significant differences relative to control in the investigated respiratory states (Figure 2A-E). Oxygen consumption in succinate-supported mitochondria in the presence of ADP exhibited a sharp peak (Figure 2A), which was followed by a decline and plateau. These phenomena were potentially attributed to the accumulation of oxaloacetate [154;155], the physiologic inhibitor of succinate dehydrogenase.

4.1.1.3. Respiration of α-glycerophosphate (α-GP) supported mitochondria

Similarly to succinate, α-GP also donates electrons to coenzyme Q in the ETS without using Complex I during the forward electron transfer. α-GP, unlike succinate, does not enter the mitochondrial matrix, it is oxidized on the outer surface of the mitochondrial inner membrane by α-GPDH [156-158]. Furthermore, the α-GPDH shuttle can mediate the oxidation of cytosolic (glycolytic) NADH in the mitochondria. Brain mitochondria possess a high α-GPDH activity [158;159], therefore, using this substrate could serve as a good control for the effects of the KGDHC mutations on the ETS. In line with the results on succinate oxidation, there were no significant differences between the groups of transgenic animals compared to the control under the conditions applied (Figure 3).

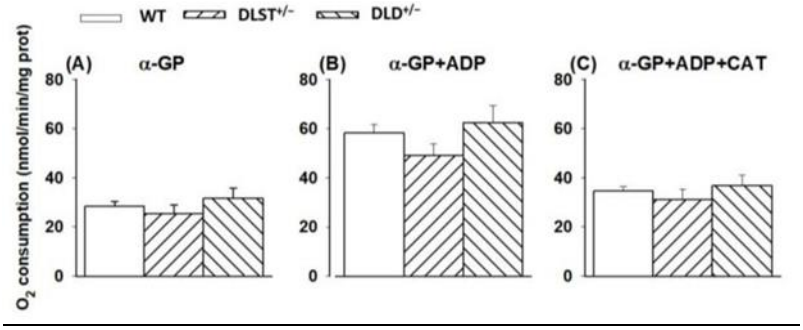

Figure 3. . Oxygen consumption of mitochondria isolated from wild-type and KGDHC subunit heterozygote KO mice respiring on α-glycerophosphate in the absence (A) or presence (B,C) of ADP, and after the addition of carboxyatractyloside (CAT), (C). -GP (20 mM), ADP (2 mM); (B,C) and CAT (2 M); (C) were given as indicated. All other conditions and representations are as in Figure 1.

4.1.2. H_2O_2 production of mitochondria

Considering that the KGDHC subunits are capable of producing ROS, even in the absence of the other subunits of the enzyme [160], the aim of the experiments was to determine the effects of reduced expression of the subunits on the mitochondrial ROS production. Formation of H_2O_2, the most stable form of ROS, was studied according to the following experimental setup: i) mitochondria were energized with a single substrate (α-KG, succinate, or α-GP); ii) ADP was added to generate a high rate of respiration via decreasing the ΔΨm and supplying substrate for the ATP synthase; iii) an ANT inhibitor was given to re-establish the hyperpolarized ΔΨm and prevent the transport of extramitochondrial ADP to the matrix; iv) rotenone, an inhibitor of the Complex I, was

administered to inhibit the reverse electron transfer (RET) evoked by either succinate or α-GP; and v) antimycin A was added to block electron transport at Complex III.

4.1.2.1. H_2O_2 production of α-ketoglutarate-supported mitochondria

In α-KG-supported mitochondria (with no other additions), the following tendency in ROS production was found: WT>DLST$^{+/-}$>DLD$^{+/-}$, but there was no statistically significant difference among the WT and transgenic groups (Figure 4B). As it was expected, in the presence of ADP, there was a slight decrease in H_2O_2 production in every group, however, significant differences could still not be detected in the transgenic groups compared to the control (Figure 4A and B). Inhibition of ANT by CAT elevated the rate of H_2O_2 formation owing to hyperpolarization of ΔΨm. In the DLD$^{+/-}$ transgenic groups H_2O_2 formation was significantly decreased relative to the control after CAT addition (Figure 4D). Adding CI inhibitor rotenone, H_2O_2 production was accelerated in every group, and differences between the control and the transgenic groups became significant. Inhibition of Complex III by antimycin A added after rotenone only slightly stimulated the H_2O_2 formation, while H_2O_2 production rate was again significantly smaller relative to control in all the transgenic groups in the presence of antimycin A. It is worth mentioning that the decrease of the rate of H_2O_2 production was the smallest in the DLST$^{+/-}$ mitochondria. Therefore, most of the decrease in H_2O_2 production could be ascribed to the lost allele of DLD.

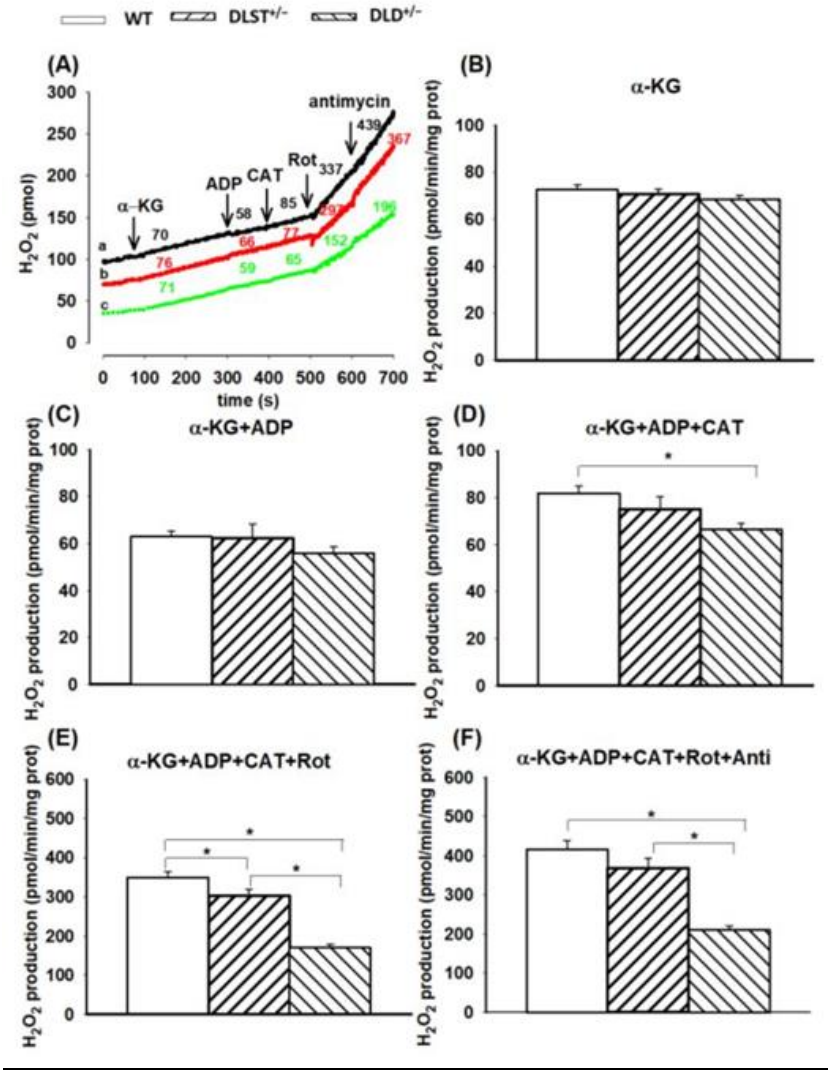

Figure 4. Hydrogen peroxide production in wild-type and KGDHC-subunit-KO mitochondria respiring on α-ketoglutarate. H_2O_2 production was measured with the Amplex UltraRed assay as described under Materials and Methods. (A) Traces represent single independent experiments and are offset for clarity. Trace a (black): WT, trace b (red): $DLST^{+/-}$, trace c (green): $DLD^{+/-}$. Mitochondria (0.1 mg/mL) were incubated in the standard medium which was followed by the addition of α-KG (5 mM), ADP (2 mM), CAT (2 M), rotenone (Rot, 250 nM), and antimycin A (1 M), as indicated. Numbers on the traces represent rates of H_2O_2 generation expressed in pmol/min/mg protein. H_2O_2 production was measured in the presence of α-KG (B) and after adding ADP (C–F), CAT (D–F), Rot (E,F) and antimycin A (F). Results are expressed as means of rates of H_2O_2 production in pmol/min/mg protein S.E.M. (N = 4–14). All other representations are as in Figure 1. Statistically, significant differences are indicated by asterisks; * $p < 0.05$

4.1.2.2. H_2O_2 production of succinate-supported mitochondria

Succinate-evoked ROS formation has several components. Succinate dehydrogenase is a flavoenzyme with its own ROS-forming ability [161-163]. Under selected conditions (e.g. in the absence of ADP), electrons from succinate can flow backwards passing through Complex I in the reverse direction and reduce NAD^+ to NADH + H^+. This pathway is referred to as the reverse electron transfer (RET) [164;165] where the rate of ROS formation is an order of magnitude higher than in the normal flow from Complex II towards Complexes III and IV [150;166-168]. As it can be seen in Figure 5B, wild-type mitochondria exhibited the highest rate of H_2O_2 formation in the presence of succinate. In the KO group $DLD^{+/-}$, ROS formation was significantly lower than that of control. Addition of ADP abolished the conditions necessary for RET [168], therefore, the forward flow of electrons evoked a less intensive H_2O_2 formation. Under these conditions, there were no significant differences detected among the individual mitochondrial groups (Figure 5C). Inhibition of ADP entry into the mitochondria, applying the ANT inhibitor CAT, reestablished the conditions favorable for RET, and again, significant differences in ROS formation were detected among the WT and transgenic mitochondria (Figure 5D). After blocking the ANT, mitochondria were treated with the Complex I inhibitor rotenone. Contrary to that found with α-KG, which generates the Complex I substrate NADH, addition of rotenone to succinate-supported mitochondria decreased the rate of H_2O_2 production, which was attributed to the inhibition of RET. Similar to those results observed in the presence of ADP, the significant differences among the individual groups also disappeared (Figure 5E). The H_2O_2 production in the wild-type mitochondria after adding rotenone decreased from 1253.8 ± 318.8 to 203.6 ± 30.6 pmol/min/mg protein. A similar decrease was detected in the DLD+/- transgenic mice (from 682.2 ± 54.2 to 148.1 ± 23.0 pmol/min/mg protein) after rotenone addition. Administration of antimycin A to rotenone-treated mitochondria stimulated the H_2O_2 production to the third highest level, indicating that inhibition at the CIII increased the electron leak from CoQ and the FAD prosthetic group of SDH.

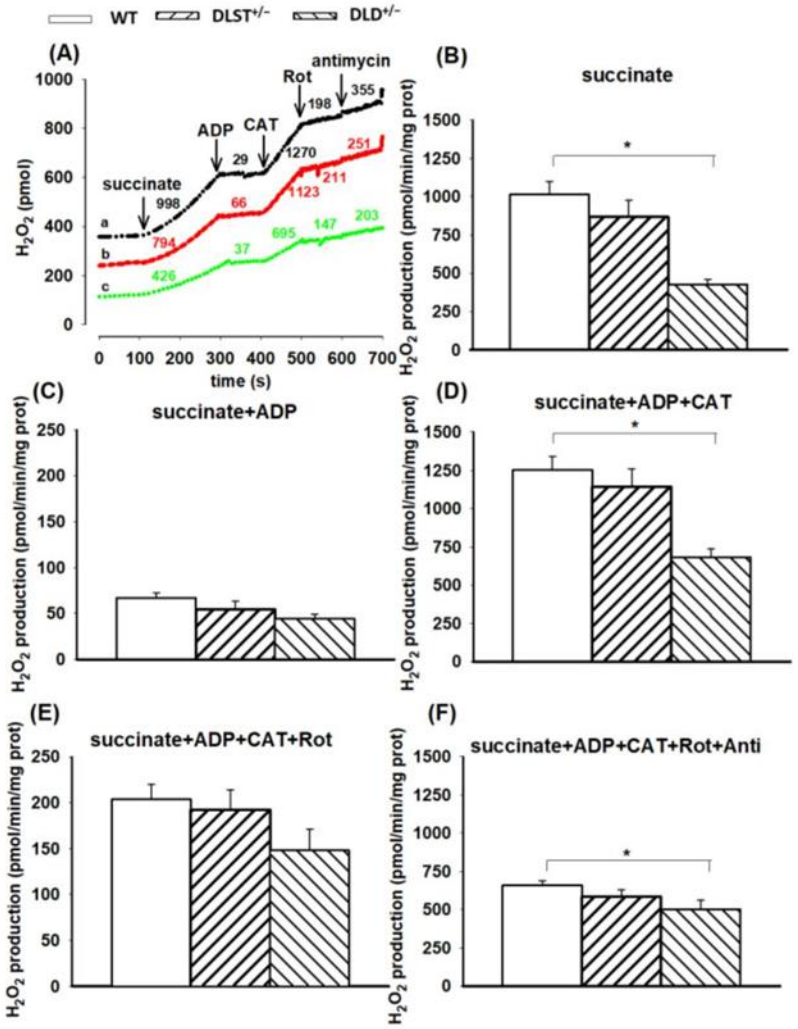

Figure 5. Hydrogen peroxide production in the wild-type and KGDHC-subunit-KO mitochondria respiring on succinate. H_2O_2 production was measured with the Amplex UltraRed assay as described under Materials and Methods. (A) Traces represent single independent experiments and are offset for clarity. Trace a (black): wild-type (WT), trace b (red): $DLST^{+/-}$, trace c (green): $DLD^{+/-}$. Mitochondria (0.1 mg/mL) were incubated in the standard medium which was followed by the addition of succinate (5 mM), ADP (2 mM), carboxyatractilozide (CAT, 2 M), rotenone (Rot, 250 nM), and antimycin A (2 M) as indicated. Numbers on the traces represent H_2O_2 production expressed in pmol/min/mg protein. H_2O_2 production was measured in the presence of succinate (B), after adding ADP (C–F), CAT (D–F), rotenone (E,F) and antimycin A (F). All other conditions and representations are as in Figure 1. Statistically significant differences are indicated by asterisks; * $p < 0.05$.

4.1.2.3. H_2O_2 production by α-glycerophosphate-supported mitochondria

We reported earlier that α-GP is capable of supporting RET in brain mitochondria [169;170] which is strongly dependent on the concentration of the substrate and the exogenous Ca^{2+}. Here, the H_2O_2 production using α-GP and succinate showed the similar pattern in the absence of ADP (Figures 5 and 6). In the α-GP resiring mitochondria, however, significant differences were found only between the WT and $DLD^{+/-}$ heterozygotes in the absence of ADP, upon inhibition of the ANT and CI inhibition (Figure 6). It is worth to note that ROS production in $DLST^{+/-}$ KO-s was always higher than that of the $DLD^{+/-}$ mitochondria.

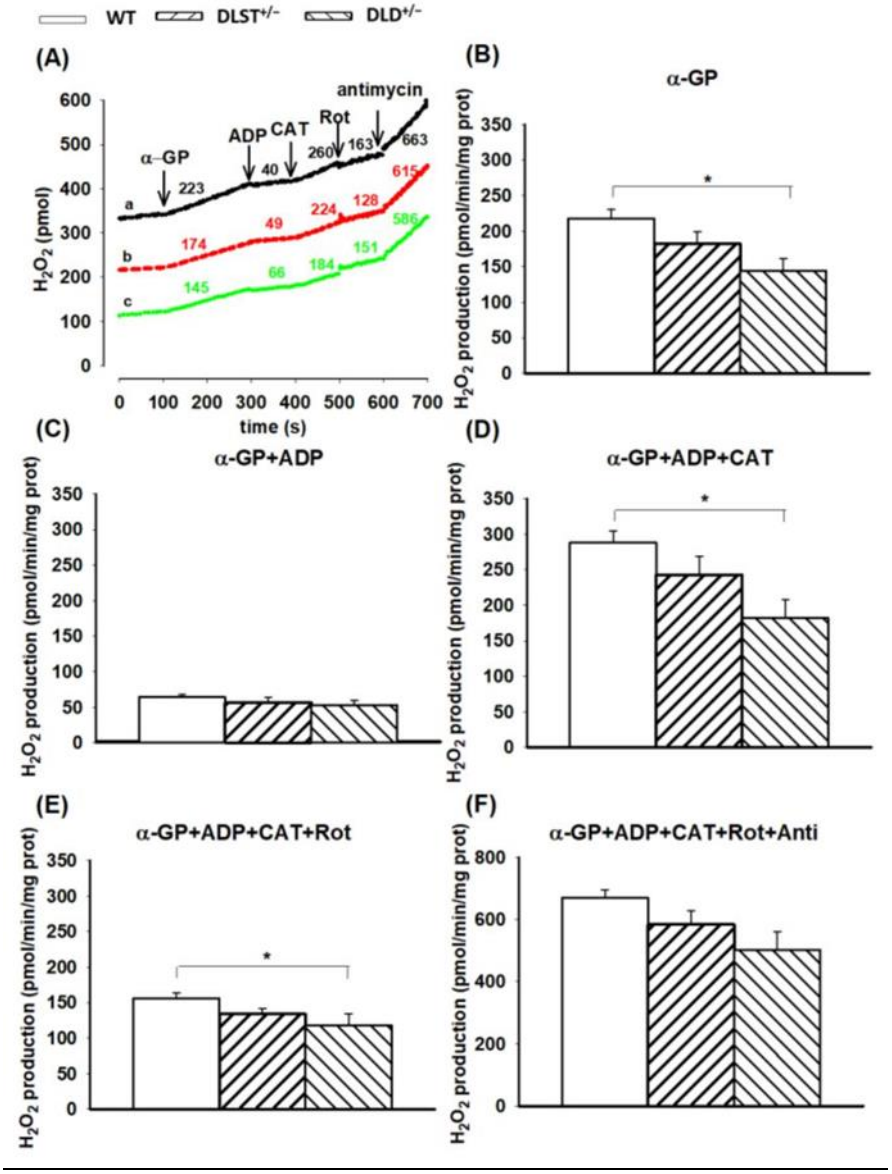

Figure 6. Hydrogen peroxide production in the wild-type and KGDHC-subunit-KO mitochondria respiring on α-glycerophosphate. H_2O_2 production was measured with the Amplex UltraRed assay as described under Materials and Methods. (A) Traces represent a single independent experiment and are offset for clarity. Trace a (black): wild-type (WT), trace b (red): $DLST^{+/-}$, trace c (green): $DLD^{+/-}$. Mitochondria (0.1 mg/mL) were incubated in the standard medium. Afterwards, α-GP (20 mM), ADP (2 mM), and carboxyatractyloside (CAT; 2 M), rotenone (Rot; 250 nM), and antimycin A (2 M) were given as indicated. Numbers on the traces represent H_2O_2 production expressed in pmol/min/mg protein. H_2O_2 production was measured in the presence of -GP (B), after adding ADP (C–F), CAT (D–F), rotenone (E,F) and antimycin A (F). All other conditions and representations are as in Figure 1. Statistically significant differences are indicated by asterisks; * $p < 0.05$.

4.1.3. Protein expression of the KGDHC subunits in the wild-type and transgenic animals

Next, we investigated how the genetic manipulations affected the protein expression of the KGDHC subunits in the mitochondria isolated from the transgenic animals compared to the controls (Figure 7-8). In Figure 8 KGDHC subunit expression levels were normalized to the expression of SOD2. As shown in Figure 8A, in all of the transgenic constructs, the E1 subunit (OGDH) expression was downregulated compared to the wild type. As it was expected, the expression level of the E2 (DLST) subunit was lower in the $DLST^{+/-}$ mitochondria, relative to control (Figure 8B). Expression of the E3 (DLD) subunit decreased in the DLD+/- animals (Figure 8C).

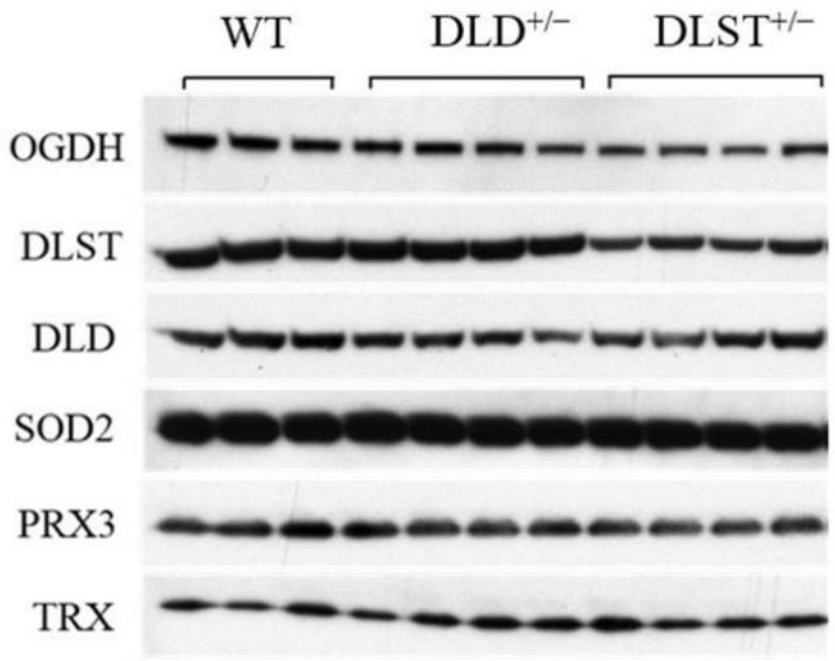

Figure 7. Immunoreactivities of alpha-ketoglutarate dehydrogenase (KGDH/OGDH), dihydrolipoyl succinyltransferase (DLST), dihydrolipoyl dehydrogenase (DLD), superoxide dismutase 2 (SOD2), peroxiredoxin 3 (PRX3), and thioredoxin (TRX) in mitochondria isolated from brains of wild-type (WT), $DLST^{+/-}$, and $DLD^{+/-}$ transgenic mice.

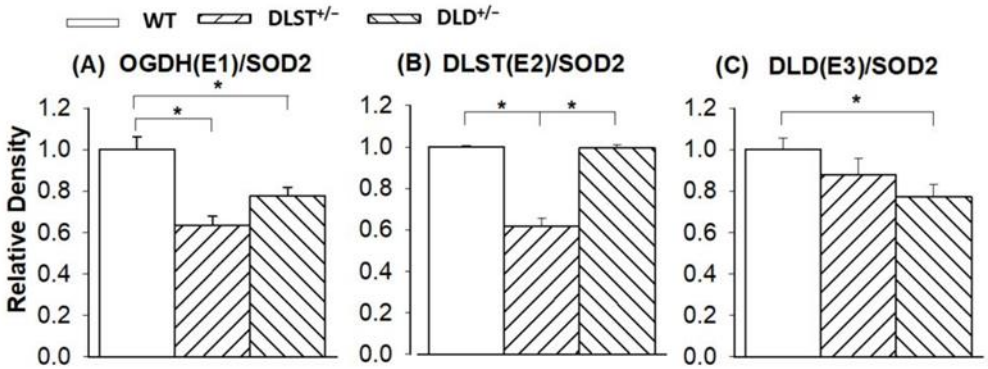

Figure 8. Western blot analysis and relative density changes for the KGDHC subunits, normalized for superoxide dismutase 2 (SOD2) protein expression, in brain mitochondria isolated from the wild-type and KGDHC-subunit-KO mice. (A) alpha-ketoglutarate/2-oxoglutarate (KGDH/OGDH) subunit; (B) dihydrolipoyl succinyltransferase (DLST) subunit; (C) dihydrolipoyl dehydrogenase (DLD) subunit. White bars: wild-type (WT); bars with left diagonal stripes: dihydrolipoyl succinyl-transferase mutation ($DLST^{+/-}$); bars with right diagonal stripes: dihydrolipoyl dehydrogenase mutation ($DLD^{+/-}$). Results are expressed as means of the relative densities S.E.M. (N = 3–4). Statistically significant differences are indicated by asterisks; * $p < 0.05$

4.1.4. The expression and activities of enzymes participating in the antioxidant system of mitochondria

It was expected that decrease of ROS production in the subunit deficient KGDHC downregulates the expression of selected mitochondrial antioxidant enzymes. In fact, the thiol-disulfide exchange reactions tightly link the lipoyl residues of the DLST enzyme to glutathione, thioredoxin, and the peroxidases using these reductants to scavenge H_2O_2 [171]. Therefore, the activities of glutathione peroxidase (GPX) and glutathione reductase (GR), as well as the expression levels of thioredoxin and peroxiredoxin were all assessed in the transgenic animals and compared to the respective controls. The GPX has a mitochondrial and a cytosolic form [172;173], however, the western blot assays were carried out with mitochondrial lysates, hence detecting only the mitochondrial isoform (Figure 9). In all the transgenic groups, there was a notable decrease in the GPX expression (normalized for CypD) compared to the control group suggesting the downregulation of GPX when less ROS was produced in the transgenic animals. Similarly, GR activity normalized for CypD showed a decrease in the transgenic groups compared to the WT mitochondria (Figure 10A). However, no significant differences were detected in the thioredoxin (TRX)/SOD and peroxiredoxin (PRX)/SOD expression ratios normalized for SOD (Figure 10 B, C).

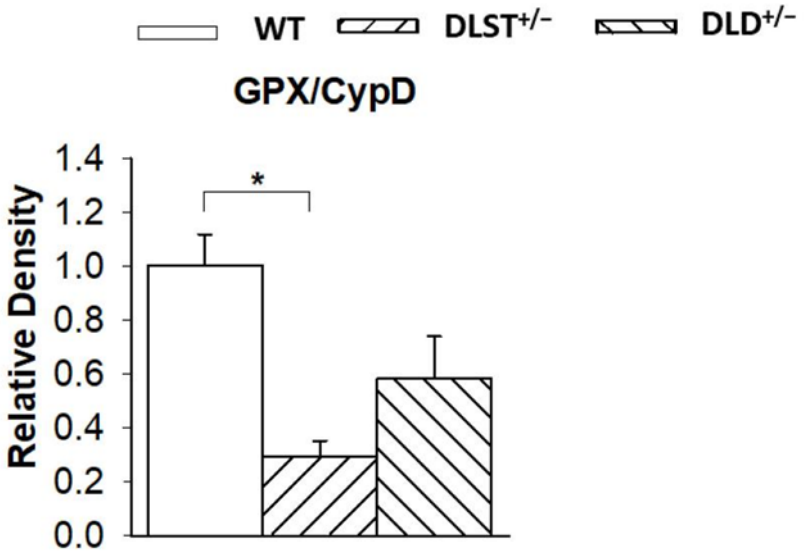

Figure 9. Western blot analysis and relative density changes for glutathione peroxidase (GPX), normalized for cyclophilin D (CypD) protein expression, in brain mitochondria isolated from the wild-type and KGDHC-subunit-deficient mice. White bars: wild-type (WT); bars with left diagonal stripes: dihydrolipoyl succinyltransferase mutation ($DLST^{+/-}$); bars with right diagonal stripes: dihydrolipoyl dehydrogenase mutation ($DLD^{+/-}$). Results are expressed as means of the relative densities S.E.M. (N = 3–4). Statistically significant differences are indicated by asterisks; * $p < 0.05$.

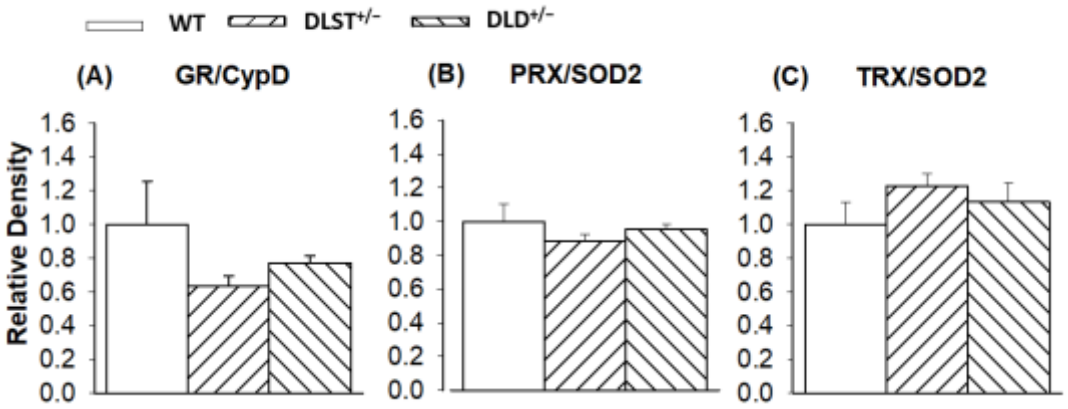

Figure 10. Western blot analysis and relative density changes for protein expression in mitochondria isolated from wild-type and KGDHc-subunit-deficient mice. (A) Glutathione reductase (GR) normalized for cyclophilin D (CypD); (B) peroxiredoxin (PRX) normalized for superoxide dismutase 2 (SOD2); (C) thioredoxin (TRX) normalized for SOD2. White bars: wild-type (WT); bars with left diagonal stripes: dihydrolipoyl succinyltransferase mutation ($DLST^{+/-}$); bars with right diagonal stripes: dihydrolipoyl dehydrogenase mutation ($DLD^{+/-}$);. The results are expressed as means of the relative densities±S.E.M. (N=3-4).

4.2. Mitochondrial effects of methylene blue

In order to dissect the beneficial effects of methylene blue (MB) we examined the mitochondrial oxygen consumption, ATP synthesis, membrane potential, calcium uptake and ROS release in isolated brain mitochondria from guinea pig

4.2.1. The effect of MB on the respiration of mitochondria

Given the hormetic pharmacological effect of MB meaning that it could have opposite effects in high and low doses ([174], we used MB in a concentration range (100 nM – 1 μM), which is relevant to cellular studies, in particular to those reporting neuroprotective effects of MB [125;126;175]. When mitochondria are respiring on glutamate plus malate electrons enter in the respiratory chain from NADH via complex I, whereas with succinate or α-glycerophosphate (α-GP) electrons from succinate dehydrogenase or α-glycerophosphate dehydrogenase (α-GPDH), respectively, reduce CoQ bypassing complex I. The rate of state 4 respiration of isolated brain mitochondria supported by glutamate plus malate in the absence of added ADP was stimulated from 375±20.6 to 618.3±36.2 pmol/sec/mg protein upon addition of 1 μM MB (Fig.11); the effect of MB was significant at already 300 nM concentration (463±24.6 pmol/sec/mg protein). The ADP-stimulated (state 3) respiration was unaffected by MB, but the stimulation of the state 4-like respiration was again evident after the addition of carboxyatractylozide (CAT), which inhibits the adenine nucleotide translocator (ANT) preventing the entry of ADP into the matrix, therefore mitochondria behave as if in the absence of ADP (Fig.11). The rate of respiration, as expected was reduced by rotenone and addition of ADP was without an effect, but MB was able of stimulating respiration also under this condition (Fig.11, bars at the right end).

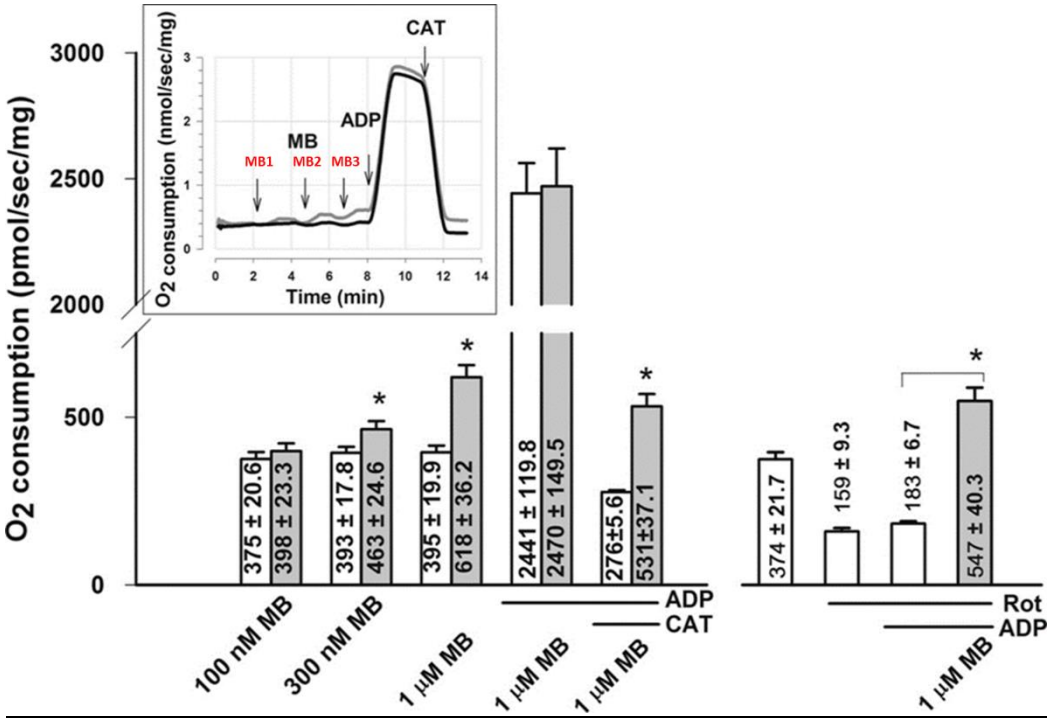

Figure 11. The effect of MB on the rate of oxygen consumption in glutamate plus malate supported mitochondria. Mitochondria (0.05 mg/ml) were incubated in the standard medium as described in Methods. MB (100 nM, 300 nM and 1 μM final concentrations), ADP (2 mM) then carboxyatractylozide (CAT; 2 μM) were given as indicated in the inset (grey trace; no MB addition for black trace). The effect of MB (grey bars) are compared to controls (white bars; no MB, only buffer added) measured for each individual experiment. Data from similar experiments performed in the presence of rotenone (0.5 μM) are shown separated on the right. Results are expressed as mean oxygen consumption in pmol/sec/mg protein ±SEM n>4 and written on the bars. Asterisks indicate significant differences ($p<0.05$) from the corresponding MB-free controls

4.2.3. ATP production is partially restored in respiration-impaired mitochondria

Stimulation of respiration itself is not informative as to the bioenergetic competence of mitochondria. Uncouplers stimulate directly the respiration and decrease the electrochemical proton gradient without the production of ATP. To address this, ATP production in mitochondria respiring on glutamate plus malate or succinate was measured. In fully functional mitochondria ATP generation was found to be unaffected by MB applied in 2 μM concentration (data not shown), however, significant effects were observed in respiration compromised mitochondria. As expected, the rate of ATP generation initiated by addition of ADP in glutamate plus malate-supported mitochondria was drastically decreased by rotenone treatment (from 1100±44 to 46±1.5

nmol/min/mg protein). The slow rate of ATP generation was significantly increased by 1 or 2 μM MB (67±2.4 or 93±4.6 nmol/min/mg protein, respectively) (Fig.2/A, C). In succinate-supported mitochondria the rate of ATP generation was reduced by the complex III inhibitor, antimycin A from 910±76 to 46.5±1.5 nmol/min/mg protein. ATP generation from this low level was slightly but significantly stimulated by 1 or 2 μM MB (to 59.2±2.1 or 63.4±2.5 nmol/min/mg protein, respectively) (Fig.12/B,D).

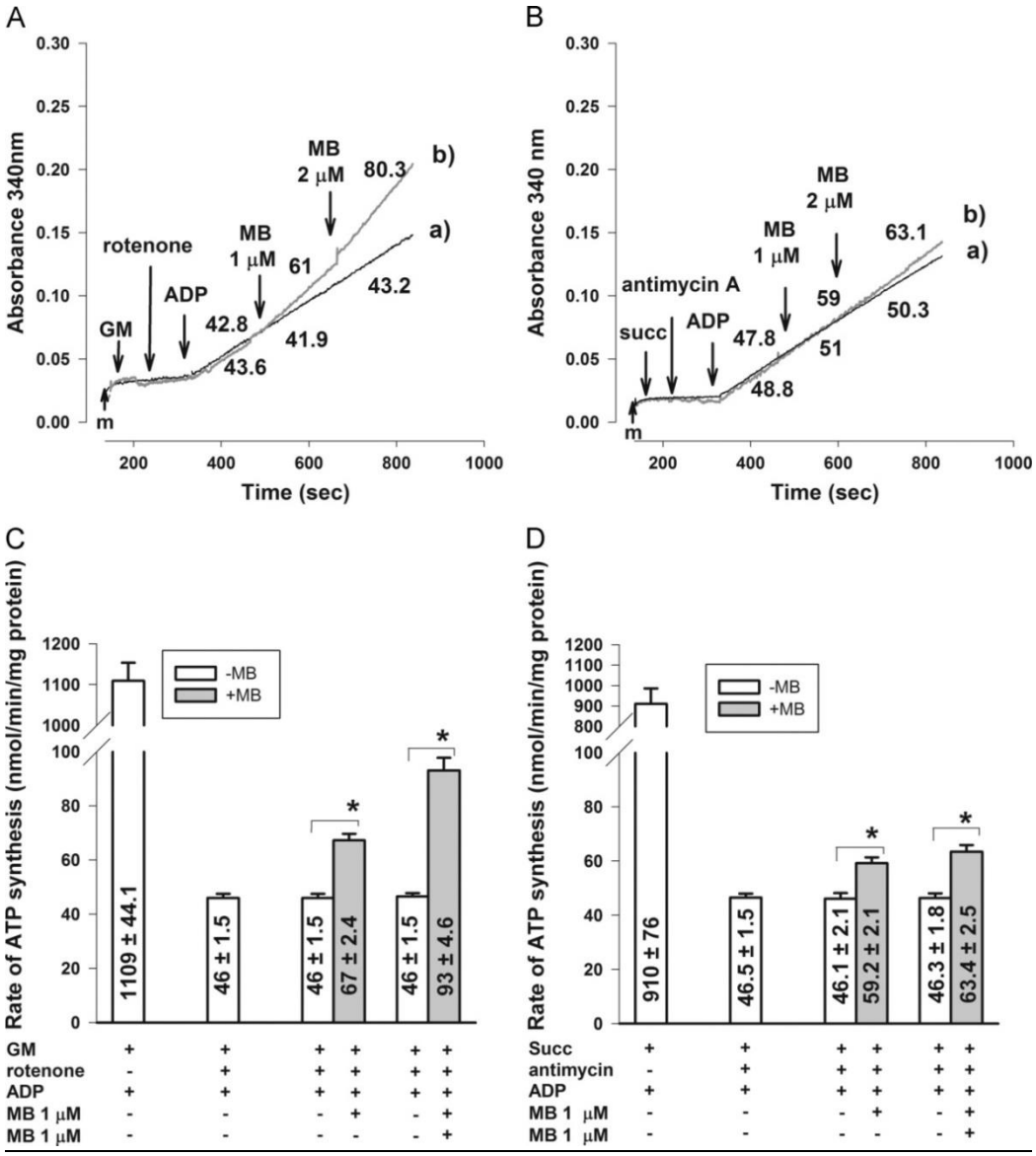

Figure 12 The effect of MB on the rate of ATP production in respiration-impaired mitochondria. In glutamate plus malate-supported mitochondria (A,C) rotenone (0.5 μM) was used to inhibit complex I; in succinate-supported mitochondria (B,D) complex III was inhibited by antimycin A (0.1 μM). Original traces are shown in panels A and B with numbers calculated as rate of ATP synthesis in nmol/min/mg in these particular experiments; additions were as indicated (MB 1 and 2 μM final concentrations). For traces (a) no MB was given. In panels C and D bars represent average ATP production rates in nmol/min/mg protein ± S.E.M from at least 3 experiments. Asterisks indicates significant differences ($p<0.05$) from the corresponding controls measured in the absence of MB

4.2.4. The effect of MB on ΔΨm in resting and respiration-compromised mitochondria

ΔΨm is another key parameter that is essential for the bioenergetic performance of mitochondria. In highly energized mitochondria in the presence of glutamate plus malate (5 mM each) ΔΨm was unchanged by MB as detected by safranin fluorescence (Fig.13 traces a,b). With insufficient amount of substrates, in the presence of 50 or 10 μM glutamate plus malate, mitochondria cannot be fully energized due to the limited availability of NADH as indicated by ΔΨm set at a depolarized value (Fig.13 traces c and e, respectively). Diversion of electrons from NADH to MB under these conditions is likely responsible for the evident further loss in ΔΨm (traces d and f), which is not apparent when substrates are abundant (traces a and b). Similar results were obtained with mitochondria respiring on succinate (not shown).

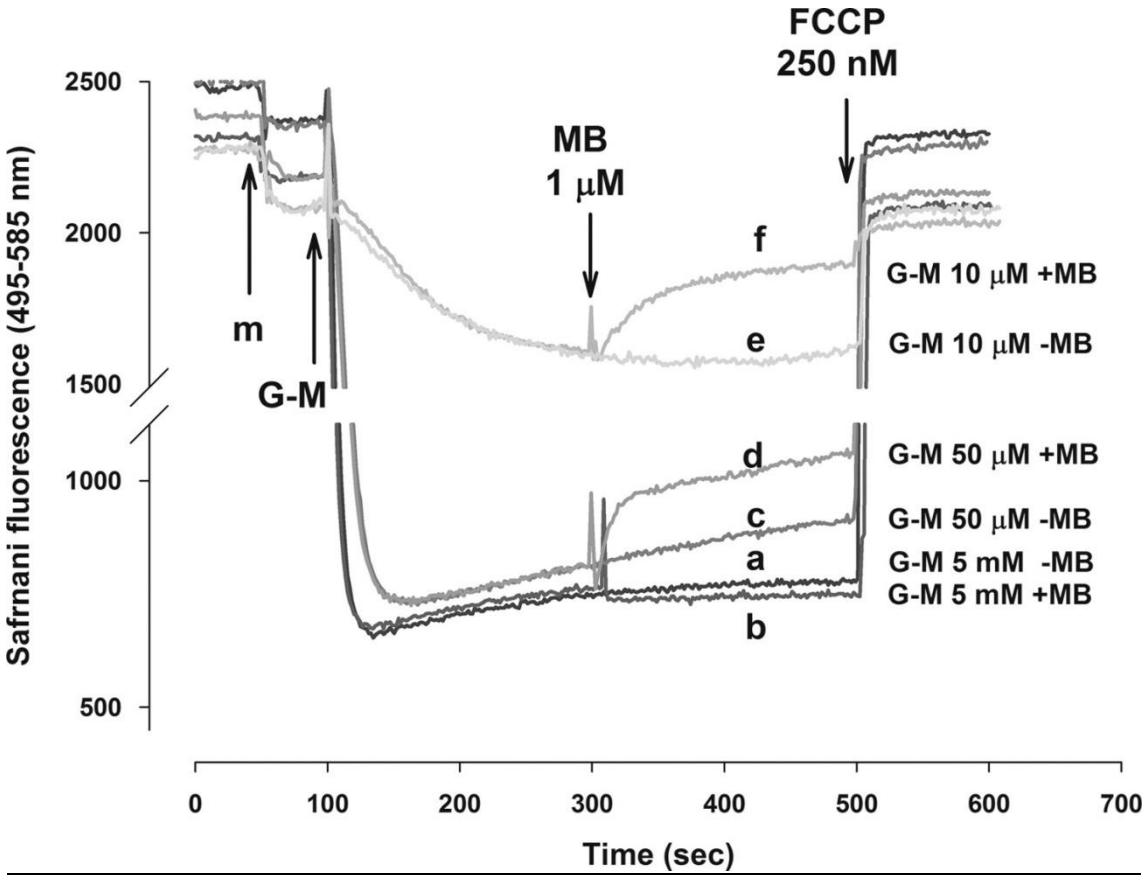

Figure 13 The effect of MB on safranine fluorescence indicating ΔΨm in mitochondria respiringon glutamate plus malate (G-M, 5 mM for traces *a, b;* 50 μM for traces *c, d;* and 10 μM for traces *e, f*). MB (1 μM) was added as indicated for traces *b, d, f.* Traces are representatives of experiments performed in three replicats.

In glutamate plus malate-supported mitochondria rotenone induced an abrupt drop in ΔΨm, which recovered after addition of 1 μM MB and then these mitochondria, similar to competent ATP-synthesizing mitochondria, reacted with depolarization to the addition of ADP followed by recovery of ΔΨm in response to the ANT inhibitor, CAT (Fig.14/A trace b). ΔΨm was not rescued by MB when complex IV was inhibited by KCN (Fig.14/A traces c). ATP synthesis can also be blocked by oligomycin, instead of CAT, given after ADP (Fig. 14/B, trace b), but with this, smaller repolarization was observed than with CAT (compare traces b in Fig.14/A versus Fig.14/B). In mitochondria without MB addition, oligomycin given after ADP resulted in a small further depolarization (Fig.14/B trace a), whereas CAT slightly repolarized ΔΨm (Fig.14/A trace a). The explanation lies in the different reversal potential of the adenine nucleotide translocase and that of the ATP synthase. At low ΔΨm ATP synthase can operate in reverse, but ANT still works in the forward mode. Under this conditions application of oligomycin depolarizes ΔΨm because it stops proton pumping, but inhibition of ANT would hyperpolarize mitochondria [176]. Similarly, rescue of ΔΨm by MB, though to a smaller degree, was observed with succinate (Fig.14/C trace b) or α-GP (Fig. 14/D trace b) as a substrate, when respiration was inhibited and ΔΨm was highly depolarized with the complex III inhibitor, antimycin. Contrary to this, in mitochondria poisoned by cyanide, MB was unable to rescue ΔΨm (Fig.14/A,C,D traces c), indicating that MB donates electrons to the respiratory chain proximal to cytochrome oxidase. These results indicate that MB supports the maintenance of ΔΨm in mitochondria subjected to inhibitors of complex I.

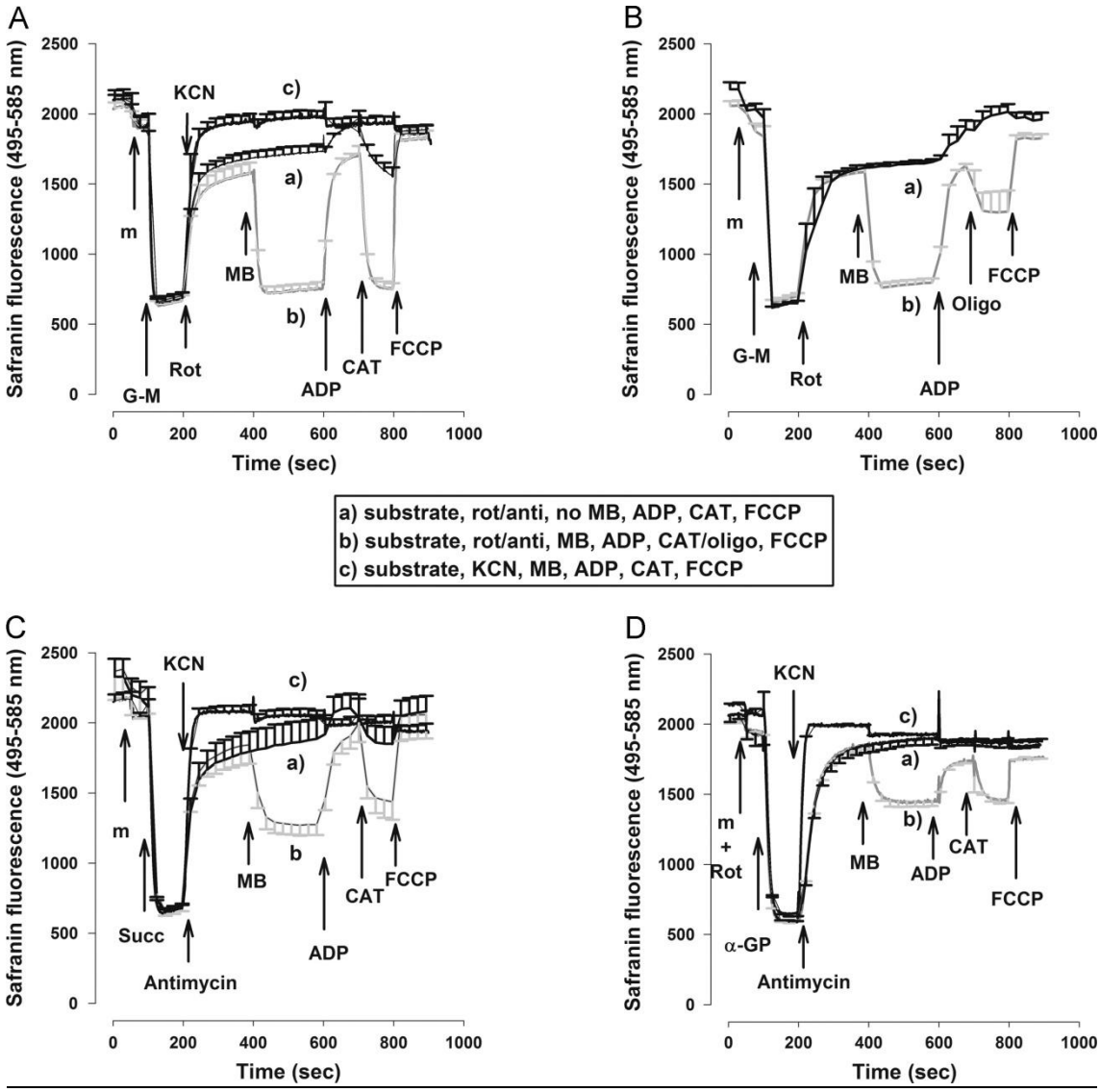

Figure 14 The effect of MB (1 μM) on m in respiration-impaired mitochondria supported by glutamate plus malate (5 mM each; A,B), succinate (5 mM; C) or α-GP (20 mM; D). Additions are indicated by arrows and the order of additions for traces *a, b or c* is also shown in the frame. For traces *a)* no MB was given. For each trace mitochondria (m) then respiratory substrate were given then the order of additions was as follows: traces *a)* rotenone (0.5 μM, for A and B) or antimycin (0.1 μM, for C and D), ADP (2mM), CAT (2 μM), FCCP (250 nM); traces *b)* rotenone (A and B) or antimycin (C and D), MB (2 μM), ADP, CAT (A,C and D) or oligomycin (2 μM, B), FCCP; traces *c)* , KCN (2 mM), MB, ADP, CAT, FCCP. Each trace represents an average ±SEM of at least three independent experiments

4.2.5. Ca^{2+} uptake capacity is increased by MB in energetically compromised mitochondria

Ca^{2+} uptake is also a key function of mitochondria and it is well established that mitochondrial Ca^{2+} uptake is an energy- and m-dependent process [177;178]. The effect of MB on the mitochondrial Ca^{2+} uptake was investigated in resting and energetically compromised mitochondria. The rate of Ca^{2+} uptake was decreased in the presence of 1 μM MB in resting mitochondria energized with glutamate plus malate (Fig.15/A, upper trace). Contrary to this, Ca^{2+} uptake reduced in rotenone-treated mitochondria was stimulated by MB (Fig.15/B, lower trace) consistent with the partially restored m observed under similar conditions.

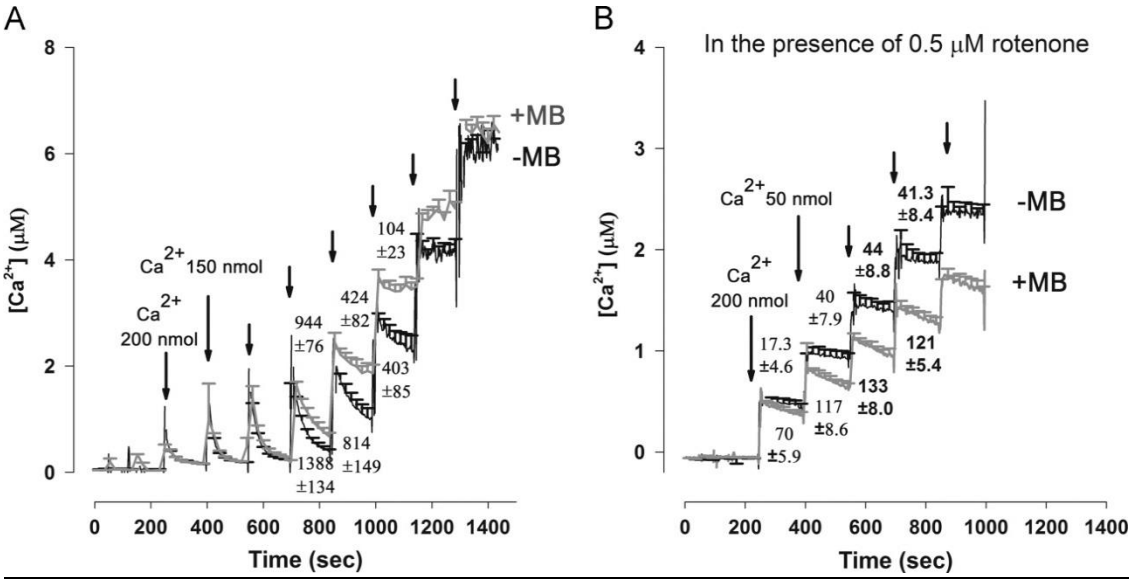

Figure 15 The effect of MB on the Ca^{2+}-uptake in energized (A) and in rotenone-treated mitochondria (B) respiring on glutamate plus malate. Ca^{2+} pulses (150 nmol each for A except the first addition, which was 200 nmol ; 50 nmol each for B except the first addition, which was 200 nmol) were given in every 100 seconds in the absence (lower trace in A; upper trace in B) or in the presence of 1 μM MB (upper trace (grey line) in A; lower trace (grey line) in B). Lower Ca^{2+} concentrations in the presence of rotenone were used to avoid permeability transition pore opening under this condition. At the end of the experiments Ca^{2+} was added at saturating concentrations. Numbers on the traces represent average rates of changes in Ca^{2+} concentration in the medium (nM/min). Traces are averages ±SEM from at least four independent experiments.

4.2.6. Enhanced H_2O_2 generation by MB in both energized and respiration-impaired mitochondria

Given the controversy in the available data as to the prooxidant versus antioxidant response of different cells to the treatment with MB [125;126;179-183], we compared the H_2O_2 generation in brain mitochondria in the absence and presence of MB. The use of different substrates to support respiration in these experiments is justified by the fact that due to the different site of entry of electrons in the respiratory chain ROS are generated by distinct mechanisms. H_2O_2 formation was measured using Amplex UltraRed horseradish peroxidase fluorescent system as described in the Method**.**

4.2.6.1 H_2O_2 formation in mitochondria energized with glutamate plus malate

In mitochondria fueled with NADH-linked substrates complex I, the major site of entry of electrons into the respiratory chain [66] and/or α-ketoglutarate dehydrogenase [63;64;184;185] appear to be the predominant sites of ROS generation. Complex III in these mitochondria contributes to ROS production mainly when the respiratory chain is blocked at a downstream site [186].

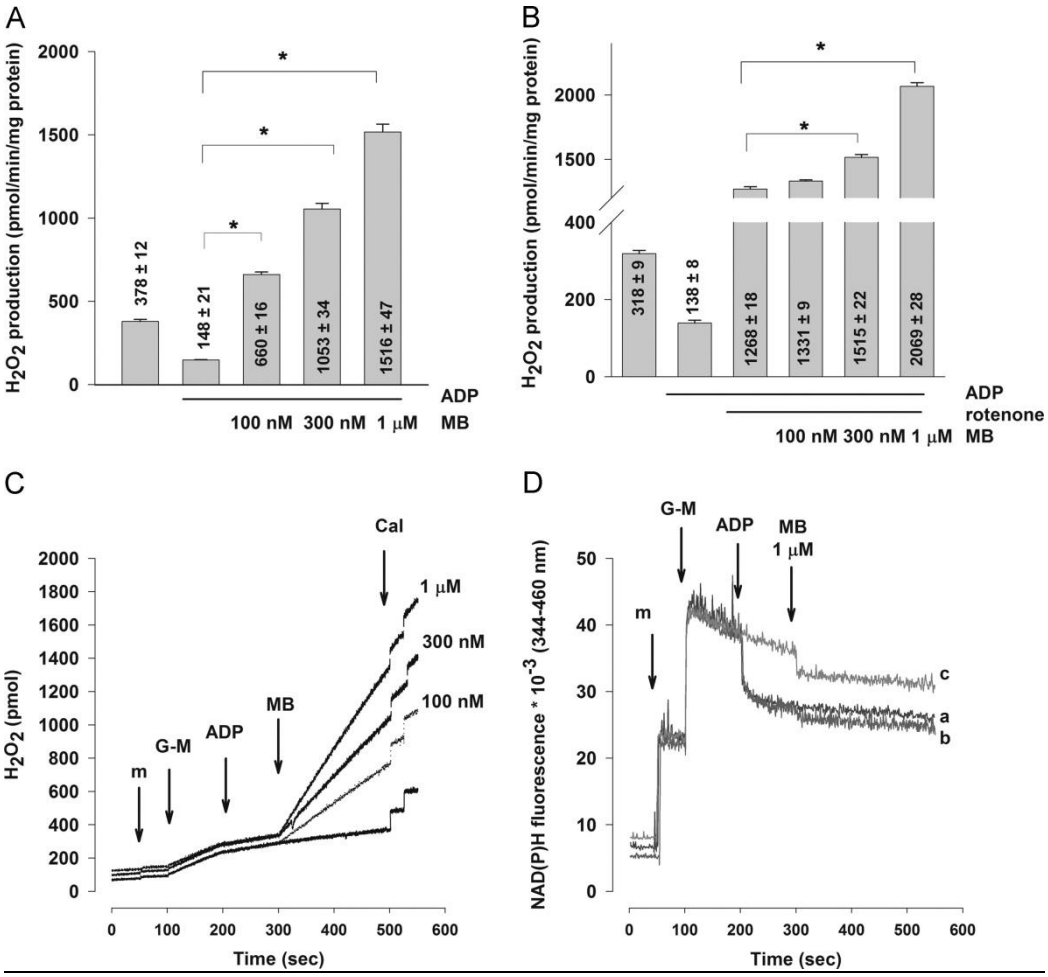

Figure 16 The effect of MB on H_2O_2 production (A,B,C) and NAD(P)H steady state (D) in glutamate plus malate-supported mitochondria.
Mitochondria (m; 0.1 mg/ml), ADP (2 mM), MB and 0.5 μM rotenone (for B) were given where indicated. Representative experiments with original traces are shown in C and D. Data on H_2O_2 generation in pmol/min/mg protein in panels A and B represent the average ±SEM from at least four experiments. Asterisks indicate significant differences.

As demonstrated in Fig.16, MB caused a remarkably large stimulation of H_2O_2 production in the presence of glutamate plus malate. The stimulation of H_2O_2 formation by MB was evident both in resting mitochondria (in the absence of ADP), where the rate of H_2O_2 generation was increased from 378±12 to 1792±53 pmol/min/mg protein by 1 μM MB (not shown) and in ATP-synthesizing mitochondria (in the presence of ADP, where the rate of H_2O_2 generation is smaller than that without ADP due to depolarization of $\Delta\Psi m$) (Fig.16/A, C). The rate of H_2O_2 formation under the latter condition was significantly increased by as low as 100 nM MB from 147.8±21 to 660±16 pmol/min/mg protein and further increased to 1516±47 pmol/min/mg by 1 μM

MB. NAD(P)H fluorescence was decreased by MB in parallel to the stimulation of H_2O_2 generation (Fig.16/D trace c); an effect less apparent in the presence of ADP, when the level of NAD(P)H was already low (Fig.16/D trace b as compared to trace a, where no MB was given). In order to address H_2O_2 formation in energetically impaired mitochondria rotenone (0.5 μM) was given after ADP, followed by addition of MB (100 nM, 300 nM or 1 μM). Rotenone, as documented earlier [168;186-189] resulted in a huge stimulation of H_2O_2 formation (from 138±8 to 1268±18 pmol/min/mg protein) and 1 μM MB was able to induce a significant further increase in the rate of H_2O_2 generation to 2069±28 pmol/min/mg protein (Fig.16/B).

4.2.6.2. H_2O_2 formation in mitochondria energized with succinate

In succinate-supported mitochondria succinate dehydrogenase [161] and complex III could contribute to the overall ROS generation (see [190] and when sufficient protonmotive force is generated, RET is considered as a major ROS-forming mechanism [65;187]. The RET-related high rate of H_2O_2 generation observed in fully polarized mitochondria (in the presence of 5 mM succinate) [65;191-193] in the present experiment was found to be 2219±180 pmol/min/mg protein (Fig.17/A). Addition of 1 μM MB induced an additional increase in the rate of H_2O_2 formation to 3321±113 pmol/min/mg protein. When RET was prevented by rotenone the rate of H_2O_2 generation was dropped to 451±15.9 pmol/min/mg protein, but MB also under this condition resulted in a large stimulation of H_2O_2 generation (to 1887±83 pmol/min/mg protein). In parallel experiments the level of NAD(P)H was found to be moderately decreased by MB (Fig.17/C trace b). The sharp drop in the NAD(P)H fluorescence by rotenone (Fig.17/C trace b) reflects the elimination of RET and NADH generation by complex I and was parallel with a decrease in H_2O_2 formation as demonstrated in Fig.17/A. At low succinate concentration (50 μM), which is insufficient to support RET, the rate of ROS formation was low but addition of 1 μM MB produced a sixfold elevation in the rate of H_2O_2 generation (from 41.3±4 to 274±21 pmol/min/mg protein, which was further stimulated by rotenone (Fig.17/B). There was only a small and slow NAD(P)H formation here (Fig.17/C traces c,d) unrelated to RET, as addition of rotenone significantly increased NAD(P)H level (Fig.17/C trace c) suggesting that NADH generation due to succinate oxidation in the Krebs cycle is reflected in the NAD(P) signal under this condition.

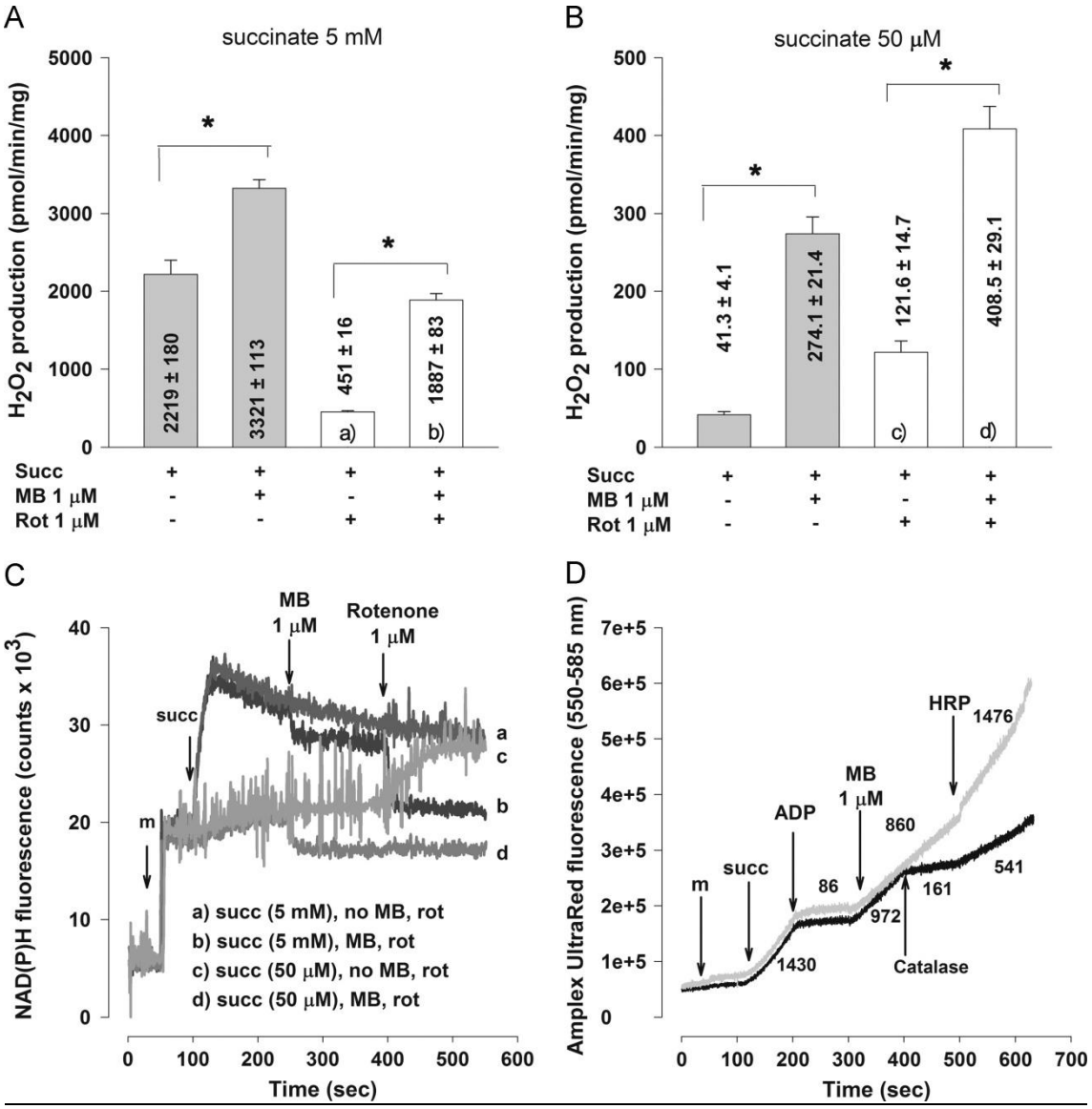

Figure 17 The effect of MB on succinate-supported H_2O_2 production (A,B) and NAD(P)H level (C). Succinate (5 mM for A and for traces a,b in panel C; 50 μM for B and for traces c,d in panel C) was given to mitochondria then 300 sec later 1 μM MB was added where indicated followed by addition of rotenone (0.5 μM) after 300 sec, where indicated. Numbers on bars in panels A and B indicate the average rate of H_2O_2 production in pmol/min/mg±S.E.M. from at least four independent experiments. Panel D shows the effect of catalase on the MB-induced Amplex UltraRed fluorescence. Horseradish peroxidase activity at the beginning of these experiments was 20 times lower than in other Amplex experiments. Numbers indicate the rate of fluorescence changes/sec. Traces are representative of three similar measurements. Asterisks indicate significant differences.

In order to demonstrate that with Amplex UltraRed fluorescence H_2O_2 is specifically detected, we added catalase to the incubation medium containing MB. The MB induced rise in the fluorescence signal was abruptly halted by catalase, which then reacted to the

addition of HRP. This shows that the elevated signal in the presence of MB indeed reflect H_2O_2 generation (Fig.17/D). It is to note that in this experiment lower activity of horseradish peroxidase was used to allow an efficient competition between HRP and catalase for scavenging H_2O_2 explaining the smaller rate of H_2O_2 formation found in this particular experiment. H_2O_2 generation in the experiments with MB in mitochondria respiring on glutamate plus malate or α-GP was also verified with catalase (not shown).

4.2.6.3 H_2O_2 generation in α−GP-supported mitochondria

As previously characterized [169;170;194;195] ROS generation at high α-GP concentration (20 mM) can be attributed mainly to complex I receiving electrons from the oxidation of α-GP via RET [169;170;191]. Oxidation of α-GP at small α-GP concentration (5 mM) is unable to generate sufficient protonmotive force for RET; electrons for H_2O_2 generation under this condition was suggested to originate largely from α-GPDH or, in case of a blockage of electrons at complex III, they could be provided by CoQ or complex III [169;196;197].

In mitochondria respiring on 20 mM α-GP, MB induced a large stimulation of H_2O_2 generation (Fig.18/A); the rate of H_2O_2 formation was increased from 554±40 to 1368±31 pmol/min/mg protein (Fig.18/C). Consistent with the involvement of RET and RET-related NADH generation in this effect, both rotenone and ADP, which decreased α-GP-supported ROS generation by preventing RET [169;195] decreased the MB-induced H_2O_2 formation (Fig.18/C).

H_2O_2 generation stimulated by MB was paralleled by a drop in the NAD(P)H fluorescence (Fig.8/B trace b) suggesting that for H_2O_2 formation MB takes electrons from NAD(P)H generated by RET. MB, however, remained capable of enhancing H_2O_2 formation in the total absence of RET (Fig.18/C). This is evident not only because MB increased H_2O_2 formation in 20 mM α-GP-supported mitochondria in the presence of rotenone or ADP, but also because mitochondria respiring on 5 mM α- GP, when no RET is possible, also responded with a huge increase in H_2O_2 generation to a treatment with MB (from 67±10.9 to 459±51 pmol/min/mg; Fig.18/D). This shows that for MB-mediated H_2O_2 generation electrons could be provided by α-GPDH as well.

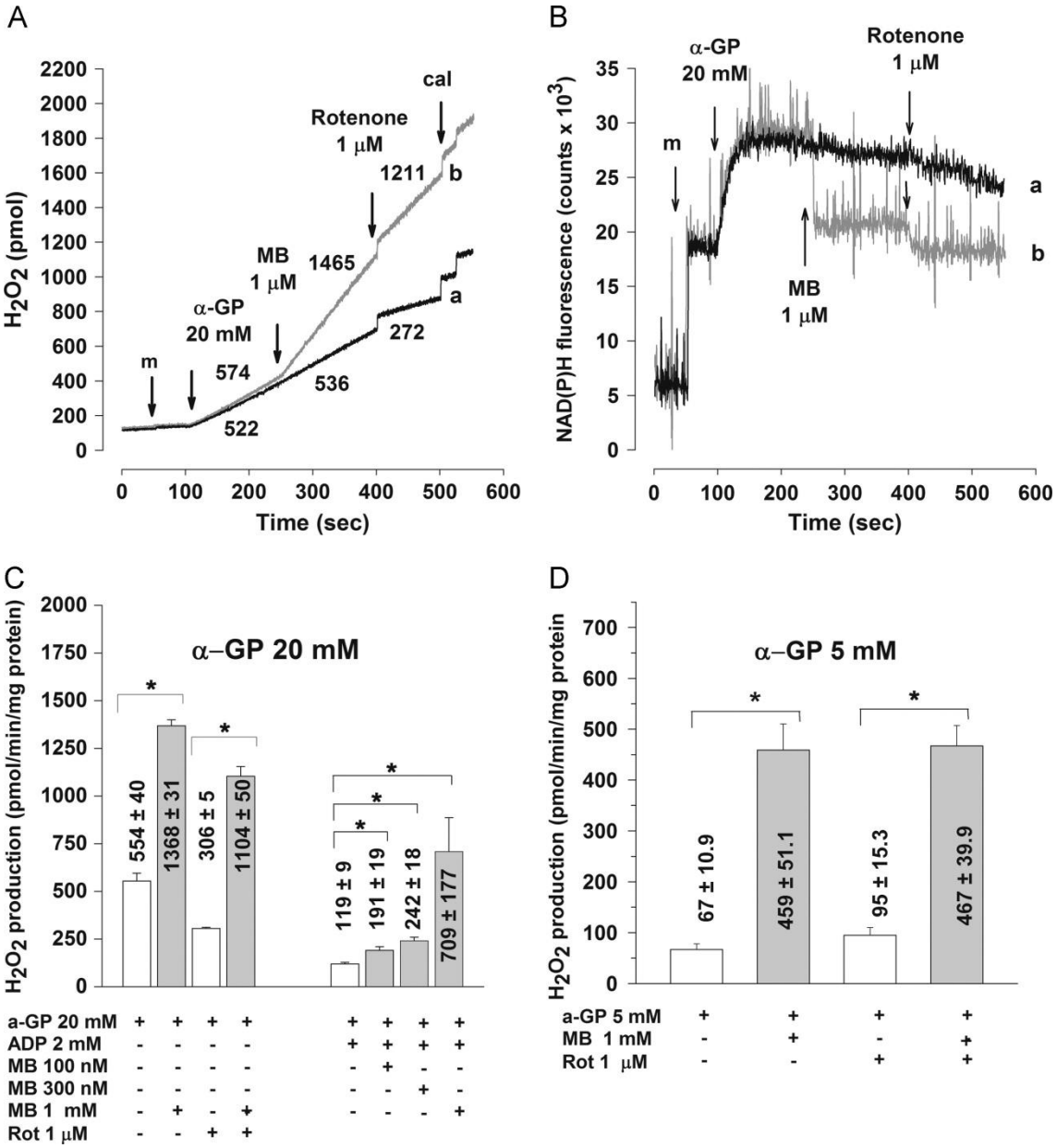

Figure 18. The effect of MB on H_2O_2 production (A, C, D) and NAD(P)H level (B) in α−GP supported mitochondria. Mitochondria (m), MB (1 μM; for traces b) or rotenone were given as indicated. Original traces from one experiment are shown in A and B. Experiments with rotenone were as shown for A, but in the experiments with ADP, this was given 150 sec after α-GP followed by MB addition 150 sec later. Quantitative data from at least four experiments observed in the presence of 20 mM (C) or 5 mM α−GP (D) in pmol/min/mg ± S.E.M. from at least four experiments are shown in C. Asterisks indicate significant differences.

4.2.7. Enhanced H_2O_2 signal in the presence of MB is unrelated to illumination during the experiment

MB is used in photodynamic therapy and MB-mediated phototoxicity was suggested to be, at least partially, ROS-dependent [181;198;199]. It is important to demonstrate that the enhanced H_2O_2 signal in the presence of MB presented above is not due to the illumination during excitation in our experiments. Thus, in order to exclude false positive results with MB, key experiments were repeated without using continuous illumination of the samples. For this, excitation light of the PTI Deltascan fluorescence spectrophotometer was switched on and off using a shutter. When the illumination time was reduced to 1/8th of the control, Amplex UltraRed fluorescence signal in response to MB decreased only by 9 % and there was no difference in the mitochondrial NAD(P)H fluorescence either (Fig.19). We can safely conclude that more than 90 % of H_2O_2 production observed in the presence of MB is independent of the illumination of the samples. Similarly, measurements on mitochondrial oxidation in the presence of MB were also repeated in the dark providing essentially similar results to those presented above in this report (not shown).

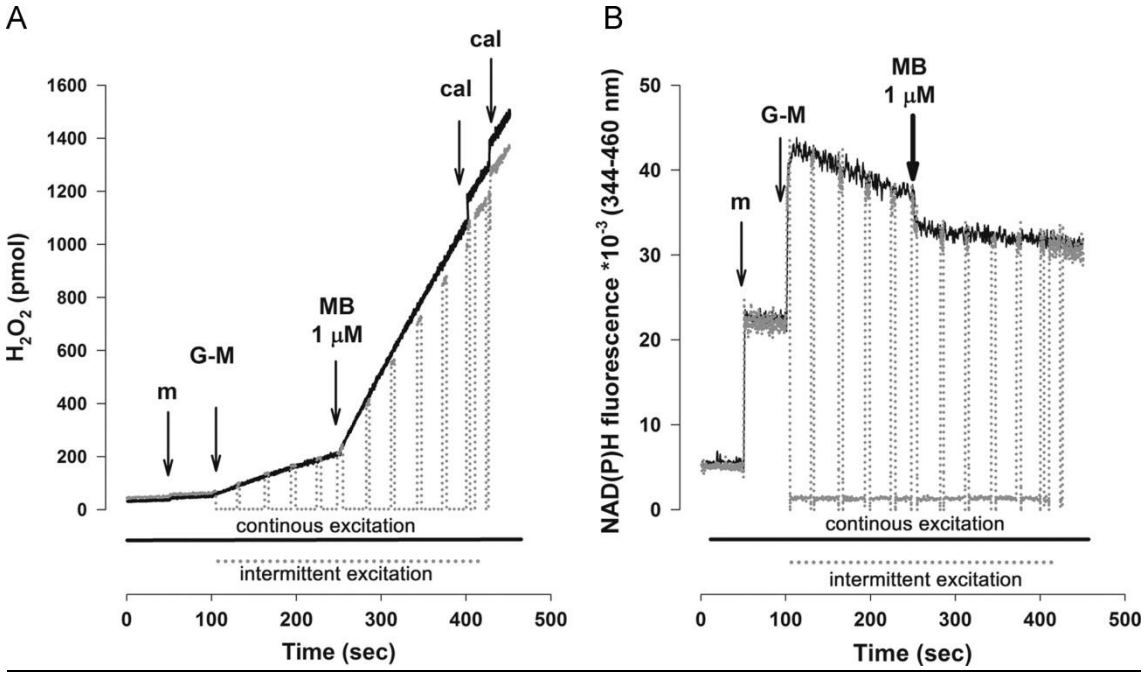

Figure 19. The effect of intermittent excitation light on MB induced changes of H_2O_2 production (A) and NAD(P)H level (B). Under control conditions (black continuous line) mitochondrial samples in the cuvette were exposed to continuous excitation light (550 nm for A and 340 nm for B). In the period from 100-400 seconds (grey, dotted line) samples were subjected to 35 sec dark and 5 sec of illumination cycles by closing and opening the shutter for excitation light. Additions were as indicated on the graphs. Traces are representative of three experiments.

5. DISCUSSION

5.1. Dimished mitochondrial functions in α-KGDH E2 or E3 Heterozygous KO animals

The KGDHC catalyzes the rate-limiting step in the TCA cycle, a crossroad of many metabolic processes [7]. Therefore, it is not surprising that reduced activity of the enzyme has even been identified in many age-related neurodegenerative diseases such as Alzheimer's disease [18;106;200]. The aim of the present study was to reveal the role of the KGDHC and its components/subunits in mitochondrial bioenergetics and redox homeostasis. In particular, ROS generation in brain mitochondria isolated from wild-type and $DLD^{+/-}$ and $DLST^{+/-}$ transgenic mice was investigated. Based on our results, we could conclude that the α-KG-dependent O_2 consumption was affected in these transgenic animals. It was not the case though with succinate and α-GP, which do not demand a fully functional KGDHC for their oxidation. Importantly, in all the KGDHC-subunit-deficient mitochondria the H_2O_2 production was decreased relative to the respective controls not only in the α-KG energized mitochondria but also in mitochondria respiring with succinate or α-GP. The experimental setup we used is entirely different from that used in studies dealing with mutations in the genes responsible for the expression of KGDHC subunits[19;201]. The common feature of the mutations is that they alter both the catalytic and the ROS forming properties of the subunits. Depending upon the type of mutations ROS production can be higher or lower than that of the controls.

5.1.1. Substrate-dependent alterations in mitochondrial oxygen consumption

O_2 consumption is a sensitive parameter for the mitochondrial bioenergetic status, hence, mitochondrial respiration might well reflect on the impairments or enzyme mutations in the ETS or TCA cycle. Specific respiratory pathways get activated in isolated mitochondria (or other types of mitochondrial preparations, like permeabilized cells) when applying fuel substrates such as α-KG, succinate or α-GP in the presence or absence of ADP and specific inhibitors of the ETS [202] respiring on succinate and α-GP. Throughout the present study, the O_2 consumption was monitored in the investigated mitochondria applying various respiratory substrates.

5.1.1.1. Mitochondrial O_2 consumption in α-KG-supported mitochondria

In accord with earlier reports [24;63;203], O_2 consumption was not lower in mitochondria isolated from either $DLD^{+/-}$ or $DLST^{+/-}$ transgenic mice in the absence of ADP (Figure 1). This might be explained by the fact that there are endogenous respiratory substrates present which could at least partially compensate for the decrease in the KGDHC activity in the $DLST^{+/-}$ and $DLD^{+/-}$ mice. In the presence of ADP, the respiration proved to be highly stimulated. This effect could be attributed to the ADP-dependent depolarization of $\Delta\Psi_m$, the stimulatory effect of ADP on the KGDHC [204], and ADP-dependent disinhibition of KGDHC by succinyl-CoA due to participation of ADP in the succinyl-thiokinase reaction. Apart from the disinhibition, the succinyl-CoA discharge in the presence of ADP also releases the KGDHC substrate CoA for the enzyme turnover [185]. Owing to the ADP-induced decrease in $\Delta\Psi_m$, the proton pump activities of the respiratory complexes got stimulated, which led to an augmented O_2 consumption. As was depicted in Figure 1B, the O_2 consumption rate significantly decreased in all the KGDHC-subunit-deficient mitochondria compared to the controls after ADP addition. Nevertheless, there were no significant differences in the RCR values (or P-L control efficiency) compared to WT, suggesting that these transgenic mitochondria could still produce ATP, as was previously reported by Kiss et al.[24]. The effect of ADP was suspended with the ANT inhibitor CAT, leading to a strong decline in O_2 consumption (Figure 1D). This phenomenon indicates the presence of a normal ADP- and likely $\Delta\Psi_m$ -dependent regulation of the substrate oxidation, not only in the WT, but also in the transgenic mitochondria.

5.1.1.2. Mitochondrial O_2 consumption in mitochondria supported by succinate and α-glycerophosphate.

Succinate and α-glycerophosphate are two mitochondrial energy donor substrates not having any direct connection with α-KG or KGDHC, therefore it was not surprising that their oxidation was not different in the KO animals.

5.1.2. H_2O_2 production in mitochondria using various respiratory substrates

The mitochondrion is a major source of ROS under selected pathological conditions [65-67]. A growing body of evidence reveals that matrix dehydrogenases, such as the KGDHC, are capable of producing ROS under specific conditions when the

$NADH/NAD^+$ ratio is rather high [62;64;185;205;206]. In this study, one of the research objectives was to explore how deletion of the E2 or E3 subunit of the KGDHC affects the ROS-producing sites in brain mitochondria. For this to be accomplished, we utilized various respiratory substrates such as α-KG, succinate, and α-GP, and specific inhibitors of the ETS.

5.1.2.1. H_2O_2 production in mitochondria respiring on α-ketoglutarate

In agreement with earlier studies, H_2O_2 production was decelerated, relative to the respective controls, in KGDHC- E3 subunit-deficient mitochondria with α-KG as respiratory substrate [63] (Figure 4). As was expected, the addition of ADP lowered the ROS production rate likely due to the decrease (depolarization) of $\Delta\Psi_m$, and $NADH/NAD^+$ ratio [63;64;167;168] (Figure 4C). CAT, an inhibitor of the ANT, reestablished the high $\Delta\Psi_m$ and $NADH/NAD^+$ ratio and enhanced the H_2O_2 production (Figure 4D). In the presence of CAT, the H_2O_2 production significantly decreased in the $DLD^{+/-}$ transgenic mitochondria compared to the controls. Rotenone blocked the electron flow at Complex I and further elevated the H_2O_2 formation (Figure 4E). The reduced rate of H_2O_2 production in the transgenic mitochondria refers to the determining roles of the KGDHC subunits in mitochondrial H_2O_2 production. Antimycin A, acting at Complex III, exerted only a minimal stimulatory effect on the H_2O_2 production, indicating that in the presence of rotenone only a relatively small percentage of the electrons could reach the ROS-forming site(s) of the Complex III (Figure 4F).

Considering the multienzyme nature of the KGDHC, this finding is in agreement with a previous reports [17;68;160] that all the three catalytic subunits of the KGDHC participate in the ROS-producing activity thus it is also a possibility that in the absence of DLST the production of free radicals on the OGDH subunit could be accelerated.

5.1.2.2. H_2O_2 production in mitochondria respiring on succinate and α-glycerophosphate

In the absence of ADP, succinate-supported mitochondria exhibited an extremely high rate of ROS production which can be attributed to the reverse electron transfer (RET) [150;165;166]. During RET, electrons from Complex II flow back towards Complex I and reduce NAD^+ to NADH at high $\Delta\Psi_m$. Similar to that detected in the α-KG-

supported mitochondria, the H_2O_2 production rate decreased in the transgenic mitochondria, which was most pronounced in the $DLD^{+/-}$ mitochondria, indicating that under this condition indeed the DLD is the most important player in the course of ROS production. In the presence of ADP, there was a dramatic decrease observed in the rate of H_2O_2 formation, which could be attributed to the ADP-induced depolarization of $\Delta\Psi_m$ and thus the abolishment of RET [166;167] (Figure 6C). Importantly, the decrease in the H_2O_2 production rate upon ADP addition was more pronounced in succinate-supported mitochondria as compared to the ones respiring on α-KG. This could be explained by the fact that the RET is more sensitive to $\Delta\Psi_m$. In the presence of CAT, the inhibition of ANT hyperpolarizes the mitochondrial inner membrane, hence the conditions get favorable for the RET, and mitochondrial ROS production gets stimulated (Figure 4D). Under this condition, in the $DLST^{+/-}$ and $DLD^{+/-}$ KO animals, the rate of ROS production decreased compared to the control. ROS production was the slowest for $DLD^{+/-}$ indicating again that the DLD subunit is the most important ROS producer when the RET is active (Figure 5B,D). Similar observations were made with the mitochondria energized by α-GP (Figure 6B,D), where all of the NADH formed in the mitochondrial matrix originated from the RET [169]. Inhibition of the ANT brought the ROS formation back to the level detected before the addition of the ADP, consequently, the electrons must at least partially flow in the reverse direction. Administration of rotenone ceased RET and decreased the ROS production indicating that RET was the major source of ROS in the presence of succinate.

5.1.3. Protein expression levels

Surprisingly, in our study the $DLST^{+/-}$ heterozygote KO-s decreased not only the protein expression level of DLST, but also that of the OGDH subunit (Figure 8). Contrary to our results, Yang et al. [203] demonstrated only a decreased expression of the DLST subunit in the $DLST^{+/-}$ animals. This difference might be explained by the different reference proteins and different antibodies used for normalization in these studies.

Cellular and mitochondrial antioxidant enzymes play key roles in the regulation of redox homeostasis. Under physiological conditions, antioxidants keep the ROS level low preventing the cell or mitochondria from a burst of oxidative stress. Thus, their expression levels reflect a potential redox imbalance and let us conclude on the rate of

ROS formation. Therefore, we investigated the protein levels of selected antioxidant enzymes such as GPX, GR, TRX, and PRX3 in mitochondria isolated from the wild-type and KGDHC subunit deficient mice. Surprisingly, only the protein levels of GPX and GR, but not of TRX and PRX, were reduced in the KGDHC subunit deficient transgenic animals (Figure 9 and Figure 10). Conversely, Yang et al. [203] have revealed no difference in the protein levels of the mitochondrial antioxidant enzymes (GPX, GR, MnSOD) in the $DLST^{+/-}$ heterozygotes compared to the controls. These discrepancies might again be explained by the different reference proteins used for normalization in these studies. It is noteworthy that reduced protein levels of GPX and GR support the data on the decreased rate of ROS formation in the KGDHC-subunit-deficient transgenic groups. The lowered expression levels of glutathione-dependent antioxidant enzymes can be considered as an adaptation to the lower rate of ROS production in the mitochondria.

5.1.4. Relevance of Our Data in Ischemia-Reperfusion Injury

Ischemia-reperfusion injury is associated with disturbed mitochondrial metabolism. During ischemia, succinate accumulates owing to the reversal of succinate dehydrogenase [207] or due to the canonical operation of the Krebs cycle partially supported by aminotransferase anaplerosis [208]. Reperfusion is associated with high ROS production supported by reverse electron transfer induced via the oxidation of the accumulated succinate. As it has been reported by others [209-211], the FMN subunit of Complex I might be responsible for most of the ROS produced during RET. Hereby we demonstrate that heterozygous KO of KGDHC subunits decreased the succinate- and α-GP- evoked H_2O_2 production under conditions favoring RET. Our data indicate that heterozygous KO of the DLD subunit resulted in a 58% decline in ROS production with succinate and 34% with α-GP. Therefore, these results suggest that the DLD subunit can contribute to the RET-evoked ROS production (Figure 20). Our data confirm the results of Starkov et al. 2004 emphasizing the role of NADH-dependent KGDHC in contribution to RET-evoked ROS formation [63].]. To the best of our knowledge, we have shown for the first time that α-GP-induced RET, which can occur in the brain having high α-GPDH activity, was also sensitive to heterozygous KO of DLD subunit. This might have an importance during ischemia when α-GP can accumulate as proven by Ben-Yoseph et al., 1993 [212] and Nguyen et al. 2007 [213] in ischemic in rat brain.

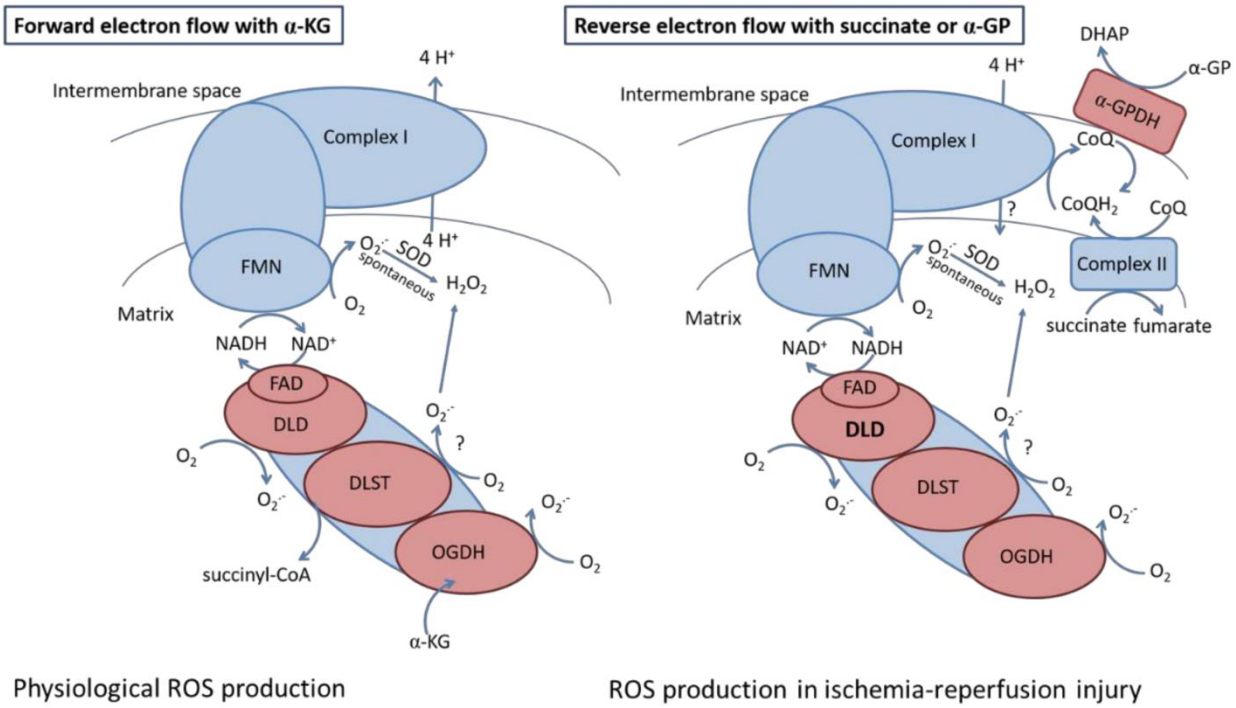

Figure 20. The role of KGDH in ROS production observed during forward and reverse electron transfer in ischemia-reperfusion injury. Abbreviations: α-GP: α-glycerophosphate; α-GPDH: α-glycerophosphate dehydrogenase; α-KG: α-ketoglutarate; CoQ: oxidized coenzyme Q; CoQH2: reduced coenzyme Q; DHAP: dihidroxiaceton-phosphate; DLD: dihidrolypoil dehydrogenase; DLST: dihydrolipoamide succinyltransferase; FAD: flavin adenine dinucleotide; FMN: flavin adenine mononucleotide; OGDH: oxoglutarate dehydrogenase; SOD: superoxide dismutase.

5.2 Beneficial effects of methylene blue in isolated brain mitochondria

Addressing several key bioenergetic parameters in this study, we aimed to dissect the mitochondrial effects of MB, which could determine its beneficial effects found in several pathological conditions [125;175;179;180;214-216]. In addition, release as well as elimination of H_2O_2 was addressed to reveal the pro-oxidant/antioxidant effect of MB that accompanies the mitochondrial bioenergetic alterations.

5.2.1 Methylene blue stimulated respiration in isolated brain mitochondria

We established that MB stimulated respiration in isolated brain mitochondria and, by this, confirmed several earlier reports demonstrating MB-stimulated oxygen consumption in different mitochondria and cellular models [125;126;217-219]. Stimulation of respiration by MB has been assigned to its ability to shunt electrons in the respiratory chain. It was first described in the 1960s and 1970s that MB could be reduced in isolated mitochondria by electrons from NADH and transfer them to cytochrome c, bypassing coenzyme Q in the respiratory chain [220;221]. Although reduction of MB by flavoenzymes such as xanthine oxidase, NADH cytochrome c reductase, and NADPH cytochrome c reductase has been reported [135;222], generally NADH has been considered a major electron donor for MB in mitochondria [125;126;175]; recently it has also been found in isolated rat heart mitochondria that cytochrome c reduction was increased with NADH as an electron donor but not with succinate [217]. It is evident from our study that respiration was improved by MB not only with NADH-linked substrates but also with succinate or α-GP, suggesting that electrons from succinate dehydrogenase or α-GPDH could also reduce MB. This conclusion is supported by data obtained with diphenyleneiodonium (DPI), which was shown to inhibit complex I by keeping the flavin groups reduced [223]; MB in DPI-treated succinate-supported mitochondria significantly increased (by 10%) the State 3 respiration of mitochondria (not shown). Importantly, our study shows that in functional mitochondria only the resting oxygen consumption is stimulated; the ADP-stimulated respiration is unaffected by MB. Because mitochondria in vivo are functioning in the presence of ADP, this finding allows the conclusion that in vivo MB might not influence the respiration of normal ATP-synthesizing mitochondria. However, the significant stimulation of oxygen consumption in respiration-impaired mitochondria supports the suggestion that MB could partially restore respiration when the flux of electrons in the respiratory chain is inhibited either at complex I or at complex III, evident in this study with NADH-linked substrates in the presence of rotenone (Figure. 11) or with succinate in the presence of antimycin (not shown).

5.2.2 Methylene blue stimulated ATP synthesis in isolated brain mitochondria

Accelerated respiration alone would not imply an improved bioenergetic performance of mitochondria, but only when this would result in an increased ATP generation. We report here, as a new finding, that in respiration-impaired mitochondria, in the presence of complex I or complex III inhibitors, the severely inhibited ATP synthesis was increased by MB (Figure 12). The rate of ATP synthesis in rotenone-inhibited mitochondria was doubled in the presence of 2 μM MB, but was still modest compared to that observed in mitochondria not treated with rotenone. Importantly, ATP synthesis in normally respiring isolated brain mitochondria was unchanged by MB. This finding seems to be contradictory to that by Wen et al. [126] showing an increased cellular ATP level in HT-22 cells. However, in this report the ATP level in the transformed HT-22 cells was only moderately decreased by strong mitochondrial drugs such as the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone or the complex IV inhibitor cyanide [126], implicating a largely nonmitochondrial ATP generation.

5.2.3 Methylene blue partially restore membrane potential in isolated brain mitochondria

It is also revealed in this study that MB, although not influencing $\Delta\Psi_m$ in fully respiring functional mitochondria, supports the maintenance of $\Delta\Psi_m$ when complex I or complex III is inhibited (Figure 14). The significance of this finding is that with a partially maintained ΔΨm mitochondria become more resistant against PTP induction [224-227]; therefore, the chances for survival of these mitochondria are highly increased. This suggestion is supported by data from Ca^{2+}-uptake measurements in this study (Figure 15) showing an improved Ca^{2+} uptake capacity in MB-treated mitochondria. Rescued $\Delta\Psi_m$ by MB in rotenone-treated mitochondria could contribute to a decreased rotenone-induced neurodegeneration observed in HT-22 cells [126;175] and retinal ganglion cells [221]. The finding that ADP depolarized $\Delta\Psi_m$ in rotenone-treated mitochondria in the presence of MB (Figure 14A and B) indicates that the rescued $\Delta\Psi_m$ is able to drive ATP synthesis. Depolarization of HT-22 cells by a neurotoxic amount of glutamate was also attenuated by MB [175], but HeLa cells were depolarized in the presence of MB [199]. Depolarization in our study was seen only when substrates were used in suboptimal concentrations (Figure 13), decreasing the amount of NADH available for complex I,

and under this condition diversion of electrons from NADH by MB could critically decrease proton pumping.

Our results show that mitochondria modestly but significantly benefit from the ability of MB to transfer electrons between NADH and cytochrome c or between α-GPDH or succinate dehydrogenase and cytochrome c, bypassing blocks in the respiratory chain at either complex I or complex III. The beneficial effects of MB are consistent with the modestly improved ATP synthesis and maintained $\Delta\Psi_m$ in respiration-impaired mitochondria shown in this report. The polarizing action of CAT in rotenone- and MB-treated mitochondria (Figure 14) indicates that MB prevents ANT from functioning in reverse, which could be critical to save the glycolytic ATP from entering mitochondria and being hydrolyzed by ANT. The reversal of ANT is dependent upon $\Delta\Psi_m$ and the concentrations of ADP and ATP on both sides of the inner membrane [176]. These data extend our understanding of the bioenergetic consequences of stimulated respiration by MB reported earlier by many studies [125;126;215;218;221;228], which alone would be insufficient to suggest improved mitochondrial function.

5.2.4 Methylene blue increased H_2O_2 release in isolated brain mitochondria

We found in this study a remarkable increase in the rate of H_2O_2 release from mitochondria by MB. The extent of stimulation of H_2O_2 release by MB was unusually large; a greater than fourfold increase was induced already at 100 nM MB in ATP-synthesizing glutamate plus malate-supported mitochondria (Figure 16A). The enhanced H_2O_2 generation was observed in respiring, as well as in respiration-impaired mitochondria, under resting as well as ATP-synthesizing conditions, and with each substrate combination. These observations were somewhat surprising considering literature reports on the antioxidant effects of MB [125;126;175;229-231] MB, in the presence of cytochrome c, decreased the paraquat-induced superoxide production [222] and suppressed the superoxide generation in the xanthine oxidase reaction [232]. These effects were clearly related to a competition between MB and oxygen for electrons resulting with the two-electron reduction of MB to MBH_2, rather than the one-electron reduction of O_2 to O_2^-. As an explanation for the suppressed superoxide generation in the xanthine oxidase reaction by MB the possibility of reduction of superoxide to H_2O_2 by MBH_2 has been raised [232]. Similarly, Poteet et al. [175] assumed that MBH_2 might directly scavenge superoxide, contributing to the decrease in the glutamate-evoked ROS

generation observed with the nonselective fluorescent ROS indicator H_2DCF-DA in HT-22 cells, and then H_2O_2 could be eliminated by catalase or peroxidases, without the accumulation of harmful reactive oxygen species. However, the actual H_2O_2 generation or accumulation in the presence of MB has never been addressed in a biological system, in particular in mitochondria, where MB exerts its major cellular effects. Furthermore it has not been considered that H_2O_2 is one of the reactive oxygen species having its own damaging effect due to interaction with iron–sulfur centers and protein SH groups, though with less reactivity than superoxide (see [233]). H_2O_2 has sensitive targets within mitochondria [63;86;87;234-236] and because it is a membrane-permeative ROS its effect in vivo is likely to extend beyond mitochondria.

For the ROS measurements in cellular studies suggesting antioxidant effects of MB, DCF-DA [126;175], a nonselective ROS sensor, or MitoSOX [126], detecting superoxide, has been used. With these dyes H_2O_2 formation is not detected or could be masked by a reduced superoxide generation in the presence of MB. In fact, when we used MitoSOX to repeat crucial experiments done with Amplex red no increase in superoxide signal was observed in the presence of MB (not shown). H_2O_2 measurements with Amplex fluorescence in mitochondria are generally done with the understanding that it reflects primarily generation of superoxide, which is dismutated to H_2O_2. In the particular case with MB, however, H_2O_2 could be generated without superoxide formation. Our results are in line with the effect of MB as an alternative electron acceptor in mitochondria taking electrons from complex I [125], but also from complex II and α-GPDH, as shown in this study, resulting in the formation of MBH_2. We suggest that MBH_2 would reduce not only cytochrome c but also O_2, generating H_2O_2 and recycling back to MB. This interpretation is consistent with the standard redox potential of MB → MBH2 ($E^{o'}$+10 mV) favoring electron donation for the ½O_2 → H_2O_2 reaction ($E^{o'}$ +300 mV) under standard conditions. The remarkably large H_2O_2 generation implies that a significant number of electrons from MBH_2 reduce O_2 instead of cytochrome c, decreasing the efficiency of the improvement in the bioenergetic competence of MB-treated mitochondria. This could explain the relatively modest increase in the ATP synthesis in MB-treated respiration-impaired mitochondria (1.5-fold increase; Fig. 12C), whereas respiration under the same conditions is stimulated 3-fold (Fig. 11). In addition to stimulating the generation of H_2O_2, MB also attenuates the

elimination of H_2O_2 (Fig. 19). This could also result, at least partly, from the electron-acceptor property of MB oxidizing glutathione directly ($E^{o'}$ 2GSH → GSSG −230 mV) as demonstrated in red blood cells [237]. Furthermore, the decrease in NAD(P)H level by MB could impair the glutathione peroxidase and thioredoxin system, the major mechanisms responsible for H_2O_2 elimination. In a mitochondrion-free experiment we could indeed demonstrate an MB-dependent oxidation of NADPH, but no direct inhibition of glutathione reductase by MB (data not shown).

The huge H_2O_2 release from mitochondria in the presence of MB observed in our study clearly indicates an oxidative burden, which should be balanced when MB is applied in vivo. The need for an increased resistance to H_2O_2 may be reflected in the enhanced expression of thioredoxin reductase measured in HepG2 cells treated with MB [125].

In MB-treated piglets increased transcription of antioxidant enzymes in the brain was also found in vitro [238]. Likewise in a recent study MB improved among others the oxidative damage in P301S mice and upregulated the prosurvival Nrf2/ARE genes [239]. As Nrf2 is known to be upregulated by H_2O_2 [239;240] it is likely that the stimulated H_2O_2 production demonstrated in the present study contributes to the upregulation of Nrf2/ARE genes.

In summary, our results demonstrate significant bioenergetic improvement by MB in isolated respiration-impaired mitochondria, in particular a modest, but significant increase in ATP synthesis and a restoration of $\Delta\Psi_m$, which could be important in the beneficial in vivo neuroprotective and cognitive-enhancing action of MB. However, the highly elevated H_2O_2 generation observed in our *in vitro* study has to be considered in the estimation of the overall oxidative state in vivo in mitochondria under treatment with MB.

6. CONCLUSIONS

The most important finding of this thesis is that the KGDHC subunits play an important role in the mitochondrial ROS formation. Decreased ROS production was detected in $DLST^{+/-}$ or $DLD^{+/-}$ KO animals not only in the forward mode of the enzyme (in the presence of α-KG as substrate) but also under the circumstances of reverse electron flow (when NADH is formed by the reverse flow of electrons via CoQ to Complex I). This latter finding highlights the importance of KGDHC subunits in the reperfusion-mediated oxidative tissue damage. In the transgenic animals investigated, not only the ROS production was decelerated, but the expression levels of the studied antioxidant enzymes were also attenuated.

Our results demonstrate significant bioenergetic improvement by MB in isolated respiration-impaired mitochondria, in particular a modest, but significant increase in ATP synthesis and a restoration of $\Delta\Psi_m$, which could be important in the beneficial in vivo neuroprotective and cognitive-enhancing action of MB. However, the highly elevated H_2O_2 generation observed in our *in vitro* study has to be considered in the estimation of the overall oxidative state in vivo in mitochondria under treatment with MB.

7. SUMMARY

Our results highlight the importance of the KGDHC subunits in the mitochondrial ROS formation. Decreased ROS production was detected in $DLST^{+/-}$ and $DLD^{+/-}$ KO animals not only in the forward mode of the enzyme (in the presence of α-KG as substrate) but also under the circumstances of reverse electron flow (when NADH is formed by the reverse flow of electrons via CoQ to Complex I). This latter finding highlights the importance of KGDHC subunits in the ischemia/reperfusion-mediated oxidative tissue damage. In the transgenic animals investigated, not only the ROS production was decelerated, but the expression levels of the studied antioxidant enzymes were also attenuated.

Addressing several key bioenergetic parameters in our study, we aimed to determine the beneficial mitochondrial effects of MB. We observed that MB stimulated respiration in isolated brain mitochondria. Importantly, our study shows that in functional mitochondria only the resting oxygen consumption is stimulated; the ADP-stimulated respiration is unaffected by MB. However, the significant stimulation of oxygen consumption in respiration-impaired mitochondria supports the suggestion that MB could partially restore respiration when the flux of electrons in the respiratory chain is inhibited. Accelerated respiration also improved bioenergetic performance of mitochondria, which was detected in the increased ATP generation, maintenance of membrane potential and an improved Ca^{2+} uptake capacity in complex I inhibited but MB-treated mitochondria. In addition, both the release and the elimination of H_2O_2 was addressed to reveal the pro-oxidant/antioxidant effect of MB.

ÖSSZEFOGLALÁS

Eredményeink rávilágítanak a KGDHC alegységek kulcsfontosságú szerepére a mitokondriális ROS képződés tekintetében. Csökkent mitokondriális ROS termelést észleltünk $DLST^{+/-}$ ès $DLD^{+/-}$ KO állatokban, nemcsak az α-KG mint szubsztrát jelenlétében, hanem a reverz elektrontranszportot támogató szubsztrátok (szukcinát és α-glicerofoszfát) esetében is. Ez utóbbi eredményeink rávilágítanak a KGDHC alegységek fontosságára az ischemia-reperfúzió által közvetített oxidatív szövetkárosodásban. A vizsgált transzgenikus állatok mitokondriumaiban nemcsak a ROS termelése csökkent, hanem a vizsgált antioxidáns enzimek expressziós szintje is mérséklődött.

Vizsgálataink során a metilénkék számos jótékony, a mitokondriumok bioenergetikai paramétereire kifejtett hatását figyeltük meg. Kísérletekben a MB izolált agyi mitokondriumokban stimulálta a légzést. Eredményeink szerint a MB csak a nyugalmi oxigénfogyasztást fokozta; az ADP-stimulált légzést nem befolyásolta. A MB szignifikánsan növelte az oxigénfogyasztást a rotenonnal gátolt mitokondriumokban, ami alátámasztja azt a feltételezést, hogy az MB részben helyreállíthatja a légzési lánc működését, amennyiben a légzési lánc elektronjainak áramlása az I komplexben gátolt. Az oxigénfogyasztás mellett a MB a mitokondriumok számos egyéb bioenergetikai paraméterét is pozitívan befolyásolta. Így kísérleteink során megnövekedett ATP termelést, magasabb membránpotenciált illetve jobb Ca^{2+}-felvevő kapacitást mértünk komplex I gátolt, MB-kel kezelt mitokondriumokban. Ugyanakkor azonban fokozott H_2O_2 felszabadulást és csökkent eliminációt mértünk a MB hatására.

8. References

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