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SIRT1 is increased in affected brain regions and hypothalamic metabolic pathways are altered in Huntington disease

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Abstract

Aims: Metabolic dysfunction is involved in modulating the disease process in Huntington disease (HD) but the underlying mechanisms are not known. The aim of the present study was to investigate if the metabolic regulators sirtuins are affected in HD.

Methods: Quantitative real time polymerase chain reactions were used to assess levels of *SIRT1-3* and downstream targets in postmortem brain tissue from HD patients and control cases as well as after selective hypothalamic expression of mutant huntingtin using recombinant adeno-associated viral vectors in mice.

Results: We show that mRNA levels of the metabolic regulator SIRT1 are increased in the striatum and the cerebral cortex but not in the less affected cerebellum in postmortem HD brains. Levels of *SIRT2* are only increased in the striatum and *SIRT3* is not affected in HD. Interestingly, mRNA levels of *SIRT1* are selectively increased in the lateral hypothalamic area (LHA) and ventromedial hypothalamus (VMH) in HD. Further analyses of the LHA and VMH confirmed pathological changes in these regions including effects on SIRT1 downstream targets and reduced mRNA levels of orexin (hypocretin), prodynorphin and melanin-concentrating hormone (*MCH*) in the LHA and of brain-derived neurotrophic factor (*BDNF*) in the VMH. Analyses after selective hypothalamic expression of mutant huntingtin suggest that effects on BDNF, orexin, dynorphin and MCH are early and direct, whereas changes of SIRT1 require more widespread expression of mutant huntingtin.

Conclusions: We show that SIRT1 expression is increased in HD affected brain regions and that metabolic pathways are altered in the HD hypothalamus.

Abbreviations

aa	amino acids
BDNF	brain-derived neurotrophic factor
BMI	body mass index
C	control

CART	cocaine and amphetamine regulated transcript
CREB-1	cAMP responsive-element binding-1
D2R	dopamine receptor D2
DYN	dynorphin
FOXO3	Forkhead box O3
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HD	Huntington disease
HTT	huntingtin
LHA	lateral hypothalamic area
MCH	melanin-concentrating hormone
MRI	magnetic resonance imaging
NAD+	nicotinamide adenine dinucleotide
OX2R	Orexin receptor 2
PET	positron emission tomography
PolyQ	polyglutamine
PVN	paraventricular nucleus
qRT-PCR	quantitative real time polymerase chain reaction
rAAV	recombinant adeno-associated viral
RIN	RNA integrity number
Sir2	silent information regulator 2
SIRT	sirtuin
SON	supraoptic nucleus
TH	tyrosine hydroxylase
TRH	thyrotropin-releasing hormone
VGAT	vesicular GABA transporter
vGLUT2	vesicular glutamate transporter
VMH	ventromedial hypothalamus

Introduction

Huntington disease (HD) is a fatal hereditary neurodegenerative disorder caused by an expanded CAG repeat in the *HTT* gene that expresses the huntingtin (HTT) protein [1]. It is clinically diagnosed when a person manifests with typical motor symptoms in combination with a positive gene test, which usually occurs in midlife [2], but

circadian rhythm, sleep and other hypothalamic disturbances are observed as early as 15 years before clinical diagnosis [3]. HD continues to progress leading to premature death around 20-25 years after motor onset. Weight loss is present in advanced stages and interestingly, it has been shown that individuals with a higher body mass index (BMI) at clinical diagnosis have a slower disease progression [4]. As there are no disease-modifying treatments available, understanding the underlying mechanisms of the metabolic alterations in HD could reveal effective targets for therapeutic interventions to slow the disease course.

In HD, the neuropathology in the striatum and the cerebral cortex has been extensively studied and well documented [5-9], and a number of reports now also showing significant hypothalamic pathology [10-13]. In one report, hypothalamic degeneration occurred prior to striatal and cortical degeneration [13], as may be expected based on preclinical features (see above). The nutrient sensors sirtuins are the mammalian orthologues of the evolutionary conserved Sir2 (silent information regulator 2) family which maintain whole-body energy metabolism. They are nicotinamide adenine dinucleotide (NAD+) –dependent protein deacetylases and all seven mammalian sirtuins (SIRT1-7) function through transcriptional regulation [14]. Transcriptional dysregulation of key factors involved in cellular homeostasis is thought to be important in the pathogenesis of HD [15]. Importantly, SIRT1 has been shown to regulate metabolism and longevity through hypothalamic circuitries [16-18] and also regulates the maturation of hypothalamic peptide hormones controlling energy balance and their processing enzymes [19]. Sirtuins, in particular SIRT1, SIRT2 and SIRT3, have been suggested to play a role in HD but the published results are conflicting and the effects on expression levels in HD patients are not well known [20-29]. Interestingly, it has been shown that treatment with SIRT1 inhibitors is neuroprotective in Drosophila, mammalian cells and mouse models of HD [30]. Also, treatment with the SIRT1 inhibitor selisistat for 14 days was shown to be safe and tolerable in a first clinical trial in early HD patients with the objective to identify pharmacodynamic markers to further develop the drug [31]. A more comprehensive evaluation of these sirtuins in HD is warranted, particularly as SIRT1 is also known to maintain neuronal genomic stability [32], and genes involved in DNA repair are known to modify the clinical course of HD [33, 34]. We therefore examined mRNA levels of *SIRT1*, *SIRT2* and *SIRT3* in relevant brain regions from postmortem tissue from HD patients (with dopamine receptor D2 (*D2R*) and brain derived neurotrophic

factor (*BDNF*) as known HD-affected transcripts [35-38]). We also examined downstream targets of SIRT1 and relevant hypothalamic peptide hormones. Based on the data obtained, targeted injections of recombinant adeno-associated viral (AAV) vectors expressing mutant huntingtin into the brains of mice was used to identify the earliest effects on affected pathways. The data show that SIRT1 pathways are affected in HD with relevance for hypothalamic metabolic pathways.

Materials and methods

Post mortem human tissue

Tissue from the striatum, the cerebral cortex and the cerebellum from 17 HD cases and 10 control cases were collected in blocks and were frozen on dry ice upon autopsy and stored at -80^{o}C for quantitative real time polymerase chain reactions (qRT-PCR) analysis. Tissues from the entire hypothalamus from 4 HD cases and 4 control cases were frozen on dry ice upon autopsy and stored at -80^{o}C for qRT-PCR analysis. Demographic data is shown in Table 1. Formalin fixed coronal sections from 4 HD and 3 control cases from a previously described cohort of postmortem hypothalamic tissue were used for immunohistochemistry [12, 13]. Demographic data is shown in Supplemental Table 1. The human postmortem tissue (see exception below) was obtained from Victorian Brain Bank Network and the Sydney Brain Bank at Neuroscience Research, Australia, after approval of the project by their Scientific Review Committee (PID167). All persons had given their informed consent prior to the donation of their brains and the brain donor programs were approved by Institutional Human Research Ethics Committees. The human postmortem tissue of the HD case with Vonsattel grade 0 was obtained from the Department of Neuropathology in Lund, Sweden. The analysis was approved by the region ethical review board at Lund University (reference number 2014/466) and written informed consent was obtained by the patient's relative before analysis of the tissue.

Animals

Female mice of the FVB/N strain (The Jackson Laboratories, Bar Harbor, MA, USA) were stereotactically injected at 2 months of age into the hypothalamus with recombinant AAV vectors expressing either a mutant or a wild-type HTT variant. The

viral vectors have been described in detail previously [39, 40]. Briefly, we used pseudotyped rAAV2/5 vectors, where the HTT gene fragment was flanked by inverted terminal repeats of the AAV2 packaged in an AAV5 capsid (referred to as rAAV5 elsewhere). The human HTT gene expressed the first 853 amino acids (aa) (obtained from Dr Nicole Deglon; [41]) with either a disease-causing polyglutamine length (79Q) or a normal variant (18Q) and was expressed under the neuronal specific human Synapsin-1 promoter. Mice were bilaterally injected in the hypothalamus with 0.5 µl/side of rAAV5-HTT853-79Q or rAAV5-HTT853-18Q under 2% isoflurane in oxygen/nitrous oxide (3:7) anaesthesia. The stereotaxic coordinates for the hypothalamic region were 0.6 mm posterior and 0.6 mm lateral to the bregma (Franklin and Paxinos brain atlas). A pulled glass capillary (outer tip diameter ≈ 80 µm) attached to a 5 µl Hamilton syringe (Nevada, USA) was used to inject 0.5 µl of viral vectors at the depth of 5.2 mm ventral to the dura mater. The viral vectors were administered with 0.05 µl injections in 15 s intervals, subsequently to an initial injection of 0.1 µl of viral vector solution. After the injection, the glass capillary was left in target for additional 5 minutes to allow absorbance of the virus by the tissue. The vector concentrations were 2.1 x 10^{14} genome copies (GC)/ml for rAAV5-HTT853-79Q and 1.6 x 10^{14} GC/ml for rAAV5-HTT853-18Q. The animals were kept at 12 hours night and day cycle with free access to water and normal chow diet.

The mice were killed either 6 or 18 weeks post-injection using cervical dislocation (n = 7-9/group and time-point). The experimental procedures performed on mice were carried out in accordance with the ethical permit approved by the Lund University Animal Welfare and Ethics committee in the Lund-Malmö region (ethical permit numbers M20-11 and M65-13).

Dissection of hypothalamic nuclei

Prior to all tissue handling, all equipment and tools were cleaned with RNaseZap (Ambion). Frozen human hypothalamic tissue and whole fresh frozen mouse brains were positioned in the frozen state in a coronal position using OCT Cryomount (Histolab), and serially sectioned from a rostral to caudal direction at -15^{o}C on a Cryostat (Microm HM 560). The tissue was cut at a thickness of 100 µm (human) or 200 µm (mouse) and collected on pre-cooled glass slides and stored at -20^{o}C over night before being processed for micro dissection. The anatomical positions of the

lateral hypothalamic area (LHA), the ventromedial hypothalamus (VMH), the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) were identified in accordance with the relevant brain atlas (the Allen Mouse Brain Atlas: http://mouse.brain-map.org/; and the human brain atlas of Mai et al. 2008 [42]). To assist with this identification in the human tissue, a 20 μm section was collected every 1 mm, processed for Nissl stain (0.5% cresyl violet or CV, ICN Biomedicals Inc, stabilized with 10% acetic acid), scanned using a light microscope, and printed out on A4 paper for orientation purposes. The tissue from these nuclei was collected with either a 1 mm (mouse) or 2 or 3 mm (human) disposable biopsy punch (Miltex) and stored in RNA-free tubes that were immediately frozen on dry ice and kept in -80^{o}C until processed for RNA isolation.

qRT-PCR analysis

Total RNA was isolated from all tissue samples using RNeasy Lipid Tissue Kit (Qiagen) with an on-column DNase digestion (RNase-free DNase set, Qiagen) according to supplier's recommendations. RNA quantity was measured on a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA integrity number (RIN), an indicator for appropriate preservation of RNA integrity, was used to assess the RNA quality of the human postmortem tissue [43-45]. RNA samples were analysed by SCIBLU Genomics, Affymetrix unit at Lund University using Agilent 2100 Bioanalyze and RNA integrity was determined for all samples before proceeding with the analyses (Tables 1 and 2).

cDNA was generated using random hexamer primers and SuperScript IV Reverse Transcriptase (Invitrogen) according to supplier's recommendations. qRT-PCR reaction was performed on a LightCycler 481 in a two-step protocol using SYBR Green I Master mix (Roche). The specificity of the amplification was determined by melting curve analysis. Data were quantified using the ΔΔCT-method and were normalized to the expression of the two housekeeping genes β-actin and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). All primers were designed with Beacon DesignerTM (Premier Biosite). All values are presented as ratios to the mean of the control group. The primer sequences used for the gene expressions analyses are found in Supplementary Tables 2 and 3.

Immunohistochemistry

One coronal section cut at 50 μm at the level of bregma 5.4-6.7 mm from 4 HD and 3 control cases was processed for immunohistochemistry with an antibody against melanin concentrating hormone (MCH: MCH antibody made in rabbit, used at a concentration of 1:20 000, H-070-47, Phoenix Pharmaceuticals, Burlingame, CA, USA) using a previously described protocol [13](Supplemental Table 1). The total number of MCH neurons as well as their cross-sectional areas was then estimated in a well-defined area of the LHA from one section of 4 HD cases and 3 controls using stereological principles as previously described [12] (Supplemental Table 1). One coronal section of the hypothalamus cut at 50 μm from 3 HD and 3 control cases was processed for immunohistochemistry with an antibody against SIRT1 (antibody made in rabbit, used at a concentration of 1: 200, Sigma Aldrich)(Supplemental Table 1). Sections were counterstained with CV as previously described [12]. One coronally cut series of sections (section thickness 30 μm, cut in 6 series) from a previously described cohort of AAV-vector injected mice was processed for MCH immunohistochemistry according to a previously described immunohistochemical protocol (MCH antibody made in rabbit, used at a concentration of 1: 4000, Phoenix Pharmaceuticals; [39]). The total number of MCH neurons in the mouse hypothalamus was assessed using stereology as previously described [39].

Statistical analysis

All data are presented as mean ± SEM. Parametric tests were used for normally distributed data and non-parametric tests were used if the data was not normally distributed. For the human samples, the non-parametric Mann-Whitney test was performed for the hypothalamic samples and the parametric Student *t* test was used for the striatal, cortical and cerebellar samples, with significant statistical difference considered at $p < 0.05$. For the mouse samples, either one-way ANOVA or Kruskal Wallis test were performed, followed by Tukey's or Dunn's multiple comparisons tests for post-hoc analyses as appropriate.

Results

Increased *SIRT1* expression in all HD-affected brain regions

Before assessing hypothalamic regions important for metabolic control, we assessed the mRNA levels of the sirtuins *SIRT1*, *SIRT2* and *SIRT3* in the HD-affected striatum

and cortex compared to the less affected cerebellum in the postmortem human tissue from 17 HD patients and 10 age and sex-matched control cases. *D2R* and *BDNF* levels were also assessed as internal controls (known to be significantly reduced in HD affected areas, [35-38]). qRT-PCR analyses showed significant increases of *SIRT1* mRNA levels in the striatum and the cerebral cortex in HD compared to control cases but no changes in the cerebellum (Figure 1a). *SIRT2* mRNA levels were significantly **increased** in the striatum in HD cases but not in the other two brain regions compared to controls (Figure 1b). mRNA levels of *SIRT3* were not differentially expressed in the striatum, the cerebral cortex or the cerebellum in HD cases compared to control cases (Figure 1c). Confirmation of reduced *D2R* (Figure 1d) and *BDNF* (Figure 1e) in HD compared to controls gives confidence in the changes observed in *SIRT1*, *2* and *3*.

Increased *SIRT1* expression in hypothalamic regions important for metabolic control in HD

mRNA levels of *SIRT1*, *SIRT2* and *SIRT3* were examined in postmortem tissue from four hypothalamic nuclei from four HD cases and four controls. qRT-PCR analyses revealed a significant increase in the mRNA levels of *SIRT1* in both the LHA and the VMH of HD cases compared to controls (Figure 2a). There was no difference in the levels of *SIRT1* between HD and control cases in the PVN and SON (Figure 2a). We found no differences in the expression levels of *SIRT2* or *SIRT3* between HD cases and controls in any of the examined hypothalamic nuclei (Figure 2b, 2c). Hence, *SIRT1* is selectively increased in the LHA and VMH in HD patients. We also processed hypothalamic tissue from 3 HD cases and 3 control cases for immunohistochemistry against SIRT1. The number of cells expressing SIRT1 appeared more and the staining was more intense in the LHA and VMH of HD cases compared to control cases (Figure 4g). Interestingly, one of the HD cases had a Vonsattel grade 0 and had not yet manifested with overt motor symptoms [13]. Similarly, the number of cells and the staining intensity after immunohistochemical processing for SIRT1 were increased in the HD case of grade 0 compared to control cases, opening up for the possibility that increased SIRT1 may be an early event in the disease (Figure 4h).

Effects of increased *SIRT1* expression on SIRT1 downstream targets in the LHA and VMH in HD

We explored effects on mRNA levels of downstream targets of SIRT1 in the HD-affected hypothalamic regions. There was a significant increase of mRNA levels of Forkhead box O3 (*FOXO3*), an intracellular stress regulator, in both LHA and VMH in HD cases compared to controls ($p<0.05$ for both regions, Figure 2d). Furthermore, mRNA levels of *FOXO1* showed a trend towards increase in HD cases compared to controls (LHA and VMH: $p= 0.0571$, Figure 2e). Upregulation of *SIRT1* has been shown to regulate metabolism and longevity in mice through upregulation of Orexin 2 receptor (OX2R) via the transcription factor NK2 homeobox 1 (Nkx-2.1) specifically in LHA and VMH [18]. Here, on the contrary, we detect a reduction of *OX2R* (LH: $p= 0.0571$, VMH: $p<0.05$; Figure 2f) and *NKX2-1* (VMH: $p<0.05$; Figure 2g) in HD, indicating a disruption of this pathway in HD. Finally, we show that mRNA levels of the gene for SIRT1 regulated transcription factor cAMP responsive-element binding (*CREB*)-1 are increased in the VMH in HD cases compared to controls (Figure 2h). Taken together, there are selective effects on downstream targets of SIRT1 in the LHA and VMH in HD postmortem tissue.

Reduced expression of dopamine receptors D2 selectively in the LHA in HD

Imaging studies using PET have previously shown reductions in D2R in the whole hypothalamus in prodromal HD [46], but it is not known which hypothalamic nuclei are affected. Here we show that mRNA levels of *D2R* were significantly reduced in the LHA of HD cases compared to controls, whereas there were no significant differences between the two groups in the VMH, the PVN or the SON (Figure 3). Hence, mRNA levels of *D2R* are altered selectively in the LHA in HD.

Pathological changes in the LHA in postmortem HD tissue

Further analyses of the LHA revealed significant reductions in mRNA levels of orexin as well as prodynorphin, which is usually co-expressed with orexin, in HD cases compared to controls (Figure 4a). mRNA levels of genes encoding for other neuropeptides also expressed in the LHA such as cocaine and amphetamine regulated transcript (CART), enkephalin, thyrotropin-releasing hormone (TRH), neurotensin and nociceptin or for markers of glutamatergic and GABAergic cells were not affected (Figure 5b and 5c). mRNA levels of tyrosine hydroxylase (*TH*) showed a

strong trend towards a statistically significant reduction in HD compared to controls (Figure 4b). Interestingly, we found a significant reduction in mRNA levels of melanin-concentrating hormone (*MCH*) in HD cases compared to control (Figure 4c). We then analysed hypothalamic tissue from HD cases and controls immunoprocessed for MCH (Figure 4e, f, g). Assessments of the number and cross-sectional areas of MCH-immunopositive cells in a well-defined area of the LHA from one section of 4 HD cases (166 ± 18 cells, 331± 37 μm^2) and 3 controls (136 ± 19 cells, 411 ± 28 μm^2) showed no significant difference between the groups ($p > 0.05$ using Mann-Whitney test). Hence, the reduction in mRNA levels of *MCH* is not due to a loss of MCH immunopositive neurons in HD.

Effects of direct expression of mutant HTT in the hypothalamus of mice using rAAV5-vectors

We investigated whether the changes found in the clinical material can be caused by the expression of mutant HTT in the hypothalamus. We therefore injected a set of mice with rAAV5 vectors expressing either an 853 aa HTT fragment with 79Q (corresponding to a disease-causing polyQ length) or with 18Q (corresponding to a normal range polyQ length) selectively in the hypothalamus and analysed mRNA levels of the altered genes at 6 and 18 weeks post-injection in dissected tissue from either the LHA or the VMH. qRT-PCR analysis confirmed similar overexpression levels of the 79Q and 18Q fragment at both 6 and 18 weeks post-injection in the LHA (79Q at 6 weeks: 777±37 folds relative to mean of uninjected; 18Q at 6 weeks 680±82; 79Q at 18 weeks 886±130; 18Q at 18 weeks 665±82) and in the VMH 79Q at 6 weeks: 264±27; 18Q at 6 weeks 229±30; 79Q at 18 weeks 485±112; 18Q at 18 weeks 370±53).

We show that expression of mutant HTT leads to a reduction of mRNA levels of D2r in the LHA compared to uninjected mice already at 6 weeks and compared to mice with overexpression of the 18Q HTT fragment at 18 weeks post-injection (Figure 5a). We further show a reduction of orexin and prodynorphin mRNA levels in the 79Q group compared to both the 18Q and the control group as well as in the 18Q group compared to the uninjected control group (Figure 5b, c). This is in line with our previously reported results in this model [39, 40]. There was also a significant reduction in mRNA levels of TH in the 79Q group (Figure 5d). In contrast, we found no differences in the mRNA levels of *Sirt1* in the LHA of mice injected with rAAV5

vectors into the hypothalamus at 6 weeks post injection and a difference only between 79Q and controls at 18 weeks (Figure 5e). These results suggest that reductions of D2R, orexin, prodynorphin and TH could be a direct, early effect of mutant HTT expression in the hypothalamus. However, an increase of SIRT1 levels appears to require more widespread HTT expression or might be a later phenomenon that was not developed within the time frame studied in animals. It is also possible that the weight gain known to occur in mice after hypothalamic expression of mutant HTT, and similar to transgenic mice expressing full length mutant HTT, may mask effects on SIRT1 [39]. Furthermore, the larger expansion of the polyglutamine tract in HD mice compared to the common disease range of 40-55 polyglutamines in HD patients may also be involved in the discrepancies between the SIRT1 findings in clinical and experimental tissues.

Interestingly, we also found a reduction in the mRNA levels of *Mch* in both the 79Q and the 18Q group compared to the uninjected control group already at 6 weeks post-injection (Figure 5f). This persisted up to the 18 weeks examined (Figure 5f). When assessing the number of MCH immunopositive neurons at 18 weeks post-injection in another set of mice injected with the same vectors, we found a reduction in MCH immunopositive cells in the 79Q group compared to uninjected controls but not compared to the 18 Q group (Figure 5g and h). Hence, overexpression of both mutant and wild-type HTT fragments in the hypothalamus rapidly leads to downregulation of mRNA levels of *Mch* in mice. Hence, it is possible that wild-type HTT normally exerts effects on neuronal activity in the hypothalamus and that overexpression of also the wild-type form of the protein alters hypothalamic functions. However, it does not appear to be a general effect as many mRNA levels affected by mutant HTT are not affected by overexpression of the wild-type form.

Reduced *BDNF* in the VMH

BDNF is expressed in the LHA, VMH and PVN in the hypothalamus where it has been implicated in the regulation of energy homeostasis [47]. In the VMH, we found a trend for a reduction in mRNA levels of *BDNF* in the HD cases compared to controls (Figure 6a; $p = 0.0571$), but no differences in the LHA or the PVN. Interestingly, reduction in mRNA levels of *BDNF* in the VMH may be a direct effect of mutant HTT as there was a significant reduction in mRNA levels of *Bdnf* in the VMH of the 79Q group compared to both the 18Q group and uninjected controls at 18

weeks post-injection in mice (Figure 6b). Similar to the LHA in mice, we found no differences in the mRNA levels of *Sirt1* in the VMH of mice injected with rAAV vectors into the hypothalamus up to 18 weeks post injection (data not shown).

Discussion

The present study has shown that mRNA levels of *SIRT1* are selectively increased in the striatum and the cerebral cortex in HD, and also in hypothalamic regions important for metabolic regulation (the LHA and VMH; main results summarized in Figure 7). In contrast, *SIRT2* levels are increased only in the striatum, and *SIRT3* levels remain unchanged in the HD brain. In the hypothalamic regions affected, we confirm that downstream targets of SIRT1 and relevant hypothalamic peptide hormones are affected in patients with HD. Using animal modelling with viral vectors of mutant HTT injected into the hypothalamus we show that the earliest local effects are on hypothalamic hormone levels and not *Sirt1*, indicating that more widespread expression of mutant HTT impacts on hypothalamic SIRT1 in HD.

The importance of assessing sirtuins in HD is that they are key regulators of metabolism through hypothalamic circuits [14, 48], as the hypothalamus is one of the early focal regions of degeneration in HD [13]. There is evidence that greater metabolic dysfunction at clinical onset slows the progression of HD [4] and trial information suggesting SIRT1 inhibitors are safe [31]. Our novel data showing an increase of SIRT1 in HD-affected brain regions and specifically in the LHA and VMH in postmortem hypothalamic tissue from HD patients produces somewhat of a dilemma – to treat increased SIRT1 and potentially produce metabolic dysfunction in HD? We also show that not all HD hypothalamic regions display increased *SIRT1* and our animal experiments suggest that the more widespread effect of mutant HTT in the brain is important for the regionally selective increase of SIRT1 in the hypothalamus. While overall this data gives evidence for the potential use of SIRT1 inhibitors in HD, knockdown of SIRT1 in unaffected brain regions may have unintended longer-term consequences and titrating therapy to maintain optimal metabolism may prove difficult. Regardless, our data indicate that an increase of SIRT1 may be a signature of selectively affected brain areas in HD that is likely to play a role in the metabolic symptoms found in HD.

In terms of therapeutically manipulating sirtuins in HD, there is controversy as to whether activation or inhibition of sirtuins would be beneficial [14, 49, 50]. This may not be surprising as this group of proteins exerts a wide range of different key functions in many organs of the body. For HD, animal studies have shown conflicting results regarding effects of SIRT1 modulation. Two studies have shown that normalizing reduced SIRT1 levels in HD mice is beneficial [26, 27] and others studies demonstrated the beneficial effects of decreasing already increased SIRT1 [30, 51]. Furthermore, inhibition of SIRT1 in transgenic Drosophila and R6/2 mice resulted in a selective reduction in the levels of mutant HTT protein [51]. Interestingly, mice that have been experimentally engineered to overexpress SIRT1 in the striatum develop depression and anxiety-like behaviours as well as impaired motor function [52, 53]. Taken together, the studies assessing increased SIRT1 are in line with the evidence we have obtained from human brain tissue. As discussed, one safety clinical trial in HD patients using an agent that inhibits SIRT1 activity has been completed with supportive results [31]. Combined with our new data, we suggest that the idea of normalising increased SIRT1 levels is HD patients has some merit, although one cannot exclude the possibility that increased levels of SIRT1 represent a compensatory and protective phenomenon. This later scenario seems less likely as the beneficial effects of increasing SIRT1 are not present in HD –these include improved insulin sensitivity (not different in HD; [31, 54]), increased mitochondrial number and function (decreased number and function in HD; [55, 56]) and enhanced survival.

The assessment of a potential metabolic role for sirtuins in the HD hypothalamus showed specificity for SIRT1 increases in the relevant metabolic pathways in the LHA and VMH, as levels of *SIRT2* and *SIRT3* were not affected. SIRT1 regulates metabolism as well as aging and longevity in these hypothalamic nuclei [16-18]. To assess whether increased levels of SIRT1 would be associated to effects on related targets, we confirmed that several downstream factors of SIRT1 are also altered on the mRNA level in HD. FOXO transcription factors such as FOXO1 and FOXO3 act as cellular sensors to survival signals and are increased in response to intracellular stress. mRNA levels of *FOXO3* have previously been found increased in the striatum of HD patients and mice [57, 58] and a few studies have suggested a neuroprotective role for FOXO3 in HD [59-61]. Here we found that mRNA levels of *FOXO3* were increased in both the LHA and the VMH in HD cases. In contrast to SIRT1 overexpressing mice [18], we detected a reduction rather than an increase of

orexin receptor type 2 (OX2R) in the LHA and the VMH in HD, but this could be due to the reduction of *NKX2-1* in the VMH in HD, as Nkx-2.1 is a critical transcription factor for the SIRT1 mediated upregulation of OX2R [18]. Furthermore, SIRT1 regulates CREB-1 mediated transcription [62], which has recently been shown to be increased in adipose tissue from HD patients [63]. Here we show increased levels of *CREB-1* also in the VMH in HD. Taken together, the findings in the present study support the idea of SIRT1 involvement in HD and demonstrate that SIRT1 pathways are affected in two metabolically relevant areas in the hypothalamus.

Previous neuropathological analyses of postmortem hypothalamic tissue from HD patients have revealed loss of somatostatin neurons in the lateral tuberal nucleus, orexin (hypocretin) in the LHA, as well as of oxytocin and vasopressin neurons in the paraventricular nucleus (PVN) in HD compared to controls [10-13, 64-66]. In prodromal HD patients, imaging studies using magnetic resonance imaging (MRI) and positron emission tomography (PET) have shown that the hypothalamus appears affected before motor onset and that the pathological changes include loss of D2R [46, 67, 68]. Importantly, HD patients suffer from a range of non-motor features that represent disturbed functions typically regulated by the hypothalamus [3, 69]. These include psychiatric symptoms such as irritability, aggression, apathy and depression, sleep problems, changes in circadian rhythm and body weight changes. Specific alterations in hypothalamic circuitries as indicated by the present results may play a role in the development of these non-motor features in HD. Experimental studies using rAAV vectors to either express or inactivate mutant HTT in the hypothalamus in mice have provided support for a causative link between hypothalamic dysfunction and non-motor phenotypes such as metabolic dysfunction and depressive-like behaviour [39, 70]. Further studies are now needed to determine the impact of the present findings with regards to the development of non-motor features including any classic metabolic alterations in HD.

SIRT1 influences a number of neuropeptide systems in the hypothalamus to impact on metabolism. SIRT1 upregulates the expression of OX2R specifically in the LHA and the VMH [17, 18], whereas the expression of orexin and MCH is reduced in the hypothalamus [71]. SIRT1 also inhibits the active state of orexin neurons [72]. Here we show a reduction of mRNA levels of orexin and *MCH* in HD cases. Previous neuropathological studies [10-12], including our present immunohistochemical analyses of the human hypothalamus, have detected around 30% loss of the lateral

hypothalamic neuropeptide orexin, whereas the number of neurons expressing MCH is preserved. This indicates that the qRT-PCR results represent a smaller transcriptional dysregulation in *MCH* expression in hypothalamic neurons compared with the expression of either the transcript of or the actual peptide orexin. SIRT1 has previously been shown to regulate BDNF transcription through CREB-11 [26]. Despite increased levels of SIRT1 and *CREB1* in the VMH, here we found reduced expression of mRNA levels of *BDNF* specifically in this area in HD. Diminished BDNF signalling in mice results in severe hyperphagia and obesity, and BDNF haploinsufficiency and certain polymorphisms are linked to elevated food intake and body weight in humans [73-75], suggesting that this hypothalamic change in HD should have the opposing effects to those observed. We found that the expression of mutant HTT in the hypothalamus rapidly lead to reduced expression of *Bdnf* in the VMH, indicating an early event in the HD disease process, but one unlikely to impact on metabolism as observed clinically and in animal models of HD. BDNF has been implicated in the regulation of aggression with heterozygous BDNF knock-out mice showing aggressiveness and hyperphagia [76]. It is therefore more likely that the reduced BDNF in HD is related to the development of the non-motor features of irritability and aggression rather than metabolism *per se*.

Dopamine plays an important role in the LHA. Early studies have shown that local injections of the D2R antagonist sulpiride directly into the LHA can induce locomotion, feeding and drinking as well as reward and dopamine release into the nucleus accumbens [77, 78]. Later studies have shown that dopamine inhibits activity of orexin neurons in a D2R–dependent fashion [79]. In the present study, we show that mRNA levels of *D2R* are selectively reduced in the LHA in HD cases compared to controls. It is possible that a reduction of D2R occurs on the orexin neurons that are affected in HD. Furthermore, the experiments performed in mice indicate that the reduction in mRNA levels of *D2r* is an early and direct effect of the expression of mutant HTT. This is also in line with previous PET studies indicating that a presymptomatic reduction in D2R in the hypothalamus in HD [46].

One limitation of the present study is the relatively low number of cases that were available for the analyses we carried out on the human postmortem hypothalamic tissue. The reason for this is that frozen entire hypothalami that can be used for anatomical visualization necessary for accurate dissection of specific nuclei are extremely rare in brain banks. Nevertheless, the present tissue appeared

representative of HD patients as previous findings of reduced orexin in neuropathological studies and reduced D2R in neuroimaging studies were reproduced**.** [10-12, 46]. Further analyses of a larger cohort of tissue, if and when available, would be important to validate the present findings. Furthermore, in the present study, we only report an association between increased levels of SIRT1 and altered levels of downstream factors regulating metabolism. Studies using clinical postmortem tissue are limited by the fact that a number of factors besides the expression of the mutant HTT gene can influence the results and causative relationships cannot be established. There could be a potential impact of differences in causes of death between HD cases and controls and the variation in RIN. Also, increased levels of mRNA may reflect an increased total production of a particular protein but may also represent the selective survival of certain cells expressing the particular factor. This has not been addressed in the present study. Furthermore, the focus of this study was on analysis of mRNA levels. Changes in mRNA levels do not always predict changes in protein expression. Previous studies using immunohistochemistry have shown reduced numbers of orexin- immunopositive neurons in HD, which is in line with the reduced mRNA of orexin reported here. Immunohistochemical analyses of SIRT1 are in line with the increased mRNA levels detected in HD cases. Other markers have not yet been validated on the protein level. Overall, the functional impact of increased levels of SIRT1 on molecular pathways in the hypothalamus as well as on systemic metabolism in HD needs to be studied to establish whether there is indeed a clinically relevant causative relationship.

In summary, this study demonstrates that *SIRT1* is increased at the mRNA level in HD-affected brain regions. Moreover, SIRT1 levels are also increased in the LHA and VMH of postmortem hypothalamic HD tissue, and importantly, downstream targets are also affected. Taken together, our study shows that the metabolic regulator SIRT1 is affected in relevant brain regions in HD and supports the concept that SIRT1 should be considered as a therapeutic target for disease-modifying treatments.

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Ethical approval

The human postmortem tissue was obtained from Victorian Brain Bank Network and the Sydney Brain Bank at Neuroscience Research, Australia, after approval of the project by their Scientific Review Committee (PID167). All persons had given their informed consent prior to the donation of their brains and the brain donor programs were approved by Institutional Human Research Ethics Committees. The human postmortem tissue of the HD case with Vonsattel grade 0 was obtained from the Department of Neuropathology in Lund, Sweden. The analysis was approved by the region ethical review board at Lund University (reference number 2014/466) and written informed consent was obtained by the patient's relative before analysis of the tissue. The experimental procedures performed on mice were carried out in accordance with the ethical permit approved by the Lund University Animal Welfare and Ethics committee in the Lund-Malmö region (ethical permit numbers M20-11 and M65-13).

Author contributions

ÅP, GH, DK, BB and SG conceived and designed the study. GH, CM and EE provided the clinical material. BB, SG, RSK, RYC and JBH performed the experiments and analyzed the data. ÅP, GH and DK interpreted the results. ÅP, GH, SG and BB wrote the manuscript. BB, SG and RSK prepared the figures. All authors were involved in editing the manuscript and approved the final version.

Disclosures

The Editors of Neuropathology and Applied Neurobiology are committed to peer-review integrity and upholding the highest standards of review. As such, this article was peer-reviewed by independent, anonymous expert referees and the authors had no role in either the editorial decision or the handling of the paper. Authors declare no conflicts of interest.

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Legends to Figures

Figure 1. Analyses of *SIRT1*, *SIRT2*, *SIRT3* and known disease-affected mRNA levels in Huntington disease (HD) compared to controls (C).

qRT-PCR analyses of mRNA levels of *SIRT1* (a), *SIRT2* (b), *SIRT3* (c), dopamine receptor D2 (d. *D2R*) and brain derived neurotrophic factor (e. *BDNF*) in the striatum

(Str), the cerebral cortex (Ctx) and cerebellum (Cb) from 17 HD cases and 10 controls. Data is expressed as mean ± SEM. * = p< 0.05, Student's t-test.

Figure 2. Analyses of mRNA levels of sirtuins and downstream targets in selected hypothalamic nuclei in Huntington disease (HD) compared to controls (C).
qRT-PCR analyses of mRNA levels of *SIRT1* (a), *SIRT2* (b) and *SIRT3* (c) in the lateral hypothalamic area (LHA), ventromedial hypothalamic area (VMH), paraventricular hypothalamic nucleus (PVN) and supraoptic hypothalamic nucleus (SON) of HD cases and controls (n = 4/group). qRT-PCR analyses of the genes encoding for FOXO transcription factors FOXO3 (d) and FOXO1 (e), orexin receptor type 2 (f. *OX2R*), the transcription factor homeobox Nkx-2.1 (g. *NKX2-1*) and cAMP responsive-element binding-1 (h. *CREB-1*) in the lateral (LHA) and ventromedial hypothalamic (VMH) areas of HD cases and controls (n = 4/group). Data is expressed as mean ± SEM. * = p< 0.05 using Mann-Whitney test.

Figure 3. Analyses of the general pathological marker of dopamine receptor D2 in affected hypothalamic nuclei in Huntington disease (HD) compared to controls (C).
qRT-PCR analyses of mRNA levels of the dopamine receptor D2 (*D2R*) in the lateral hypothalamic area (LHA), ventromedial hypothalamic area (VMH), paraventricular hypothalamic nucleus (PVN) and supraoptic hypothalamic nucleus (SON) of HD cases and controls (n = 4/group). Data is expressed as mean ± SEM. * = p< 0.05 using Mann-Whitney test.

Figure 4. Analyses of neuropeptide mRNA levels in the lateral hypothalamus (LHA) of Huntington disease patients (HD) compared to controls (C)
qRT-PCR analyses of mRNA levels of genes encoding for neuropeptides, enzymes and neuronal markers expressed in the LHA of HD cases and controls (n = 4/group): orexin and prodynorphin (*PDYN*) (a); cocaine and amphetamine regulated transcript (*CART*), enkephalin, tyrosine hydroxylase (*TH*), thyrotropin-releasing hormone (*TRH*), neurotensin, nociception (b); markers for GABAergic (*VGAT*) and glutamatergic neurons (*VGLUT2*) (c); melanin-concentrating hormone (d. *MCH*). Illustration of a coronal section of the hypothalamus where the dashed lines indicate the area in the right lower corner over the LHA where MCH immunopositive neurons

were counted, representing the area where the LHA punch for mRNA analyses was taken (e). Immunohistochemistry of MCH in postmortem tissue from a control and HD case (f). Scale bar = 100 μm. Immunohistochemistry of SIRT1 in postmortem hypothalamic tissue from a control and HD case (grade 2) with cresyl violet as a counterstain (g). Scale bar = 1 mm and 100 μm. Immunohistochemistry of SIRT1 in postmortem hypothalamic tissue from a control and an HD case of Vonsattel grade 0 (h). Scale bar = 100 μm. Dashed crosses indicate the location for the high magnification photomicrographs of the LHA and VMH respectively. Data is expressed as mean ± SEM. * = $p < 0.05$ using Mann-Whitney test.

Figure 5. Analyses of mRNA levels of *Sirt1*, neuropeptide and pathological markers in the lateral hypothalamus (LHA) in mice experimentally injected with AAV vectors to express mutant huntingtin (HTT) compared with control mice at 6 and 18 weeks.

qRT-PCR analyses of mRNA levels of genes encoding for different neurochemical factors at 6 and 18 weeks after delivery of AAV vectors expressing an 853 aa HTT fragment with either 79Q or 18Q in the LHA of mice: dopamine receptor D2 (a. *D2r*), orexin (b), prodynorphin (c), tyrosine hydroxylase (d. *Th*), *Sirt1* (e) and melanin-concentrating hormone (f. *Mch*). Stereological counts of MCH-immunopositive neurons in the LHA 18 weeks post-injection with representative photomicrographs of the area in un-injected mice and mice with either 18Q or 79Q (g, h). Data is expressed as mean ± SEM. Scale bar = 250 μm. * = $p < 0.05$, one factor ANOVA or Kruskal-Wallis test followed by Tukey's or Dunn's posthoc test.

Figure 6. Analyses of brain-derived neurotrophic factor (BDNF) in the ventromedial hypothalamus (VMH) of Huntington disease patients (HD) compared to controls (C) and in mice experimentally injected with AAV vectors to express mutant huntingtin (HTT) compared with control mice at 6 and 18 weeks

qRT-PCR analyses of mRNA levels of *BDNF* in the VMH, lateral hypothalamic area (LHA) and paraventricular hypothalamic nucleus (PVN) in HD cases and controls (a, n= 4/clinical group). qRT-PCR analyses of mRNA levels of *Bdnf* in the VMH at 6 and18 weeks post-delivery of AAV vectors expressing an 853 aa HTT fragment with

79Q compared to 18Q in the hypothalamus of mice (b, n= 7-9/experimental group). Data is expressed as mean ± SEM. * = p< 0.05 using Mann-Whitney test for the human samples and one factor ANOVA or Kruskal-Wallis test followed by Tukey's or Dunn's posthoc test for the mouse samples.

Figure 7. An overview showing increased *SIRT1* in HD affected brain regions and alterations of metabolic pathways in the lateral (LHA) and ventromedial (VMH) hypothalamus in Huntington disease.

Abbreviations: Brain-derived neurotrophic factor (*BDNF*); cAMP responsive-element binding-1 (*CREB-1*); dopamine receptor D2 (*D2R*); melanin concentrating hormone (*MCH*); orexin receptor type 2 (*OX2R*); prodynorphin (*PDYN*).

Table 1. Demographic data for HD and control cases

A. Tissue for cortical, striatal and cerebellar analyses

Case	Age/sex	CAG	DD	Cause of death	PMD	Brain	Grade	RIN: CTX	RIN: STR	RIN: CB
HD1	68/m	44	13	pneumonia	10	1184	3	7,5	6,8	7,4
HD2	69/f	42	20	cardiorespiratory failure	2	1149	2	7,0	4,8	7,7
HD3	57/f	44	22	pneumonia	22	800	4	4,8	5,9	6,4
HD4	61/m	43	17	HD endstage	17	1280	4	6,1	5,5	7,4
HD5	71/m	42	12	myocardial infarct	41	1270	2	7,0	3,6	6,9
HD6	39/m	54	13	cardiorespiratory failure	10	1047	4	7,4	5,7	6,2
HD7	39/f	46	11	HD endstage	36	680	3	8,0	7,9	8,1
HD8	58/m	46	11	HD endstage	32	1260	3	7,9	6,4	7,9
HD9	61/m	45	14	sepsis	40	1500	4	7,6	3,6	8,2
HD10	62/m	43	12	HD endstage	22	1185	3	7,8	3,1	8,8
HD11	62/m	43	10	pneumonia	24	1380	3	5,8	4,5	6,7
HD12	67/m	43	15	HD endstage	37	1200	2	6,9	4,6	8,1
HD13	67/m	45	15	pneumonia	19	952	n.d.	6,6	3,0	3,7
HD14	71/m	40	10	pneumonia	39	1150	3	4,2	3,2	6,1
HD15	72/m	43	33	pneumonia	22	940	2	5,0	3,4	7,2
HD16	74/m	40	12	cardiorespiratory failure	27	1475	2	6,5	6,0	6,4
HD17	77/m	41	20	pneumonia	9	1018	4	7,6	7,0	8,1

mean ± SD	**63 ± 11 m: 14 f: 3**		**15 ± 6**		**24 ± 12**	**1114 ± 219**		**6,7 ± 1,2**	**5,0 ± 1,5**	**7,1 ± 1,2**

Case	Age/sex	CAG	DD	Cause of death	PMD	Brain	Grade	RIN CTX	RIN CN	RIN CB
C1	69/m			pulmonary embolism	24	1290		6,9	6,7	6,8
C2	67/f			pulmonary embolism	26	1298		7,5	6,1	7,1
C3	57/m			ischemic heart disease	48	1532		6,6	7,2	7,5
C4	73/m			ischemic heart disease	43			6,4	6,8	7,3
C5	64/m			ischemic heart disease	32	1335		6,8	2,5	7,3
C6	64/m			ischemic heart disease	24	1492		7,5	7,7	7,4
C7	66/f			metastatic carcinoma	43	1233		6,8	6,5	6,7
C8	69/m			ischemic heart disease	34	1240		6,6	6,5	7,7
C9	76/m			aortic aneurysm	46	1459		7,8	7,6	7,7
C10	78/m			ischemic heart disease	46	1471		6,9	6,5	7,5
mean ± SD	**68 ± 6 m: 8 f: 2**				**37 ± 10**	**1372 ± 116**		**7,0 ± 0,5**	**6,4 ± 1,5**	**7,3 ± 0,3**

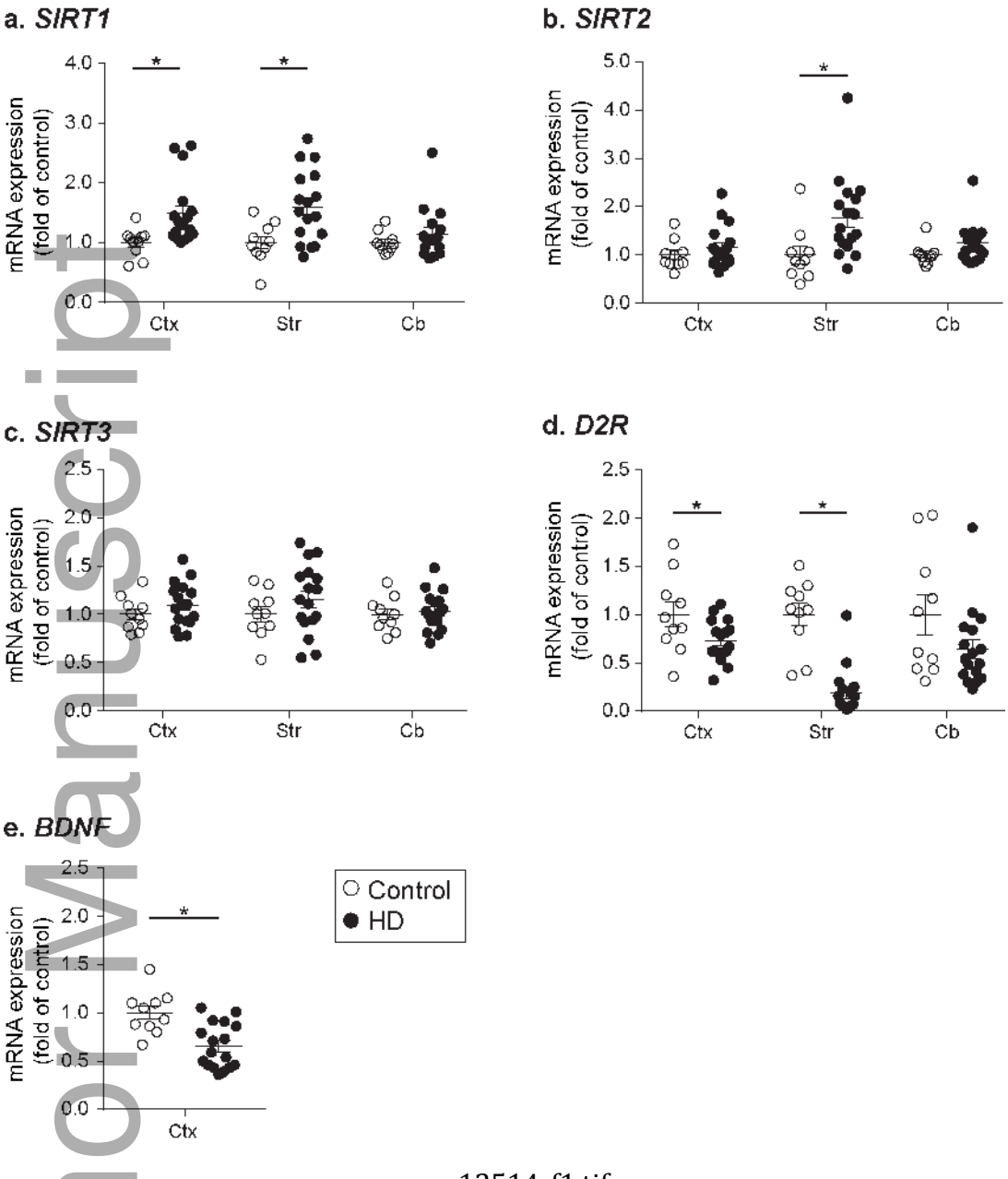
B. Tissue for hypothalamic analyses

Case	Age/sex	CAG	DD	Cause of death	PMD	Brain	Grade	RIN: HYP

HD1	68/m	44	13	pneumonia	10	1184	3	8,4
HD2	69/f	42	20	cardiorespiratory failure	2	1149	2	8,2
HD3	57/f	44	22	pneumonia	22	800	4	6,9
HD4	61/m	43	17	HD endstage	17	1280	4	7,9
mean ± SD	**64 ± 6 m: 2 f: 2**		**18 ± 4**		**13 ± 9**	**1103 ± 210**		**7,9 ± 0,7**

Case	Age/sex	CAG	DD	Cause of death	PMD	Brain	Grade	RIN HYP
C1	69/m			pulmonary embolism	24	1290		7,2
C2	67/f			pulmonary embolism	26	1298		7,4
C3	57/m			ischemic heart disease	48	1532		7,2
C4	56/f			respiratory failure	23	1360		7,2
mean ± SD	**62 ± 7 m: 2 f: 2**				**30 ± 12**	**1370 ± 112**		**7,3 ± 0,1**

Age is indicated in years. PMD (postmortem delay) is indicated in h. DD (disease duration) is indicated in years. Brain (total brain weight) is indicated in g. Grade refers to Vonsattel grade for neuropathological classification of HD [5]. RIN (RNA integrity number). HYP (hypothalamus).



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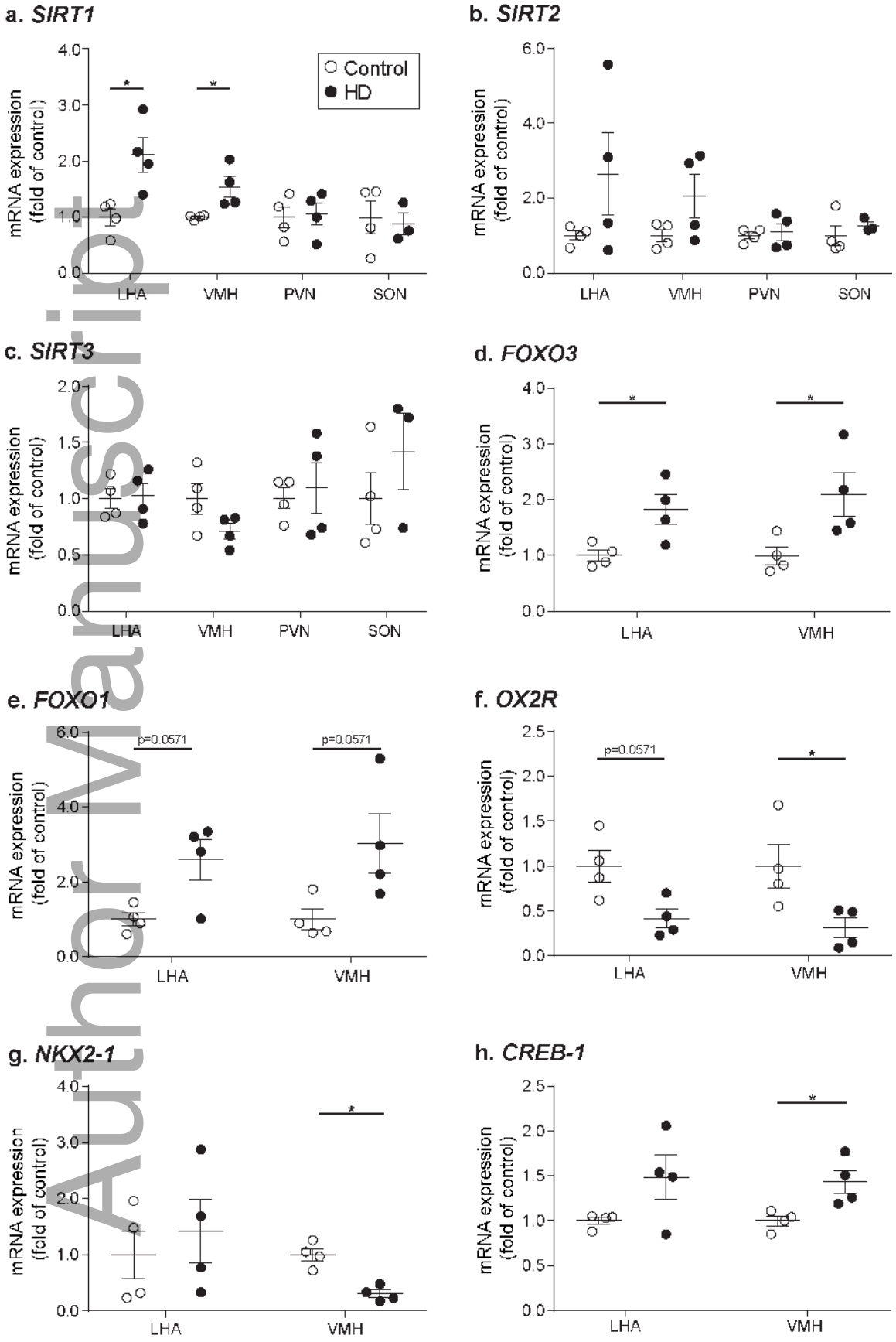

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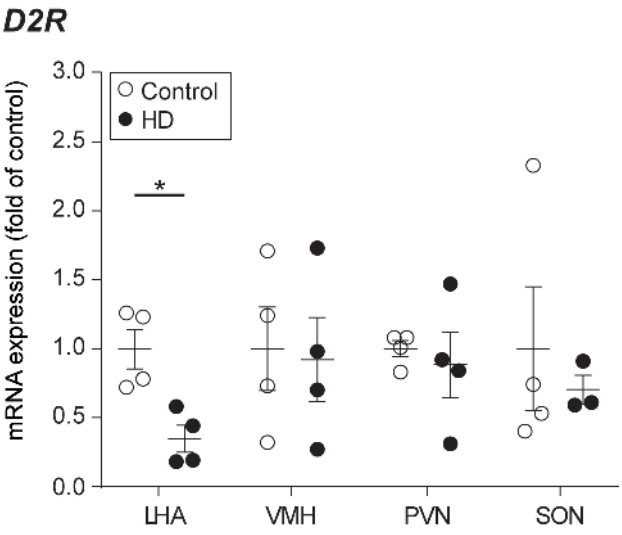

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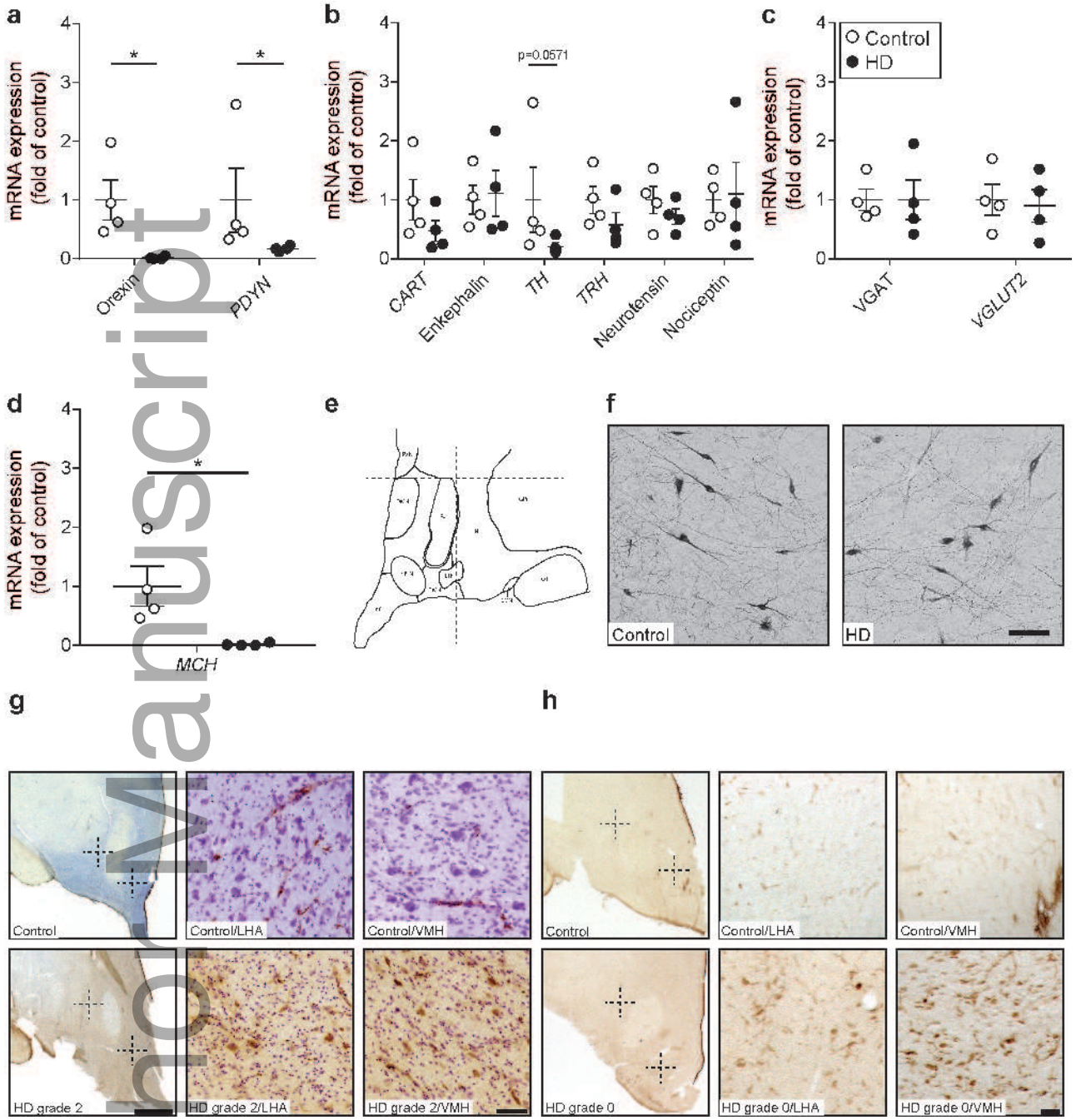

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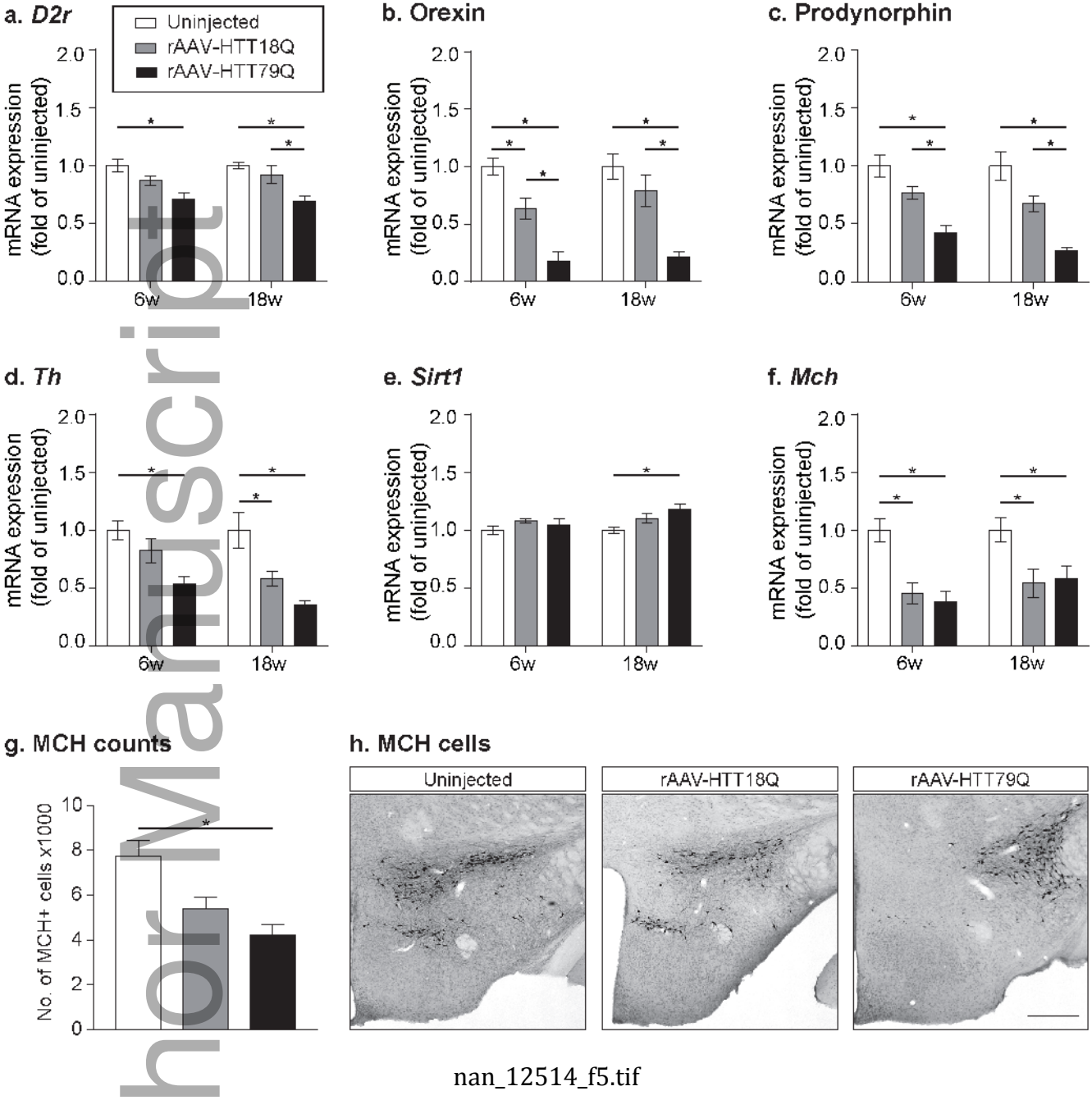

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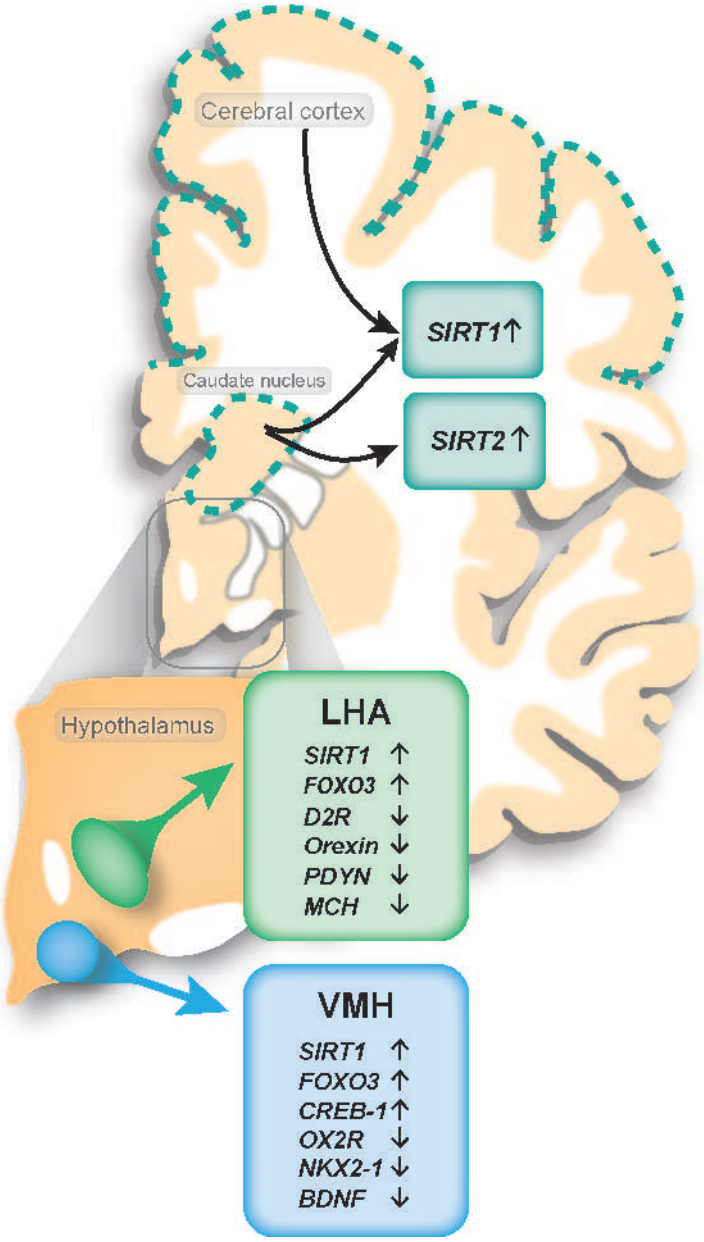

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