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Identification of elevated urea as a severe, ubiquitous metabolic defect in the brain of patients with Huntington's disease

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ABSTRACT

Huntington's disease (HD) is a neurodegenerative disorder wherein the aetiological defect is a mutation in the Huntington's gene (*HTT*), which alters the structure of the huntingtin protein through the lengthening of a polyglutamine tract and initiates a cascade that ultimately leads to dementia and premature death. However, neurodegeneration typically manifests in HD only in middle age, and processes linking the causative mutation to brain disease are poorly understood. Here, our objective was to elucidate further the processes that cause neurodegeneration in HD, by measuring levels of metabolites in brain regions known to undergo varying degrees of damage. We applied gas-chromatography/mass spectrometry-based metabolomics in a case-control study of eleven brain regions in short *post-mortem*-delay human tissue from nine well-characterized HD patients and nine controls. Unexpectedly, a single major abnormality was evident in all eleven brain regions studied across the forebrain, midbrain and hindbrain, namely marked elevation of urea, a metabolite formed in the urea cycle by arginase-mediated cleavage of arginine. Urea cycle activity localizes primarily in the liver, where it functions to incorporate protein-derived amine-nitrogen into urea for recycling or urinary excretion. It also occurs in other cell-types, but systemic over-production of urea is not known in HD. These findings are consistent with impaired local urea regulation in brain, by up-regulation of synthesis and/or defective clearance. We hypothesize that defective brain urea metabolism could play a substantive role in the pathogenesis of neurodegeneration, perhaps via defects in osmoregulation or nitrogen metabolism. Brain urea metabolism is therefore a target for generating novel monitoring/imaging strategies and/or therapeutic interventions aimed at ameliorating the impact of HD in patients.

1. Introduction

Huntington's disease is an autosomal dominant genetic disorder caused by an expanded CAG repeat in exon 1 of the *HTT* gene, which is located on chromosome 4 and encodes the huntingtin protein [1]. Biochemical pathways in which alterations have been linked to neurodegeneration in HD include cell-stress responses, apoptosis, several metabolic pathways including the ubiquitin-proteasome system and protein trafficking/endocytosis [2].

In manifest HD, progressive degeneration of the brain leads ultimately to symptoms including involuntary body movements (chorea), disturbances of speech, and progressive dementia [3]. Although the caudate-putamen and cerebral cortex are known

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primary targets of neurodegeneration in HD, it has been reported that several other brain regions are also affected in this complex disease process [4].

Besides the well-characterised neuropathology, several lines of evidence point to a widespread metabolic perturbation in HD. There is evidence that this mechanism is not limited to the basal ganglia and associated neural circuitry; rather, it is thought to be pervasive, affecting many brain regions in HD-gene carriers [5,6]. Furthermore, the existence of a systemic metabolic defect is also supported in HD [7–9].

In order to study the effects of HD on metabolism across the affected brain, we chose to apply gas chromatography/mass spectrometry-based metabolomics to detect and characterise putative alterations in metabolite levels in short *post-mortem*-delay brain tissue of patients and matched controls, using a case-control approach.

Eleven regions of the human brain were analysed here. The putamen and motor cortex were selected as they are most severely affected in advanced disease whereas sensory cortex, globus pallidus, cingulate gyrus and substantia nigra represent regions moderately affected in the HD brain [10,11]. Finally, hippocampus, entorhinal cortex, cerebellum, middle frontal gyrus and middle temporal gyrus were included as they are not usually associated with overt degeneration.

2. Materials and methods

2.1. Acquisition of human brains

Whole brains from nine HD patients and nine matched controls were obtained from the New Zealand Neurological Foundation Human Brain Bank, in the Centre for Brain Research, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand. All procedures in this study were approved by the University of Auckland Human Participants Ethics Committee with informed consent from all families. The quality of HD brain tissue acquired by the Auckland brain bank is uniformly high as has been confirmed by demonstrating mRNA integrity in representative samples of human brain tissue (data not shown).

2.2. Human brain tissue

Each brain was dissected under the supervision of neuroanatomists (SP, HJW and RLMF), who accurately identified each of the brain regions studied. We obtained tissue from eleven identified brain regions: putamen (PUT), globus pallidus (GP), substantia nigra (SN), cerebellum (CB), hippocampus (HP), middle temporal gyrus (MTG), cingulate gyrus (CG), sensory cortex (SCTX), motor cortex (MCTX), middle frontal gyrus (MFG), and entorhinal cortex (ENT). Tissue samples of 50 ± 5 mg were dissected from each region from each patient, and stored at −80 °C until analysis.

2.3. Tissue extraction

Aliquots of 50 ± 5 mg brain tissue underwent a Folch-style extraction using a TissueLyser bead homogeniser (Qiagen; UK). Each sample was extracted in 0.8 ml 50:50 (v/v) methanol:chloroform, to which a solution of the labelled internal standards in methanol had been added to achieve a final concentration of 0.016 mg/ml of each internal standard in the extraction solvent, for 10 min at 25 Hz with a single 3-mm tungsten carbide bead. Samples corresponding to each brain region were handled as single separate batches for this and all subsequent procedures. Phase separation was achieved by addition of 0.4 ml water followed by vortex-mixing (10–15 s) and centrifugation (2400 g, 5 min), and subsequent removal of the chloroform layer. Extraction blanks were prepared by including tubes without tissue samples in each the batch.

2.4. Sample preparation

From the methanol:water supernatant, 200 μl aliquots were transferred to pre-labelled tubes. A quality control (QC) pool was constructed from further 200 μl aliquots from each extraction, gently mixed, and dispensed at equal volume into tubes. After brief mixing, 200 μl aliquots of each sample were dried (30 °C, 16–18 h) in a Speedvac centrifugal concentrator (Thermo Scientific). Dried residues were then held in sealed tubes at 4 °C until derivatization for GC-MS analysis.

2.5. GC-MS analysis

The method used was based upon one that we previously described [12,13]. Briefly, this method uses methoximation and trimethylsilylation to generate a profile of polar small-molecule metabolites such as those corresponding to amino acids, simple organic acids and monosaccharides, and is typically applied to provide comparative data in a case-control experimental design [14].

Dried residues were reconstituted in 60 μl methoxylamine hydrochloride solution (20 mg/ml in dry pyridine) and heated at 80 °C for 20 min in sealed tubes. 60 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was then added and heating continued at 80 °C for a further 20 min. Finally, 10 μl of a retention-time marker solution (nine n-alkanes covering the range C12–C32 dissolved at 10 mg/ml in 1:1 hexane:pyridine) was added and the solutions transferred to autosampler vials for GC-MS analysis.

Chromatography was carried out using an Agilent/J&W DB17-MS column (30 m × 0.25 mm × 0.25 μm) with a 3 m × 0.25 mm retention gap, and helium carrier at a constant flow of 1.4 ml/min. Oven temperature was increased from 50 °C to 300 °C at 10 °C/min. 1 μl injections were performed in Pulsed-Splitless mode using an MPS2 autosampler (Gerstel; Germany), and a 7890A Gas Chromatograph with Split/Splitless inlet (Agilent; USA). Column effluent was analysed using a Pegasus HT time-of-flight mass spectrometer (LECO; UK), acquiring 10 spectra/sec over the mass range of 45–800 Da.

This study was performed in a series of single-batch experiments, wherein each specific brain region constituted a batch. Within each batch, individual HD cases and controls were randomised, and run in a sequence interleaved with injections of the pooled QC samples and extraction blanks. Extraction blanks were inspected visually to confirm absence of carryover.

2.6. Data analysis

Data were prepared using the 'Reference Compare' method within ChromaTOF 4.5 (LECO). Databases we employed were: the NIST Mass Spectral Reference Library (NIST08/2008; National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health Spectral 262 Library; NIST, Gaithersburg, MD, USA); and the Golm Metabolome Database (Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany). Chromatographic retention-time data for urea were available from our library reference standards. Matching of both mass spectra/expected retention time and peak-shape integration were manually verified by two independent investigators (SP, PB) to constitute a definitive molecular identification.

2.7. Urea measurements by GC-MS

For urea, we performed an additional identification by running a

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