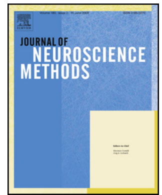
Journal of Neuroscience Methods 232 (2014) 143–149

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

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



Basic Neuroscience

Quantitative quadruple-label immunofluorescence of mitochondrial and cytoplasmic proteins in single neurons from human midbrain tissue

Anne Grünewald[a,b], Nichola Z. Lax[a], Mariana C. Rocha[a], Amy K. Reeve[a], Philippa D. Hepplewhite[a], Karolina A. Rygiel[a], Robert W. Taylor[a], Doug M. Turnbull[a,*]

[a] Wellcome Trust Centre for Mitochondrial Research, Institute of Ageing and Health, Newcastle University, Newcastle upon Tyne, UK
[b] Institute of Neurogenetics, University of Lübeck, Lübeck, Germany

HIGHLIGHTS

- We developed an assay to quantify respiratory chain deficiencies in single neurons.
- Quadruple-label immunofluorescence was combined with quantitative image analysis.
- The single-cell assay was applied to tyrosine hydroxylase-positive midbrain neurons.
- The expression of complexes I and IV was determined relative to mitochondrial mass.
- The assay proved specific in patients with known respiratory chain deficiencies.

ARTICLE INFO

Article history:
Received 6 March 2014
Received in revised form 19 May 2014
Accepted 20 May 2014

Keywords:
Mitochondria
Complex I
Complex IV
Single cell analysis
Midbrain neurons
Immunofluorescent labelling

ABSTRACT

Background: Respiratory chain (RC) deficiencies are found in primary mtDNA diseases. Focal RC defects are also associated with ageing and neurodegenerative disorders, e.g. in substantia nigra (SN) neurons from Parkinson's disease patients. In mitochondrial disease and ageing, mtDNA mutational loads vary considerably between neurons necessitating single cell-based assessment of RC deficiencies. Evaluating the full extent of RC deficiency within SN neurons is challenging because their size precludes investigations in serial sections. We developed an assay to measure RC abnormalities in individual SN neurons using quadruple immunofluorescence.

New method: Using antibodies against subunits of complex I (CI) and IV, porin and tyrosine hydroxylase together with IgG subtype-specific fluorescent labelled secondary antibodies, we quantified the expression of CI and CIV compared to mitochondrial mass in dopaminergic neurons. CI:porin and CIV:porin ratios were determined relative to a standard control.

Results: Quantification of expression of complex subunits in midbrain sections from patients with mtDNA disease and known RC deficiencies consistently showed reduced CI:porin and/or CIV:porin ratios.

Comparison with existing method(s): The standard histochemical method to investigate mitochondrial dysfunction, the cytochrome *c* oxidase/succinate dehydrogenase assay, measures CIV and CII activities. To also study CI in a patient, immunohistology in additional sections, i.e. in different neurons, is required. Our method allows correlation of the expression of CI, CIV and mitochondrial mass at a single cell level.

Conclusion: Quantitative quadruple-label immunofluorescence is a reliable tool to measure RC deficiencies in individual neurons that will enable new insights in the molecular mechanisms underlying inherited and acquired mitochondrial dysfunction.

© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/3.0/).

* Corresponding author at: Wellcome Trust Centre for Mitochondrial Research, Institute of Ageing and Health, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK. Tel.: +44 191 222 3009; fax: +44 191 222 5685.

E-mail addresses: anne.gruenewald@newcastle.ac.uk (A. Grünewald), nichola.lax@newcastle.ac.uk (N.Z. Lax), m.rocha@newcastle.ac.uk (M.C. Rocha), amy.reeve@newcastle.ac.uk (A.K. Reeve), philippa.hepplewhite@newcastle.ac.uk (P.D. Hepplewhite), karolina.rygiel@newcastle.ac.uk (K. A. Rygiel), Robert.taylor@newcastle.ac.uk (R.W. Taylor), doug.turnbull@newcastle.ac.uk (D.M. Turnbull).

http://dx.doi.org/10.1016/j.jneumeth.2014.05.026
0165-0270/© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/3.0/).

1. Introduction

The oxidative phosphorylation (OXPHOS) system comprises approximately 85 polypeptides. These polypeptides assemble (i) four respiratory chain (RC) complexes, complex I (CI) to complex IV (CIV), (ii) one complex facilitating adenosine triphosphate (ATP) generation, complex V (CV), and (iii) two mobile electron carriers, coenzyme Q (CoQ) and cytochrome *c* (Mitchell, 1961). The OXPHOS system is situated in the lipid bilayer of the inner mitochondrial membrane (Hatefi, 1985; Saraste, 1999). The passage of electrons from CI or CII via CIII and CIV catalyzes the transfer of protons from the mitochondrial matrix across the inner mitochondrial membrane to the inter-membrane space building an electrochemical gradient. This gradient is the driving force for CV, ATP synthase, to generate ATP from ADP and inorganic phosphate (Hatefi, 1985; Saraste, 1999).

RC enzyme deficiency, in particular involving CI and CIV (Greaves et al., 2010; Reeve et al., 2008; Swalwell et al., 2011), is present in patients with primary mitochondrial diseases (DiMauro and Schon, 2003). In addition, there is increasing evidence for a role of RC enzyme deficiency in ageing and neurodegenerative diseases such as Parkinson's disease (PD) (Bender et al., 2006; Langston et al., 1983; Schapira et al., 1989). In primary mitochondrial DNA (mtDNA) diseases, dysfunction of RC enzyme complexes is due to inherited mutations in the mitochondrial genome with the level of heteroplasmy (mutational burden) varying considerably between cells (Taylor and Turnbull, 2005). In PD, and normal human ageing, an accelerated accumulation of somatic mtDNA deletions in substantia nigra neurons has been reported (Bender et al., 2006; Kraytsberg et al., 2004). As a consequence of varying mutational loads, cells differ in the degree of OXPHOS deficiency which necessitates analysis on a single cell level to determine the nature of the biochemical defect. In many tissues (e.g. skeletal muscle, large intestine), information concerning RC activities and expression levels can be obtained from sequential sections (Campbell et al., 2013; Greaves et al., 2010; Rowan et al., 2012). In the CNS, however, investigations concerning RC enzyme function and abundance have so far been performed in populations rather than single cells, since the size of most neuron types, including the dopaminergic neurons of the substantia nigra, precludes serial studies.

To enable us to simultaneously explore the degree of deficiency of CI and CIV (both of which have mtDNA-encoded subunits) in single dopaminergic neurons from postmortem midbrain tissue, we have established a quantitative immunofluorescent protocol for quadruple labelling.

2. Materials and methods

2.1. Human tissue

Skeletal muscle tissue was obtained from an elderly control, following informed consent, with age-related RC deficiencies (age: 78, sex: female) and snap frozen in isopentane at −160 °C. Muscle was cyrosectioned (10 μm) and sections allowed to air dry for 60 min at room temperature (RT) before immunohistology or histochemistry was performed.

Brain tissue was provided by the Newcastle Brain Tissue Resource (NBTR) with ethical approval. To optimize the quadruple labelling immunofluorecence protocol, post-mortem brain samples were obtained from two patients with mitochondrial disease exhibiting CI and CIV deficiencies, one idiopathic Parkinson disease patient (age: 77, sex: female) and one control with no neurological phenotype (age: 55, sex: male). The mitochondrial disease patients included one individual with MERRF due to the m.8344A>G mutation (age: 58, sex: male) and one patient with Kearns Sayre syndrome (KSS) due to a single, large-scale mtDNA deletion (age: 40, sex: female) (Reeve et al., 2013). Sections of formalin-fixed paraffin-embedded midbrain blocks were cut at 5 μm using a microtome (Microm International) and mounted on to SuperFrostTM slides (Thermo Fisher Scientific).

2.2. Immunofluoresence labelling and histochemistry with muscle sections

For immunofluorescent staining, snap frozen muscle sections were dried, fixed in 4% (w/v) paraformaldehyde (PFA) for 10 min and then washed three times for 2 min in Tris-buffered saline containing 1% Tween-20 (TBST). Sections were then blocked in TBST and 1% (v/v) normal goat serum (NGS) for 1 h at RT. Monoclonal mouse antibodies against complex I-20 (NDUFB8, IgG subtype 1, Abcam, ab110242), and CIV-1 (COX1, IgG subtype 2a, Abcam, ab14705) were diluted 1:100 in 1% NGS and incubated over night at 4 °C. To remove unbound primary antibodies, sections were washed three times in TBST for 5 min. Then IgG subtype-specific secondary anti-mouse antibodies conjugated to Alexa fluor 488 and 546 (both Life Technologies, A21131 and A21143, respectively) were applied (1:100 in 1% NGS TBST) for 60 min at RT. Again, sections were washed three times for 5 min in TBST. To quench autofluorescence, Sudan black (0.3% w/v in 70% v/v ethanol) solution was applied for 10 min followed by three short washes in TBST. Finally, sections were mounted in Prolong Gold Antifade Reagent (Life Technologies).

For immunohistology with chromogens, air-dried muscle sections were fixed in 4% PFA for 10 min, followed by rinsing in TBST for 10 min. The sections were permeabilized in a graded (70%, 95%, 100%, 100%, 95%, 70% v/v, 10 min each) methanol series. Endogenous peroxidase activity was quenched by addition of hydrogen peroxide 0.3% (v/v) to 95% (v/v) methanol. Sections were rinsed in TBST and blocked in 5% (w/v) BSA TBST for 30 min at RT. Next, sections were incubated overnight with anti-CI-20 and CIV-1 antibodies (1:100 in 5% (w/v) BSA TBST) at 4 °C. Following three washes in TBST, sections were treated with the MenaPath kit according to manufacturer's protocol (Menarini Diagnostics). After five washes in TBST for 5 min, peroxidase activity was detected by incubation in 3,3′-diaminobenzadine tetrahydrochloride solution (SigmaFast DAB tablets dissolved in distilled water) for 5 min at RT.

CIV (COX) histochemical activity in muscle was detected using a published technique (Johnson et al., 1988).

2.3. Immunofluorescence with midbrain sections

To deparaffinize and rehydrate, paraffin-fixed midbrain sections were incubated at 60 °C for 30 min. This was followed by sequential washing steps in Histoclear (2 times for 5 min) (National Diagnostics), and a graded ethanol series (100%, 100%, 95%, 70% v/v, 5 min each). Next, sections were washed in distilled water. Antigen retrieval was performed in 1 mM EDTA, pH 8, in a pressure cooker for 40 min followed by another wash in distilled water and a short wash in TBST. Sections were blocked in TBST and 1% (v/v) NGS for 1 h at RT. Monoclonal mouse antibodies against CI-20, CIV-1, the mitochondrial mass marker porin (IgG subtype 2b, Abcam, ab14734) and a rabbit polyclonal antibody directed against the dopaminergic marker tyrosine hydroxylase (TH, Sigma, T8700) were used at a concentration of 1:100. Midbrain sections were incubated in the primary antibody dilution overnight at 4 °C. This was followed by three wash steps for 5 min in TBST. For quadruple immunofluorescent labelling, midbrain sections were incubated with IgG subtype-specific secondary anti-mouse antibodies conjugated with Alexa Fluor 488, 546 or 647 (A21240) and a secondary anti-rabbit Alexa Fluor 405 (A31556) antibody (all Life

Download English Version:

https://daneshyari.com/en/article/6268689

Download Persian Version:

https://daneshyari.com/article/6268689

Daneshyari.com