Dysfunctional mitochondrial respiration in the striatum of the Huntington’s disease transgenic R6/2 mouse model

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Abstract
Metabolic dysfunction and mitochondrial involvement are recognised as part of the pathology in Huntington’s Disease (HD). Post-mortem examinations of the striatum from end-stage HD patients have shown a decrease in the in vitro activity of complexes II, III and IV of the electron transport system (ETS). In different models of HD, evidence of enzyme defects have been reported in complex II and complex IV using enzyme assays. However, such assays are highly variable and results have been inconsistent. We investigated the integrated ETS function ex vivo using a sensitive high-resolution respirometric (HRR) method. The O2 flux in a whole-cell sample combined with the addition of mitochondrial substrates, uncouplers and inhibitors enabled us to accurately quantitate the function of individual mitochondrial complexes in intact mitochondria, while retaining mitochondrial regulation and compensatory mechanisms. We used HRR to examine the mitochondrial function in striata from 12-week old R6/2 mice expressing exon 1 of human HTT with 130 CAG repeats. A significant reduction in complex II and complex IV flux control ratios was found in the R6/2 mouse striatum at 12 weeks of age compared to controls, confirming previous findings obtained with spectrophotometric enzyme assays.

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Introduction
HD is a rare hereditary fatal neurodegenerative disease, with a prevalence of 5-6 per 100,000 in Europe and North America. It is caused by an expanded stretch of >36+ CAG repeats in exon 1 of the HTT gene. The expansion length inversely correlates with age of onset. Wild type huntingtin protein (Htt) is ubiquitously expressed, although the expression is especially high in the testis and brain, in particular in the striatum, cortex and hippocampus. The major neuropsychological phenotype of HD is a loss of a specific striatal neuronal subpopulation, medium spiny neurons, resulting in striatal atrophy. However, the disease also manifests systemically. Metabolic dysfunction has long been recognised as part of the pathology in HD, underlined by the weight loss of HD patients. Decreased glucose metabolism and increased lactate concentrations in several brain regions of symptomatic HD patients indicate mitochondrial involvement in the disease. Htt destabilises the mitochondrial outer membrane, which increases the sensitivity of the mitochondrial transition pore to Ca2+ and other apoptotic stimuli. Moreover, mitochondria in HD models have been shown to be dysfunctional with respect to fission and fusion, trafficking, respiration and ATP production.

Impairment of the electron transport system (ETS) in HD is an area of controversy in HD research. The ETS consists of the respiratory complexes I, II, III and IV (CI, CII, CIII and CIV), which generate the proton motive force. This is utilised mainly to generate ATP and mitochondrial Ca2+ uptake. Post-mortem examinations of the striatum have demonstrated a decrease in the in vitro enzyme activity of CII, CIII and CIV only in the late stage disease.

With the emergence of transgenic HD models, enzyme activity could be examined without the risk of post-mortem brain tissue modifications. However, conflicting results have been obtained from such studies. Whilst several studies have described dysfunction in CII and CIV, others report intact function (summarised in Table 1). In addition, several reviews mention unpublished data that purportedly could not reproduce any ETS deficiency in a number of mouse models. One of the findings disputed in these reviews is the study by Tabrizi et al. where CIV deficiency was reported in the R6/2 transgenic mouse striatum. The R6/2 mouse expresses an N-terminal fragment of mutant human HTT exon 1 with ~130 CAG repeats. The phenotype is characterised by the emergence of HD symptoms at ~9 weeks of age, with a severe phenotype at 12 weeks of age.
age and premature death at around 13 weeks of age. In the previous studies of the ETS function in R6/2 mice, the activities of the individual complexes were evaluated using enzyme assays. These methods have since been demonstrated to confer a considerable methodological variation. In addition, the ETS is organised in supercomplexes (respirasomes) and measurement of single enzyme activities does not provide an accurate description of the integrated ETS function. 

Table 1. Overview of reported ETS dysfunction in HD models.

<table>
<thead>
<tr>
<th>Model name</th>
<th>Transgenic Insert</th>
<th>Complexes affected</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD89 and HD48 mouse</td>
<td>human HTT gene coding for full-length Htt with 89 CAG repeats</td>
<td>None</td>
<td>Spectrophotometric enzyme assay</td>
<td>20</td>
</tr>
<tr>
<td>R6/2 mouse (12 weeks)</td>
<td>N-terminal fragment model with 115 CAG repeats</td>
<td>CIV, aconitase</td>
<td>Spectrophotometric enzyme assay</td>
<td>26</td>
</tr>
<tr>
<td>R6/2 mouse (8 weeks)</td>
<td>N-terminal fragment model with 115 CAG repeats</td>
<td>None</td>
<td>Autoradiogram.</td>
<td>25</td>
</tr>
<tr>
<td>N171-82Q mouse (20 weeks)</td>
<td>N-terminal fragment model with 82 CAG repeats</td>
<td>CIV</td>
<td>Spectrophotometric enzyme assay</td>
<td>37</td>
</tr>
<tr>
<td>Wistar rats (8 weeks post-injection)</td>
<td>Lentiviral N-terminal fragment model with 82 CAG repeats</td>
<td>CII</td>
<td>Autoradiogram.</td>
<td>38</td>
</tr>
<tr>
<td>Wistar rats</td>
<td>N-terminal fragment with 52 CAG repeats</td>
<td>CII+IIOXPHOS flux</td>
<td>High-resolution respirometry</td>
<td>39</td>
</tr>
<tr>
<td>HdhQ111 striatal cells</td>
<td>Knock-in mouse model carries 111 CAG repeats in endogenous HTT gene</td>
<td>None</td>
<td>Respirometry</td>
<td>47</td>
</tr>
<tr>
<td>Neonatal striatal HdhQ150 cells</td>
<td>Knock-in mouse model carries 150 CAG repeats in endogenous HTT gene</td>
<td>None</td>
<td>Respirometry</td>
<td>17</td>
</tr>
<tr>
<td>Hela cells</td>
<td>Transient expression of N-terminal HTT fragment with 40 CAG repeats</td>
<td>CII</td>
<td>Spectrophotometric enzyme assay</td>
<td>40</td>
</tr>
<tr>
<td>Rat embryonic striatal neurons</td>
<td>Lentivirally transduced with N-terminal fragment with 82 CAG repeats</td>
<td>CII subunit concentration reduced</td>
<td>Western blot</td>
<td>41</td>
</tr>
<tr>
<td>R6/2 mouse, N171-82Q YAC-72 mouse, HdhQ92 mouse, HdhQ111 mouse</td>
<td>–</td>
<td>None</td>
<td>N/A</td>
<td>22-23-24</td>
</tr>
</tbody>
</table>

In order to investigate the reported ETS dysfunction in the R6/2 mouse model using more sensitive methods, we analysed the mitochondrial function in the striatum of 12-week old R6/2 female transgenic mice and wild type littermates using HRR. In contrast to spectrophotometric enzyme assays, HRR takes into account the entire spectrum of respiratory control, compensatory mechanisms and cellular architecture affecting mitochondrial function as the respirometric measurements are carried out on non-isolated mitochondria that retain their proper cellular context. Whilst isolation of mitochondria has been shown to drastically influence function, tissue homogenate HRR has been demonstrated to constitute a sensitive method for analysing the integrated mitochondrial function.

**Materials and methods**

**Animal studies**

R6/2 mice, transgenic for exon 1 gene of the human HD gene containing approximately 130 CAG repeat units, originated from the Jackson Laboratory (Bar Harbor, Maine) and were maintained by backcrossing males to CBA/J x B6 females (Taconic, Denmark). The behavioural phenotype of the colony has been described previously. The mice were kept under specific pathogen free (SPF) conditions at a 12-hour light / 12-hour darkness cycle in standard polystyrene cages with free access to standard chow. Tail tip DNA was used for genotyping. The CAG repeat lengths of mice from the colony were around 130 throughout the experiment. In each experiment, we used six 12-week-old female R6/2 mice and six control female littermates without the HD transgene. The experiment was performed twice, yielding a total of 12 animals in each group. Experiments were performed in accordance with and approved by the Danish Animal Experiments Inspectorate.

**Sample preparation**
Experimental animals were sacrificed by cervical dislocation. The brains were excised and placed in ice-cold mitochondrial respiration medium MiR05 (EGTA 0.5 mM, MgCl2 3 mM, K-lactobionate 60 mM, tauroine 20 mM, KH2PO4 10 mM, HEPES 20 mM, sucrose 110 mM, BSA 1 g/L, adjusted to pH 7.1)35. The striatum was then dissected, weighed and homogenised in a precooled mortar with a pestle in MiR05 medium. The crude homogenate was filtered through a 40 µm cell strainer (BD Falcon, San Jose, CA, USA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

High-resolution respirometry

Mitochondrial respiration was measured in a high-resolution oxygraph (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) at 37°C. Striatum homogenates (5-10 mg) were suspended in 2 mL MiR05 medium. Oxygen concentration (µM = nmol/ml) and oxygen flux (pmol/(s·ml)) were recorded online using DatLab software version 4.3.2.7 (Oroboros Instruments, Innsbruck, Austria).

Experimental protocol

The striatum homogenate was suspended in MiR05, added to the Oxygraph-2k glass chambers and the O2 flux was allowed to stabilise. A substrate, uncoupler, inhibitor titration (SUIT) protocol was applied to assess qualitative and quantitative mitochondrial changes in R6/2 transgenic mice and unaffected controls. After stabilisation, LEAK respiration was evaluated by adding the CI substrates malate (0.8 mM), pyruvate (2 mM) and glutamate (10 mM). The maximum oxidative phosphorylation (OXPHOS) capacity with CI substrates was attained by the addition of ADP+Mg2+ (2.25 mM) (CiOXPHOS). For quality control of mitochondrial integrity cytochrome c (CytC) (10 mM) was added (CiOXPHOS+CytC). For evaluation of maximum OXPHOS capacity of the convergent input from CI and CII at saturating ADP-concentration, the CII substrate succinate (10 mM) was added (Ci+CiOXPHOS). Maximum ETS capacity was obtained by stepwise titration of the uncoupler carbonylcyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP, 1 pmol/step) (Ci+IETS). Rotenone (2.5µM) was added to inhibit CI; hence the maximal ETS capacity supported by CII alone was determined (CiETS). Residual oxygen consumption (ROX) was established by addition of the CIII inhibitor Antimycin A (2.5 mM). Finally, maximal CIV activity was determined by addition of 0.5 mM TMPD (N,N,N,N-Tetramethyl-p-phenylenediaminedihydrochloride), a substrate for the reduction of CytC, and 2 mM ascorbate (CIVmax). CIVmax was corrected for autoxidation of substrates as previously described36. ROX was subtracted from the fluxes in each run to correct for non-mitochondrial respiration. All samples were run in duplicates and the mean was used for analysis. The mean variation between duplicates was 0.9% ± 0.7% SEM for wild type and 2.3% ± 1.2% SEM for R6/2 measurements.

Citrate synthase assay

Protein was extracted from 2.5 mg striatal homogenate using CelLyticM (Sigma-Aldrich, St. Louis, MO, USA) containing 1X Complete protease inhibitor cocktail (Roche, Basel, Switzerland). Citrate synthase (CS) assay was performed in duplicate using the Citrate Synthase Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) and the mean was used for analysis. The mean variation between duplicates was 3.6% ± 3.48% SEM. Samples were analysed in a Victor3 1420 multiplate reader (Perkin Elmer, Waltham, MA, USA) at λabs= 412 nm.

Data analysis

D’Agostino & Pearson omnibus normality test was applied to all data sets. Student’s t-test was used for statistical analysis. The significance level was set at p<0.05. Differences were considered trends if p<0.1. Graphs were generated with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). All differences are given as the mean difference ± the standard error of the mean difference.

Results

The integrated ETS pathway was assessed using HRR by determining the flux control ratios (FCR) in the striatum of R6/2 transgenic mice and wild type littermates using a SUIT protocol. The FCRs were generated by normalising each respiratory state to the maximal uncoupled mitochondrial respiration, CI+IETS (State 3U). The respiratory control ratio (RCR) was generated by normalising CI+CiOXPHOS to LEAK (State 3ADP/state 4). The main findings were significant quantitative differences in CI+CiOXPHOS, CiETS and CIVmax when expressed as FCRs (Figure 1). The mean CI+CiOXPHOS FCR was decreased in the R6/2 mice relative to wild type controls by 0.035 ± 0.011 (5.2% ± 1.6%), whilst the mean CIETS FCR was decreased by 0.041 ± 0.018 (11.7% ± 5.1%). The mean CIVmax value was decreased by 0.149 ± 0.07 (11.1% ± 5.2%). No significant differences in CIOXPHOS(0.9% ± 4.6%) or in LEAK (2.1% ± 9.3%) were seen. The mean SD of the generated FCRs was 10.5%.
measurements include respiratory control, a meaningful comparison with results obtained using spectrophotometry is difficult. The decreases in ETS function we observe are small compared to results obtained using enzyme assays. However, as our studies demonstrating a deficiency in CII and/or CIV in other HD models compared to age-matched wild type littermates confirming previous findings in this model [26] and in accord with several other.

Fig. 2: Respiratory control ratios (RCR) (ratio between CI+CIIOXPHOS and LEAK or state 3/state4).

We measured the CS activity/mg tissue in the samples. We found that there was no significant difference in CS activity (p=0.74) or variation (p=0.45) in wild-type mice compared to R6/2 transgene mice. As populations were not significantly different and variances were equal, we pooled all CS activities to get an estimate of the analytical variation, and calculated the SD as ± 38.5%.

Normalising the O$_2$ flux to CS activity failed to demonstrate any significant differences in O$_2$ flux per unit CS, although a trend (p<0.1) similar to the observed decreases in R6/2 mice FCRs was seen in CIETS and CIV$_{max}$ but not in CI+IIOXPHOS (Figure 3).

Fig. 3: The absolute O$_2$ flux for each respiratory state normalized to citrate synthase (CS) activity.

n=12 for each group. CS units are defined as µmole/ml/min. No significant difference between groups was found. Error bars depict SD.

Discussion

This study demonstrates that the CI+IIOXPHOS, CIETS and CIV$_{max}$ FCRs are slightly but significantly decreased in R6/2 mice compared to age-matched wild type littermates confirming previous findings in this model [26] and in accord with several other studies demonstrating a deficiency in CII and/or CIV in other HD models [37-39,40-41].

The decreases in ETS function we observe are small compared to results obtained using enzyme assays. However, as our measurements include respiratory control, a meaningful comparison with results obtained using spectrophotometry is difficult.
Whether the modest reduction is a cause or consequence of disease pathology is not clear, but reductions in CI activity of similar magnitudes have been reported in human cardiac failure. The deficiency in CI+HOXPHOS and CI|ETS, but not in CI|OXPHOS or LEAK indicates a deficiency in coupled and uncoupled CII, but not CI. This difference is more pronounced in the uncoupled ETS state of CII, as electron input from CI has been inhibited and the physiological control of CII activity has been removed, which exacerbates the difference. The maximal capacity of CI+CII|ETS, with the excess capacity ensuring some degree of compensation for damage to CIV, whilst maintaining normal coupled mitochondrial respiratory function. Hence, a reduction in CIV is of limited physiological significance, unless the reduction is sufficiently severe to become the limiting factor in maximal respiration. Indeed, in some transgenic animals tested, the CIVmax/CI+CII|ETS ratio was close to 1, in which case the degree of CIV inhibition could potentially limit respiration.

The mean RCR ratio was not significantly decreased in R6/2 mice compared to controls, which indicates that there is no dyscoupling of the ETS or altered mitochondrial inner membrane integrity. The RCR values given in this paper are estimations only, since we use LEAK (added saturating complex I substrates, but without the addition of ADP) as an expression of state 4 respiration, where the main respiratory component is proton leakage. An accurate measure of state 4 respiration would require zero ATP synthase activity, either by inhibition or a complete ATP/ADP equilibrium. As we used semi-permeabilized cells, it is likely that residual cellular ATP, ADP and cytosolic ATPases are present when CI substrates are added, which can lead to an overestimation of the RCR.

Citrate synthase is a mitochondrial marker, and assumed to reflect mitochondrial content. The CS assay is frequently used for normalisation in mitochondrial studies, but the methodological variability – as demonstrated here – can lower the statistical resolution and obscure true differences as previously reported. The CS assay is frequently used for normalisation in mitochondrial studies, but the methodological variability – as demonstrated here – can lower the statistical resolution and obscure true differences as previously reported.

In conclusion, we find that the FCRs of coupled and uncoupled CII and the CIV in the striatum of the Huntington’s disease R6/2 transgenic mouse model at 12 weeks are significantly decreased compared to wild type littermates. Furthermore, this study highlights the strengths of HRR for the evaluation of subtle differences in the ETS.

Competing Interests

The authors have declared that no competing interests exist.

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