Biochimica et Biophysica Acta 1862 (2016) 741–753

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Adaptation within mitochondrial oxidative phosphorylation supercomplexes and membrane viscosity during degeneration of dopaminergic neurons in an animal model of early Parkinson's disease

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ARTICLE INFO

Article history:
Received 22 October 2015
Received in revised form 27 January 2016
Accepted 29 January 2016
Available online 1 February 2016

Keywords:
Mitochondrial membrane viscosity
OxPhos supercomplex assembly
Neurodegeneration
Parkinson's disease
Compensatory processes

ABSTRACT

In Parkinson's disease (PD) motor symptoms are not observed until loss of 70% of dopaminergic neurons in substantia nigra (SN), preventing early diagnosis. Mitochondrial dysfunction was indicated in neuropathological process already at early PD stages. Aging and oxidative stress, the main factors in PD pathogenesis, cause membrane stiffening, which could influence functioning of membrane-bound oxidative phosphorylation (OxPhos) complexes (Cxs) in mitochondria.

In 6-OHDA rat model, medium-sized dopaminergic lesion was used to study mitochondrial membrane viscosity and changes at the level of OxPhos Cxs and their higher assembled states—supercomplexes (SCxs), during the early degeneration processes and after it.

We observed loss of dopaminergic phenotype in SN and decreased dopamine level in striatum (STR) before actual death of neurons in SN. Behavioural deficits induced by lesion were reversed despite progressing neurodegeneration. Along with degeneration process in STR, mitochondrial Cx I performance and amount decreased in almost all forms of SCxs. Also, progressing decrease of Cx IV performance in SCxs ($I_1III_2IV_{3-1}$, I_1IV_{2-1}) in STR was observed during degeneration. In SN, SCxs containing Cx I increased protein amount and a shifted individual Cx I_1 into superassembled states. Importantly, mitochondrial membrane viscosity changed in parallel with altered SCxs performance.

We show for the first time changes at the level of mitochondrial membrane viscosity influencing SCxs function after dopaminergic system degeneration. It implicates that altered mitochondrial membrane viscosity could play an important role in regulation of mitochondria functioning and pathomechanisms of PD. The data obtained are also discussed in relation to compensatory processes observed.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder manifesting its motor symptoms in the late age, after most of the affected dopaminergic neurons in the substantia nigra (SN) have been already lost [31]. Interestingly, until the loss of approximately 70% of dopaminergic neurons in SN and subsequent 80% loss of dopamine (DA) in its target structure, striatum (STR), no major motor problems are visible, only small, diffuse, often peripheral symptoms. This makes the diagnosis of PD very difficult at early stages when degenerative processes progression could be prevented. Discovering markers of early degeneration in central nervous system, when changes are still very small, is an important issue to study nowadays.

Multiple pathological processes have been described in the course of PD progression, such as abnormal protein degradation, inflammatory processes, oxidative stress and mitochondrial dysfunction. Genetic background in hereditary forms of PD depends on genes (*PINK1* and *parkin*) that are responsible for early onset Parkinsonian syndromes and act specifically as a quality control system at the mitochondrial membrane. Also *alpha-synuclein*, *DJ-1*, and *LRRK2* involved in hereditary forms of PD have been associated with regulation of mitochondrial function and oxidative stress [12]. Thus, mitochondrial dysfunction has been suspected to be one of the main underlying causes for this disease pathology. Disturbances within mitochondria include: impairment in function of the oxidative phosphorylation (OxPhos) electron

Abbreviations: DA, dopamine; Cx, Cxs, complex, complexes; MFB, medial forebrain bundle; SN, substantia nigra; OxPhos, oxidative phosphorylation; SCx, SCxs, supercomplex, supercomplexes.

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http://dx.doi.org/10.1016/j.bbadis.2016.01.022
0925-4439/

transport chain, alterations in mitochondrial morphology and dynamics, mitochondrial DNA mutations and deletions specific for nigral neurons, as well as anomaly in calcium homeostasis and binding of alpha-synuclein to mitochondrial membrane. Besides energy production, which is fundamental for all life processes, mitochondria have also other important roles in the cell such as apoptosis regulation, hence their even slight dysfunction can have impact on the whole cell functioning [63].

Dysfunction of mitochondrial OxPhos protein complexes (Cxs) was observed in *post mortem* brains of advanced PD patients, with decreased activity of Cx I [32,45,55,56]. Lowered activities of mitochondrial Cxs were found in PD patients also outside the brain, in muscles (Cx I, II and IV) [5] and platelets (Cx I, II/III) [61]. Activities of mitochondrial Cx IV and V were reported to decline with age, which is one of main factors increasing risk of PD [43]. Different studies in humans indicated that mitochondrial dysfunction could occur already in early PD stage. Studies with phosphorus and proton magnetic resonance spectroscopy confirmed reduction of ATP and phosphocreatine in the putamen and midbrain of early and advanced PD patients [30].

In the inner mitochondrial membrane OxPhos Cxs I, III and IV form higher organised structures called supercomplexes (SCxs), containing different stoichiometric proportions of each of the three Cxs [1,59]. According to the current studies, assembly into SCxs improves enzymatic activities of individual Cxs [58,67] and facilitates electron transfer through the OxPhos chain, reducing reactive oxygen species (ROS) generation, which in turn increases individual Cxs stability and ATP synthesis efficiency [25,26,46]. Increased stress, resulting from ROS production, was one of the proposed mechanisms for the death of dopaminergic neurons in PD and this is known to increase the membrane viscosity [23].

Furthermore, OxPhos SCxs assembly, stability and activity are assumed to depend on mitochondrial membrane biophysical properties such as viscosity [1]. Aging causes significant increase of membrane viscosity in brain [64]. It is worth to note that there have been found decreased concentrations of membrane lipid precursors and degradation products, indicating reduced membrane turnover rates due to the impaired energy metabolism in the putamen of PD patients [30]. Also, dramatic decline in the contents of polyunsaturated fatty acids was observed in lipid rafts from patients frontal cortex, thus probably making the membranes less fluid [20]. There has been recently published data indicating that disrupted lipid homeostasis may be responsible for some cases of PD [68]. Importantly, those changes in membrane properties were already observed at the early stages of PD pathology. All those facts lead us to the hypothesis that degenerative process could involve changes in mitochondrial membrane viscosity, influence SCxs assembly and therefore mitochondria functioning in PD.

Our goal was to analyse if there are any changes within mitochondrial membrane viscosity and adaptations in SCxs assembly due to the dysfunction and degeneration of dopaminergic neurons and their terminals.

We present here for the first time the quantitative and detailed description of SCxs architecture and activity in mitochondria from distinct rat brain dopaminergic structures: STR and SN, at two different time-points after its selective lesioning. We have found adaptive changes within mitochondrial Cx I and Cx IV, SCxs activity and assembly as well as in mitochondrial membrane viscosity, corresponding with time-line of dopaminergic neurons degeneration in rat brain.

2. Materials and methods

2.1. Animals and stereotactic operations

Three months old male Wistar HAN rats (Charles Rivers, Germany) were kept in 12 h dark/light cycle (light from 7:00 to 19:00) with free access to food and water. The experiments were carried out in compliance with the Animal Experiments Bill of January 21, 2005; (published in Journal of Laws no. 33/2005 item 289, Poland), and according to the NIH Guide for the Care and Use of Laboratory Animals. They also received approval from the Local Ethical Committee. All efforts were made to minimize the number of animals and their suffering.

Stereotaxic brain operations were performed as before [38], under ketamine and xylasine anaesthesia (50 mg/kg and 3 mg/kg *im*, Biowet, Puławy, Poland). 30 min before lesioning desipramine was administered to protect the noradrenergic terminals (30 mg/kg ip, Sigma-Aldrich, Germany). The animals were bilaterally injected with 6-OHDA HBr (3 μg base/3 μl/side in a 0.2% ascorbic acid, Sigma-Aldrich, Germany) into the passing fibres of medial forebrain bundle (MFB) (AP: −1.6 mm, L: ± 1.6 mm, V: 8.4 mm from Bregma, according to Paxinos and Watson atlas, 1986). Control, sham operated rats received solvent. To avoid infections, the rats received an antibiotic (Lincospectin, Pharmacia, Belgium) on the day of operation and 24 h afterwards.

2.2. Behavioural analysis using automated actimeters

Rats locomotor activity (path length, time) and rearing (free, supported) were measured [34] 3 and 27 days after operation, using computerized actimeters (ACTIFRAME-SYSTEM, GERB Elektronik GmbH, Berlin, Germany with ARNO software) with 16 infrared beam transmitters and sensors. The minimum distance regarded as a locomotion event was set at 4 beams (4.44 cm). At 14:00 h animals were placed in the cages individually, with free access to food and water and behaviour was analysed until 24:00. If the same animal was tested twice, the analyses were at least 3 weeks apart.

2.3. HPLC-EC analysis of DA, its metabolite levels and turnover rates

Rats were decapitated at 4^{th} or 28^{th} day after operation. Left STR and SN were immediately dissected and frozen on dry ice. The concentration of DA and its metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), homovanillic acid (HVA) as well as noradrenaline, serotonin together with its metabolite 5-hydroxyindoleacetic acid was assessed using HPLC method with electrochemical detection as published previously [4]. Tissue samples were homogenized in 0.1 M perchloric acid with 0.05 mM ascorbic acid and injected into the HPLC system (32 °C, Hypersil Gold C18, 100 × 3.0 mm, 3 μm, Thermo Scientific, UK). The mobile phase was composed of 50 mM $NaH_2PO_4 \times 2H_2O$; 40 mM citric acid; 0.25 mM 1-octanesulfonic acid sodium salt; 0.25 mM EDTA; 1.3% acetonitrile; 2.4% methanol. The applied potential of electrochemical detector was E1 = −175 mV and E2 = +350 mV. The data were quantified using the area under the peaks and external standards with Chromeleon software (Dionex, Germany). The turnover rates were calculated as metabolites to neurotransmitter ratios.

2.4. Immunohistochemistry and stereology of dopaminergic and non-dopaminergic neurons

2.4.1. Immunohistochemistry

After the decapitation, the right hemispheres of the brains were rapidly removed, postfixed in paraformaldehyde and cryoprotected in sucrose. The brains were cut into 30 μm frontal sections (AP −4.4 to −6.84 mm from Bregma, according to Paxinos and Watson atlas, 1986) according to the stereological rules and stained as described before [38]. Free-floating sections were incubated with primary antibodies (mice anti–tyrosine hydroxylase (TH), 1:3000; Chemicon Int.; Millipore, USA), then with secondary antibodies (anti-mouse biotinylated, 1:200, Vector Laboratories, UK) and processed using an ABC-peroxidase kit (Vector Laboratories, UK) and 3,3′-diaminobenzidine as a chromogen. Subsequently, the sections with SN and ventral tegmental area (VTA) were stained with 1% cresyl violet (CV, Nissl staining).