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Differences in mitochondrial function in homogenated samples from healthy and epileptic specific brain tissues revealed by high-resolution respirometry

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ABSTRACT

Mitochondrial dysfunction and oxidative stress are strongly implicated in neurodegenerative diseases and epilepsy. Strikingly, neurodegenerative diseases show regional specificity in vulnerability and follow distinct patterns of neuronal loss. A challenge is to understand, why mitochondria fail in particular brain regions under specific pathological conditions. A potential explanation could be provided by regional or cellular specificity of mitochondrial function.

We applied high-resolution respirometry to analyze the integrated Complex I- and II (CI and CII)-linked respiration, the activity of Complex IV, and the combined CI&II-linked oxidative phosphorylation (OXPHOS)- and electron-transfer system (ETS)-capacity in microsamples obtained from distinct regions of the mouse brain. We compared different approaches to assess mitochondrial density and suggest flux control ratios as a valid method to normalize respiration to mitochondrial density.

This approach revealed significant differences of CI- and CII-linked OXPHOS capacity and coupling control between motor cortex, striatum, hippocampus and pons of naïve mice. CI-linked respiration was highest in motor cortex, while CII-linked respiration predominated in the striatum. To investigate if this method could also determine differences in normal and disease states within the same brain region, we compared hippocampal homogenates in a chronic epilepsy model. Three weeks after stereotaxic injection of kainate, there was a down-regulation of CI- and upregulation of CII-linked respiration in the resulting epileptic ipsilateral hippocampus compared to the contralateral one.

In summary, respirometric OXPHOS analysis provides a very sensitive diagnostic approach using small amounts of distinct brain tissues. In a single assay, information is obtained on numerous OXPHOS parameters as indicators of tissue-specific mitochondrial performance.

1. Introduction

The most prevalent neurodegenerative diseases share common pathophysiological mechanisms, such as mitochondrial dysfunction, oxidative stress, and cell death, yet the particular neuronal population involved dictates the symptomatic presentation of disease. Parkinson's disease and Huntington's disease, but also familial amyotrophic lateral sclerosis, Alzheimer's disease and some types of epilepsies are characterized by severe neuronal losses. Strikingly, neuronal losses show high regional specificity and frequently follow distinct patterns: Parkinson's disease affects mainly dopaminergic neurons in the substantia nigra (Hirsch et al., 1988), while Huntington's disease is associated with neuronal loss in the motor cortex and striatum (Lange et al., 1976). One research focus in our quest to understand this phenomenon is mitochondrial dysfunction and oxidative stress. Mitochondrial dysfunction may be restricted to affected regions and heterogeneity of mitochondrial parameters has been described in different brain areas (for review see Dubinsky, 2009). However, a major unanswered question is if region-specific differences in basal mitochondrial function predispose select neuronal populations to pathological stimuli, or if mitochondrial energetics are similar throughout, but the neurons are selectively vulnerable to events downstream of mitochondrial dysfunction.

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Oxidative phosphorylation (OXPHOS) is a major player in energy transformation, where the subsystems of the electron transfer system (ETS) generate the transmembrane protonmotive force and the phosphorylation system utilizes the protonmotive force. The main ETS protein complexes are located in the inner mitochondrial membrane and pump protons to drive the phosphorylation of ADP to ATP. Dysfunction of a single protein or entire complex of the OXPHOS-machinery due to genetic mutations cannot explain per se the regional specificity of neurodegenerative diseases. However, the expression of mitochondrial proteins is regulated epigenetically, therefore regional or even cellular specificity of the contribution of distinct complexes may be regulated by cell specific gene expression mechanisms.

Specifically Complexes I, II and IV (CI, CII and CIV) in distinct brain regions are presently discussed as being affected in a number of classical neurodegenerative diseases, such as Parkinson's disease (Schapira et al., 1989), Huntington's disease (Gu et al., 1996) and Alzheimer's disease (Crouch et al., 2005) as well as in several types of epilepsies (for reviews see (Baron et al., 2007; Waldbaum and Patel, 2010a), such as temporal lobe epilepsy (Kunz et al., 2000). Accordingly, the contribution of the distinct complexes to mitochondrial function needs to be studied in a regionally-specific context in order to reveal the causal relationship between mitochondrial vulnerability and pathological processes. Isolated mitochondria are often used to study mitochondrial parameters in cells or tissues (Perry et al., 2013). By contrast studying distinct mitochondrial functions in brain microsamples requires a method with low preparative loss and close to physiological tissue environments. Recently, Herbst and Holloway (Herbst and Holloway, 2015) compared the respiration of permeabilized brain tissues and mitochondria isolated from cortex. They conclude that permeabilized brain tissue offers several advantages, such as minimization of time and tissue required for measurements carried out under more physiological mitochondrial conditions.

The aim of the present study was to establish a further developed method, which would enable detailed analysis of mitochondrial functional within specific regions of the brain. Considering the ongoing discussions on mitochondrial complexes involved in neurological diseases, the main focus was put on integrated CI-, and CII-linked mitochondrial pathways under coupled and noncoupled conditions, and the single enzymatic step of cytochrome *c* oxidase (CIV; Gnaiger, 2014). A complex substrate-uncoupler-inhibitor-titration (SUIT) protocol was applied for mitochondrial phenotyping (comprehensive OXPHOS analysis) in a single incubation of small tissue samples of 2 mg fresh weight. The method was sufficiently sensitive to reveal functional differences between distinct brain areas in control animals, and between the ipsi- and contralateral dorsal hippocampi in a mouse model of temporal lobe epilepsy. The omission of detergents in the tissue preparation and our SUIT-protocol allow evaluation of several subsequentially targeted substrate and coupling states without compromising the stability of respiration. Reference states addressed within a SUIT protocol can be selected as internal functional mitochondrial markers, which have been validated in this study.

2. Materials and methods

2.1. Animals and tissue preparation

Six male C57Bl/6J mice (http://www.criver.com/products-services/basic-research/find-a-model/jax-mice-strain-c57bl-6j), 15–20 weeks old and 28–30 g on the respective experimental day, were used for experiments of different brain regions. Ten C57Bl/6 N mice (http://www.criver.com/products-services/basic-research/find-a-model/c57bl-6n-mouse), 16–20 weeks old, 27–29 g, were injected with either saline ($N = 6$) or kainic acid ($N = 4$) for experiments on temporal lobe epilepsy. The saline or kainic acid injected mice were sacrificed 2.5–3.5 or 3.0–3.5 weeks, respectively, after injections for respirometric studies. Mice were kept at 23 °C with 12/12 light/dark cycle and free access to standard laboratory rodent chow and water. All procedures involving animals were approved by the Austrian Animal Experimentation Ethics Board in compliance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes ETS no.: 123. Every effort was taken to minimize the number of animals used.

Cervical dislocation was performed between 9.30 am and 10.30 a.m. immediately before OXPHOS analysis to minimize circadian rhythm effects.

Brains were micro-dissected on ice and specimens weighed on an analytical balance (Mettler Toledo AE 160, Greifensee, Switzerland). The micro-dissected brain regions were directly transferred into ice-cold mitochondrial respiration medium (MiR06Cr in a 12 well plate) containing 280 IU/ml catalase (Pesta and Gnaiger, 2012) and 20 mM creatine.The remaining blood was removed by washing in MiR06Cr. Subsequently tissues were homogenized in the same medium in a pre-cooled glas potter (tight fit; WiseStir homogenizer HS-30E, witeg Labortechnik GmbH, Wertheim, Germany) at 1000 rpm, with 10 strokes for motor cortex and striatum, but 15 strokes for hippocampus and brainstem (pons). Resulting homogenates containing 5 mg tissue wet weight were suspended in 5 ml of ice-cold MiR06Cr and 2×2.1 ml of this suspension were used for OXPHOS-analysis in duplicates. Residual homogenates were frozen at −80 °C for protein quantification and citrate synthase activity determination.

An alternative homogenization method was tested for the experiments on epileptic hippocampal (dorsal hippocampi) tissue. Tissues were weighed, cut and homogenized in a PBI-Shredder (OROBOROS INSTRUMENTS, Innsbruck, Austria) for 10 s in position 1 and 10 s in position 2. For hippocampal tissue, highly comparable homogenate preparations could be achieved also with this method. For comparable homogenization of different tissues, the above-mentioned method for homogenization in a glass potter was superior.

Respiration was higher in potter preparations after 10–15 strokes than after fewer strokes or in first spin homogenates (in which mitochondria in the homogenate were enriched in the supernatant by a centrifugation step of 2000 g for 3 min at 4 °C). Respiratory fluxes for the optimized potter preparations were similar to shreddering.

The optimized potter preparation of brain tissue yields a high degree of permeabilization as evident by the minimal effect of digitonin titrations on OXPHOS capacity. Therefore, digitonin is not necessary for this protocol. The effect of digitonin was more evident in shredder homogenates. Homogenate preparation with 10–15 strokes of the potter resulted consistently in a variance of respiratory flux between duplicates, which was 3-fold lower than in preparations using the PBI shredder.

No cytochrome *c*-effect, indicating outer mitochondrial membrane damage, was observed for any of the tested methods. For this test, 10 μM cytochrome c were applied after addition of substrates for CI and ADP.

We cannot exclude that synaptosomes might have been present in the tissue homogenates.

2.2. Kainic acid injection

Mice were sedated with an ip injection of ketamine (160 mg/kg; Graeub Veterinary Products, Switzerland) and then deeply anesthetized with sevoflurane through a precise vaporizer (Midmark, USA). Mice were injected with 1 nmole kainic acid (20 mM; KA, Ocean Produce International, Canada) solution into CA1 region of the left hippocampus as previously described (Loacker et al., 2007).

2.3. High-resolution respirometry

Tissue homogenates were transferred into calibrated Oxygraph-2 k (O2k, OROBOROS INSTRUMENTS, Innsbruck, Austria) 2 ml-chambers. Oxygen polarography was performed at 37 ± 0.001 °C (electronic