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The hypothalamus as a key mediator of neurodegenerative disorders

Bergh, Sofia

2026

Document Version:

Publisher's PDF, also known as Version of record

[Link to publication](#)

Citation for published version (APA):

Bergh, S. (2026). *The hypothalamus as a key mediator of neurodegenerative disorders*. [Doctoral Thesis (compilation), Department of Experimental Medical Science]. Lund University, Faculty of Medicine.

Total number of authors:

1

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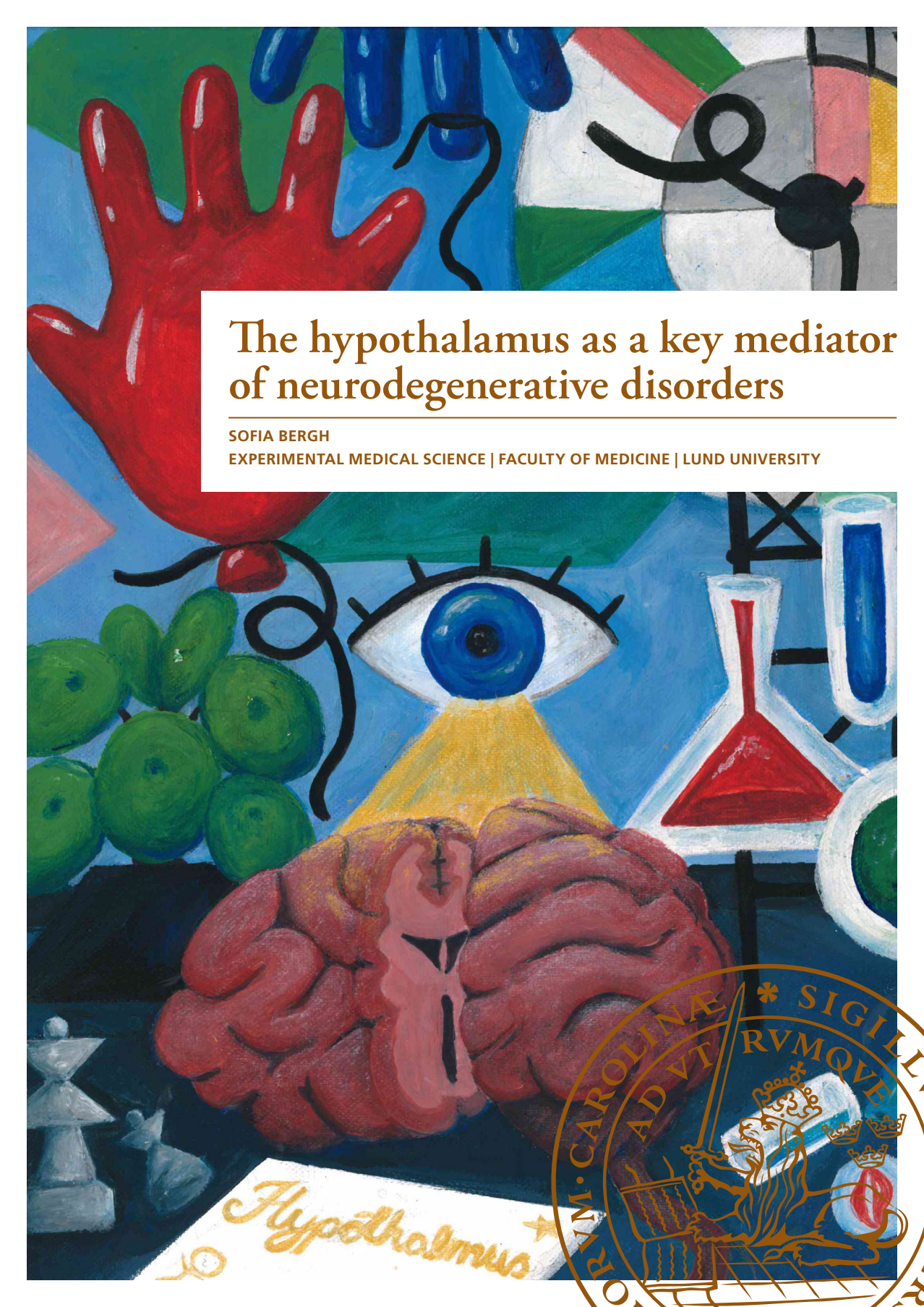
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An abstract painting featuring a large red hand reaching up from the left, a blue eye with a yellow beam of light shining down onto a red brain in the center, and various laboratory glassware like a flask with red liquid and test tubes on the right. The background is a mix of blue, green, and white with black lines. In the bottom right corner, there is a circular seal of the University of Medicine and Health Sciences, Lund University, with the text 'SIGILLUM UNIVERSITATIS MEDICINAE ET SANITATIS LUNDENSIS' and 'AD V. T. R. V. M. Q. V. E. L. L. U. N. D. E. N. S. I. S'.

The hypothalamus as a key mediator of neurodegenerative disorders

SOFIA BERGH

EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY

Hypothalamus

The hypothalamus as a key mediator of neurodegenerative disorders

SOFIA BERGH



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DOCTORAL DISSERTATION

With the approval of the Faculty of Medicine at Lund University
this thesis will be defended on May 6, 2026, at 13:00
in Belfragsalen, BMC, Lund, Sweden

FACULTY OPPONENT

Professor Gillian Bates
University College London

Organization: Lund University

Document name: Doctoral thesis

Date of issue: 2025-05-06

Author: Sofia Bergh

Sponsoring organization

Title and subtitle: The hypothalamus as a key mediator of neurodegenerative disorders

Abstract: Background: There are currently no major disease-modifying treatments for neurodegenerative disorders such as Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). HD is caused by a CAG repeat expansion in the huntingtin gene and is characterised by the accumulation of mutant huntingtin (mHTT) protein. ALS is defined by the degeneration of motor neurons connecting the brain, spinal cord and muscles, while FTD involves damage to cortical regions, primarily the frontal and temporal lobes. ALS often co-occur with FTD, with the common denominator linking these disorders being the transactive response DNA binding protein of 43 kDa (TDP-43). What connects HD, ALS-FTD and other distinct neurodegenerative disorders is pathology of the hypothalamus—a key regulator of emotion and metabolism. Clinical features such as anxiety, depression and body weight dysregulation are common across these disorders and may be linked to hypothalamic dysfunction. We hypothesize that the hypothalamus is vulnerable to mHTT and TDP-43 toxicity, and that damage in this region contributes to the observed metabolic and psychiatric phenotypes in people affected by HD or ALS-FTD. We therefore aimed to identify the structure-to-function relationship between the hypothalamus and its circuitry and the development of the psychiatric and metabolic features of HD and ALS-FTD. Results: We demonstrated that TDP-43 overexpression in the hypothalamus of mice was sufficient to induce ALS-FTD-like pathology, including the formation of cytoplasmic TDP-43 localisation with inclusions and the development of metabolic and emotional deficits (Paper I). In addition, we established the RNA/DNA autophagy protein SID1 transmembrane family member 2 (SIDT2) as a novel pathological hallmark in HD (Paper II). SIDT2 protein levels are significantly reduced in advanced HD and SIDT2-immunoreactive inclusions are present in postmortem human brain tissue from HD cases. Given that SIDT2 overexpression in vivo and in vitro reduces HTT load independent of canonical degradation pathways, the SIDT2 system represents a promising therapeutic avenue. Lastly, we showed that neurons expressing oxytocin—a neuropeptide implicated in social behaviour, anxiety and depression—are susceptible to both mHTT and TDP-43 toxicity (Paper III and IV). Interestingly, pathology in oxytocin neurons was insufficient to generate behavioural and metabolic dysfunction, suggesting that other neuronal populations may play a more prominent role in the pathogenesis of HD and ALS-FTD. Conclusion: This thesis demonstrates the involvement of the hypothalamus in neurodegenerative disorders characterised by TDP-43 and mHTT pathology. Our results indicate that hypothalamic pathology may be a convergence point of these disease processes and therefore represents a potential therapeutic target. Furthermore, the observed deficits in the SIDT2 pathway in HD suggest that SIDT2 is a promising therapeutic candidate.

Key words:

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language: English

Number of pages: 99

ISSN and key title: 1652-8220

ISBN: 978-91-8021-858-0

Recipient's notes

Price

Security classification

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The ways of the mind, 2025

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The cover art is a reflection of the academic journey, illustrating the interplay between work and play. It is an homage to the process itself and represents the balance between the disciplined effort and the sense of childlike wonder.

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Department of Experimental Medical Science

Faculty of Medicine

Lund University

Lund 2026

ISBN 978-91-8021-858-0

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University, Lund, 2026



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"Today, I consider myself the luckiest man on the face of the earth."

— Lou Gehrig, Farewell to Baseball Speech, Yankee Stadium, New York, NY, 1939

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Abstract

Background: There are currently no major disease-modifying treatments for neurodegenerative disorders such as Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). HD is caused by a CAG repeat expansion in the huntingtin gene and is characterised by the accumulation of mutant huntingtin (mHTT) protein. ALS is defined by the degeneration of motor neurons connecting the brain, spinal cord and muscles, while FTD involves damage to cortical regions, primarily the frontal and temporal lobes. ALS often co-occur with FTD, with the common denominator linking these disorders being the transactive response DNA binding protein of 43 kDa (TDP-43). What connects HD, ALS-FTD and other distinct neurodegenerative disorders is pathology of the hypothalamus—a key regulator of emotion and metabolism. Clinical features such as anxiety, depression and body weight dysregulation are common across these disorders and may be linked to hypothalamic dysfunction. We hypothesize that the hypothalamus is vulnerable to mHTT and TDP-43 toxicity, and that damage in this region contributes to the observed metabolic and psychiatric phenotypes in people affected by HD or ALS-FTD. We therefore aimed to identify the structure-to-function relationship between the hypothalamus and its circuitry and the development of the psychiatric and metabolic features of HD and ALS-FTD

Results: We demonstrated that TDP-43 overexpression in the hypothalamus of mice was sufficient to induce ALS-FTD-like pathology, including the formation of cytoplasmic TDP-43 localisation with inclusions and the development of metabolic and emotional deficits (Paper I). In addition, we established the RNA/DNA autophagy protein SID1 transmembrane family member 2 (SIDT2) as a novel pathological hallmark in HD (Paper II). SIDT2 protein levels are significantly reduced in advanced HD and SIDT2-immunoreactive inclusions are present in postmortem human brain tissue from HD cases. Given that SIDT2 overexpression *in vivo* and *in vitro* reduces HTT load independent of canonical degradation pathways, the SIDT2 system represents a promising therapeutic avenue. Lastly, we showed that neurons expressing oxytocin—a neuropeptide implicated in social behaviour, anxiety and depression—are susceptible to both mHTT and TDP-43 toxicity (Paper III and IV). Interestingly, pathology in oxytocin neurons was insufficient to generate behavioural and metabolic dysfunction, suggesting that other neuronal populations may play a more prominent role in the pathogenesis of HD and ALS-FTD.

Conclusion: This thesis demonstrates the involvement of the hypothalamus in neurodegenerative disorders characterised by TDP-43 and mHTT pathology. Our results indicate that hypothalamic pathology may be a convergence point of these disease processes and therefore represents a potential therapeutic target. Furthermore, the observed deficits in the SIDT2 pathway in HD suggest that SIDT2 is a promising therapeutic candidate.

Populärvetenskaplig sammanfattning

Introduktion: Vår kropp styrs av nervceller som reglerar allt från tankeförmåga och känslor till kroppsfunktioner som andning och matsmältning. Dessa viktiga celler är särskilt känsliga för neurodegenerativa sjukdomar, en grupp sjukdomar där skadliga proteiner klumpar ihop sig och tros bryta ner nervcellerna.

Beroende på vilka nervceller som bryts ned uppstår olika sjukdomssymptom. Vid Huntingtons sjukdom (HS), som orsakas av en mutation i huntingtin-genen (*HTT*), påverkas framförallt hjärnområdet striatum som styr motoriken. Sjukdomar som amyotrofisk lateralskleros (ALS) kan ibland uppträda i kombination med frontotemporal demens (FTD). Vid ALS bryts nervfibrer mellan hjärnan, ryggmärgen och musklerna ned, medan FTD innebär skada i områden som styr beteende och språkförmåga, främst i frontal- och temporalloben. En viktig gemensam nämnare för dessa till synes olika sjukdomar är proteinet TDP-43. TDP-43 finns i alla celler, även hos friska personer, och är viktig för cellens normala funktion. Däremot kan TDP-43 av okänd anledning leda till sjukdom.

Eftersom det i dagsläget saknas läkemedel som kan bota neurodegenerativa sjukdomar är det avgörande att förstå de gemensamma mekanismerna. Hypotalamus är en central, men liten, hjärnregion som styr våra känslor, ämnesomsättning och beteende. Symptom som ångest, depression och viktförändringar är vanliga vid sjukdomar som HS och ALS-FTD. I denna avhandling testar vi hypotesen att hypotalamus är sårbar för muterat huntingtin och TDP-43, och att skador (patologi) i denna region bidrar till de metaboliska och psykiska problemen hos patienter.

Resultat: I denna avhandling visade vi att enbart överuttryck av TDP-43 i hypotalamus hos möss var tillräckligt för att leda till ALS-FTD-liknande skador, inklusive bildning av TDP-43 proteinansamlingar. Det ledde även till tydliga brister i ämnesomsättningen och beteende (Artikel I).

Vi identifierade även den viktiga rollen av SIDT2 vid HS (Artikel II). SIDT2 som normalt är involverad i cellernas normala rengörings- och återvinningssystem (så kallad autofagi) för att avlägsna felaktiga proteiner. Proteinnivåerna av SIDT2 är kraftigt sänkta i påverkade hjärnområden i HS. När vi överuttryckte SIDT2 i möss och i celler kunde det minska mängden muterat huntingtin protein, vilket gör SIDT2-systemet till en lovande angreppspunkt för potentiell läkemedelsutveckling.

Studier bekräftade att viktiga nervceller som producerar oxytocin – det så kallade kärlekshormonet är kopplat till både socialt beteende, ångest och depression – är känsliga för både muterat huntingtin och TDP-43 (Artikel III och IV). Intressant nog räckte inte patologi enbart i dessa celler för att orsaka de typiska beteende- och ämnesomsättningsproblemen, vilket kan betyda att andra nervceller kan vara viktigare i sjukdomsförloppet av HS och ALS-FTD.

Slutsats: Denna avhandling visar att hypotalamus är viktig i sjukdomar med TDP-43 och muterat huntingtin patologi. Resultaten tyder på att patologi i hypotalamus är en viktig del i sjukdomsförloppet och kan vara ett potentiellt mål för läkemedel, och att SIDT2 kan vara en läkemedelkandidat efter upptäckten av brister av SIDT2-systemet i HS.

Abbreviations

AAV	adeno-associated virus
ACC	anterior cingulate cortex
AD	Alzheimer's disease
AGRP	agouti-related peptide
ALS	amyotrophic lateral sclerosis
bvFTD	behavioural variant frontotemporal dementia
CE	cryptic exons
Cre	Cre-recombinase
DAB	3,3'-diaminobenzidine
DEXA	dual-energy X-ray absorptiometry
DPX	distyrene plasticizer xylene
EN	extranuclear inclusions
EPM	elevated plus maze
FST	forced swim test
FTD	frontotemporal dementia
GC	genome copies
GFAP	glial fibrillary acidic protein
HD	Huntington's disease
HTT	huntingtin
Iba1	ionised calcium-binding adaptor molecule 1
ICC	immunocytochemistry
IHC	immunohistochemistry
IN	intranuclear inclusion
ITR	inverted terminal repeats
LHA	lateral hypothalamic area
MCH	melanin-concentrating hormone
mHTT	mutant huntingtin
ND	neurodegenerative disorder

NFT	neurofilament tangles
OF	open field
PCR	polymerase chain reaction
PD	Parkinson's disease
PFA	paraformaldehyde
PVN	paraventricular nucleus
Q	glutamine
qRT-PCR	quantitative reverse transcription PCR
RR	rotarod
SIDT2	SID1 transmembrane family member 2
SIT	social interaction test
SOD1	superoxide dismutase 1
SON	supraoptic nucleus
TDP-43	transactive response DNA binding protein 43 kDa
UPS	ubiquitin-proteasome system
WT	wild-type

Publications and manuscripts

Thesis publications

- I. **Bergh S**, Casadei N, Gabery S, Simonsson O, Duarte JMN, Kirik D, Nguyen HP, Petersén Å.
TDP-43 overexpression in the hypothalamus drives neuropathology, dysregulates metabolism and impairs behavior in mice
Acta Neuropathologica Communications, 2025. 13(1):119.
- II. Gabery S*, **Bergh S***, Huridou C, Cheong R, Baldo B, Scheunemann P, Schoebel ML, Holmquist Mengelbier L, Englund E, McLean C, Saft C, Kirik D, Börkqvist M, Halliday G, Petrasch-Parwez E, Nguyen H, Weber J, Petersén Å.
*Equal contribution
The DNA/RNA autophagy protein SIDT2 as a novel neuropathological hallmark in Huntington disease
Brain pathology, 2026. e70088.
- III. **Bergh S**, Gabery S, Tonetto S, Kirik D, Petersén Å*, Cheong RY*.
*Equal contribution
Effects of mutant huntingtin in oxytocin neurons on non-motor features of Huntington's disease
Neuropathology and Applied Neurobiology, 2023. 49(3):e12905.
- IV. **Bergh S***, Simonsson O*, Kirik D and Petersén Å.
*Equal contribution
Analyses of the Effects of Wild-Type TDP-43 Overexpression in Oxytocin Neurons in Mice
Neuropathology and Applied Neurobiology, 2026. 52(1):e70059.

Review article

- V. **Bergh S**, Cheong RY, Petersén Å, Gabery S.
Oxytocin in Huntington's disease and the spectrum of amyotrophic lateral sclerosis-frontotemporal dementia
Frontiers in Molecular Neuroscience, 2022. 15:984317

Publications outside of thesis

- VI. Orah J, Wagner R, **Bergh S**, Lee NJ, Kirik D, Petersén Å.
Differential effects of overexpression of mutant huntingtin and TDP-43 in agouti-related protein neurons in the arcuate nucleus of the hypothalamus in mice *Acta Neuropathologica Communications* 2025. 13(1):253
- VII. Mounier C, Pinchaud K, Lobo M, Hery M, Xue W, Le Corre L, Mendoza C, Friedrichs S, **Bergh S**, Gilles JF, Marinozzi M, Heck N, Petersén Å, Lütjohann D, Tricoire L, Pietrancosta N, Baligand C, Escartin C, Vanhoutte P, Caboche J, Néri C, Betuing S.
Liver-X-Receptor- β activation protects Huntington's disease knock-in mice from behavioral and neuropathological impairments
Preprint available at Research Square 2025.

Introduction

The thesis rationale in brief

The intricate abilities of the human mind and body are a result of a complex network, consisting of highly specialised neurons controlling everything from cognition and emotion to vital bodily functions, such as breathing and digestion. However, this sophisticated system, known as the nervous system, is susceptible to the group of diseases called neurodegenerative disorders (NDs).

While NDs share a common hallmark of progressive neuronal deterioration, each disorder primarily targets distinct parts of the nervous system. In Huntington's disease (HD) the neurons of the striatum and cerebral cortex are particularly vulnerable, impairing movement and cognition. Amyotrophic lateral sclerosis (ALS) mainly affects motor neurons, limiting movement, while frontotemporal dementia (FTD) predominantly degenerates the frontal and temporal lobes, resulting in changes in personality and language ability (aphasia). Despite these three disorders being first characterised in the 19th century, they remain irreversible and fatal with no major disease-modifying therapies.

While each ND is unique in its clinical presentation and primary affected brain regions, they share a surprising commonality. A growing body of evidence suggests that the hypothalamus, an important brain region responsible for regulating critical functions like emotion, metabolism, and motivation, is affected across a wide range of NDs, including not only HD, ALS and FTD, but also Alzheimer's disease (AD) and Parkinson's disease (PD). Furthermore, the same NDs also share a common molecular signature – pathology of transactive response DNA-binding protein 43 kDa (TDP-43). This shared vulnerability points to a potential unifying mechanism of neurodegeneration and a promising avenue for therapeutic intervention.

This thesis contributes to this critical area of research by providing new insights into the relevance of hypothalamic pathology in NDs. This work strengthens our understanding of the shared molecular mechanisms underlying diseases like HD and the ALS-FTD spectrum and could be crucial for bridging our understanding of these seemingly distinct conditions and, most importantly, for the future development of novel therapeutic strategies.

History of neurodegenerative disorders

While conditions characterised by cognitive decline, like dementia, had been noted in ancient medical texts for millennia, the first clinical description of any ND was provided in 1817 when James Parkinson published *An essay on the shaking palsy*, detailing the symptoms of what would later be named Parkinson's disease [1] (**Figure 1**). The underlying mechanisms (neuropathology) of NDs remained unknown until the 20th century. Understanding the role of proteins in neuropathology began with Alois Alzheimer, who in 1906 published his work detailing the characteristic amyloid plaques and neurofibrillary tangles found in the brain of a patient with AD [2]. Shortly thereafter in 1912, Fritz Heinrich Lewy identified the specific protein aggregates, now known as Lewy bodies, that constitute the pathological signature of PD [3]. In 2009, John Trojanowski, Virginia Man-Yee Lee and Mark Forman, discovered inclusions of TDP-43 in ALS and FTD as well as in AD [4]. These post-mortem discoveries were critical, enabling researchers to classify and differentiate disorders based on the specific type of abnormal protein aggregation, known as proteinopathies. This thesis will focus on two such proteinopathies: mutant huntingtin (mHTT) and TDP-43, mHTT pathology is exclusive to HD, while TDP-43 pathology is shared by an increasing number of NDs, most notably in ALS and FTD.

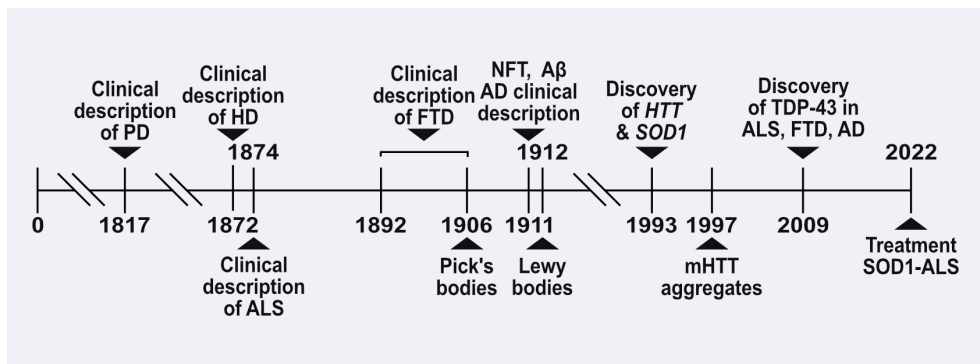


Figure 1. Timeline of milestones in the history of neurodegenerative disorders.

Parkinson's disease (PD) was described in 1817, followed by Huntington's disease (HD) (1872) and amyotrophic lateral sclerosis (ALS) (1874). Frontotemporal dementia (FTD) was described in a series of case reports between 1892 and 1906. Alzheimer's disease (AD) was described in 1906 alongside the pathological characteristics of neurofibrillary tangles (NFT) and amyloid (Aβ) plaques. The discovery of both the huntingtin (HTT) CAG repeat expansion in HD and the ALS SOD1 gene mutation happened in 1993. Furthermore, mutant HTT (mHTT) protein aggregation was identified as a neuropathological hallmark of HD in 1997. The widespread TDP-43 pathology was observed in ALS, FTD and AD (2009) and the first ever treatment of ALS caused by SOD1 was approved in 2022 [5, 6].

History of HD

George Huntington, an American physician who in 1872, published a precise description of the condition that would later bear his name in a paper titled "On Chorea" [7]. The term chorea refers to the involuntary, dance-like movements; a word derived from the Greek word for "dance". In Sweden the disorder previously went under the name *danssjukan*, which directly translates to "the dance disorder". Although he was not the first to observe the disease, his paper was the first to identify the progressive and familial nature of HD and clinical symptoms such as motor, cognitive and psychiatric disturbances. The next major breakthrough in HD research came over a century later. In 1983, a team of researchers led by Nancy Wexler identified that a region on chromosome 4 was linked to HD. This discovery was made possible by studying a large family in Venezuela with a high prevalence of HD [8]. A decade later, in 1993, the exact gene mutation was identified, revealing the underlying genetic cause of HD – a CAG repeat expansion in the huntingtin (*HTT*) gene [9]. In 1997, a group led by Marian DiFiglia and Neil Aronin identified mutant HTT (mHTT) protein aggregation in postmortem human brain tissue [10], while a separate lab led by Erich Wanker demonstrated that purified mHTT fragments could aggregate in vitro [11], these two simultaneous discoveries established the neuropathological hallmark of HD.

History of ALS

The earliest comprehensive clinical description of what would become known as ALS came from the French neurologist Jean-Martin Charcot in 1874 [12]. There are earlier descriptions, but Charcot's observations led him to distinguish ALS from other neurological disorders, characterising it by progressive muscle wasting (amyotrophy) and hardening of the spinal cord (lateral sclerosis). Decades later, the disease gained widespread public recognition through the American baseball player, Lou Gehrig. After being diagnosed with the condition in 1939, he gave an emotional farewell speech at the Yankee Stadium and brought the devastating effects of the disease to a wide audience, leading it to its colloquial name Lou Gehrig's disease. In 1993, the discovery of the first ALS-linked gene, superoxide dismutase 1 (*SOD1*), provided the initial insight into the familial aspect of the disease [13]. While sporadic ALS still accounts for most cases, *SOD1* mutations are responsible for 1–6% of ALS cases [14]. Furthermore, a breakthrough came in 2023, with the approval of Tofersen, the first treatment specifically for ALS caused by the *SOD1* mutation. For the first time, clinical reports showed that disease progression could be slowed [5, 6].

History of frontotemporal dementia

For decades, many cases of dementia were misdiagnosed as atypical AD. The first clinical distinction of what would be FTD was made in the late 1800s by Czech psychiatrist Arnold Pick [15]. In a series of case reports between 1892 and 1906, Pick described patients with progressive aphasia and personality changes, which he correctly linked to atrophy of the frontal and temporal lobes. The disorder was initially named "Pick's disease." In 1911, Alois Alzheimer identified what would be called "Pick bodies," which are an accumulation of the tau protein [16]. While the term "Pick's disease" persists, it refers to only one specific subtype of FTD. The more widely used term for this subtype is behavioural variant FTD (bvFTD) and is the subtype mainly associated with the ALS-FTD spectrum. It was not until the late 20th century that the medical community recognised Pick's disease as just one part of a larger family of related disorders, leading to the adoption of the more inclusive umbrella term, frontotemporal dementia.

Huntington's disease

HD is caused by a mutation in the huntingtin (*HTT*) gene, which involves an expansion of CAG trinucleotide repeats in exon 1 [9]. As a result, the translated huntingtin (HTT) protein contains an elongated polyglutamine (Q) tract [9]. A clinical diagnosis of HD is based on a positive genetic test, typically showing 40–50 CAG repeats, combined with the manifestation of motor disturbances [17]. For comparison, a typical tract in healthy individuals contains around 16–20 CAG repeats [18]. Individuals with more than 40 CAG repeats show full penetrance, meaning they will develop the disease. However, those with 36–39 repeats have reduced penetrance and may remain asymptomatic throughout their lives or develop the disease at a later stage [17]. The length of the CAG repeat expansion is inversely correlated with the age of onset, meaning individuals with a longer repeat tract typically develop the disease earlier in life [19, 20]. Onset typically occurs in midlife (between 30 and 55 years of age) and is followed by a disease duration of approximately 20 years. The hallmark motor disturbances include chorea, characterised by exaggerated involuntary movements, as well as rigidity, dystonia and gait imbalance [21]. A distinct juvenile-onset form of HD presents before age 20. This form is usually seen in individuals with more than 60 CAG repeats and is often dominated by a more rapid progression [22].

Beyond the motor manifestations, HD is a complex disorder with a wide range of non-motor signs and symptoms that can precede the motor onset by years or even decades. These include significant cognitive changes, such as difficulty with planning and impulse control as well as impaired social cognition [23]. Psychiatric

features are also common and include depression, anxiety, apathy and irritability [24, 25]. Patients with HD also display metabolic disturbances, with weight loss, particularly in later stages of HD but also in pre-symptomatic HD patients [26, 27].

Certain brain regions are more vulnerable to the effects of mHTT, which is thought to cause the wide range of signs and symptoms seen in HD (**Figure 2**). HD is characterised by progressive brain atrophy, which is most pronounced in the striatum of the basal ganglia and the cerebral cortex [28, 29]. The vulnerability of the striatum is believed to be the primary cause of the motor disturbances, and the severity of this atrophy is classified using the Vonsattel grading scale (0-IV) [30]. In addition, the cerebral cortex is implicated as the origin of cognitive disturbances, but also motor disturbances. Furthermore, neurodegeneration in other brain regions, such as the hypothalamus, may explain non-motor signs and symptoms, including alterations in metabolism, emotion and sleep [31].

Huntington's disease

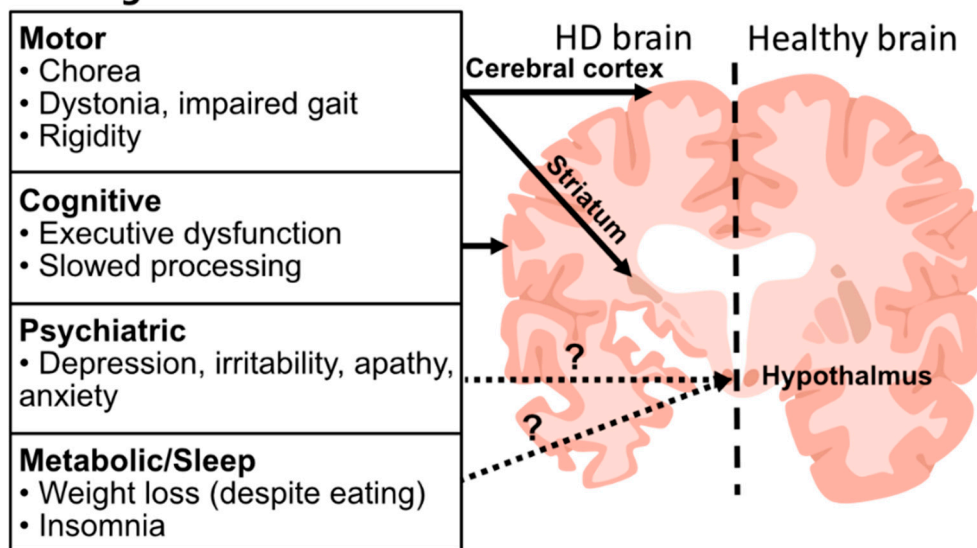


Figure 2. Neuropathology and clinical features of Huntington's disease (HD).

Huntingtin

While the cause of HD is well-established, the precise underlying mechanisms that result in neurodegeneration remain a topic of debate. Mutant HTT appears to exert toxicity across all stages of gene expression: at the DNA, RNA and protein levels. The most widely studied pathological feature is protein toxicity, characterised by the aggregation of the mHTT protein to form both intranuclear and extranuclear inclusions [10, 32]. These inclusions are believed to sequester additional cellular

proteins and disrupt normal cell function. Inclusion formation is driven by the elongated polyglutamine tract in exon 1, which causes mHTT protein misfolding and the subsequent accumulation of insoluble inclusion bodies [11, 33].

At the RNA level, the expanded CAG repeat tract within the RNA can form aberrant secondary structures. These structures can sequester RNA-binding proteins, leading to a disruption of normal RNA processing and transport [34]. Furthermore, missplicing of HTT leads to the production of exon 1 RNA, which subsequently generates exon 1 protein fragments that are highly prone to aggregation [35].

DNA toxicity is less direct but involves mHTT disrupting the transcriptional machinery, particularly DNA repair mechanisms [36]. This impairment contributes to somatic expansion, a process where the number of CAG repeats increases progressively over time in cells, and can reach hundreds of CAG repeats, which is believed to initiate and accelerate disease progression [36].

Huntingtin clearance and the SIDT2 pathway

Cells have intrinsic clearance pathways to manage and eliminate aberrant proteins, RNA and DNA species. For proteins, the major mechanisms include the ubiquitin-proteasome system (UPS), which tags faulty proteins with ubiquitin for degradation via the proteasome. Alternatively, macroautophagy is a process where proteins and larger cellular components (such as organelles) are enclosed within vesicles called autophagosomes, which then fuse with lysosomes for enzymatic degradation[37]. In both UPS and autophagy, faulty proteins are tagged with ubiquitin. Subsequently, the protein p62 delivers ubiquitinated proteins to the proteasome or the autophagosome [38]. Ubiquitin has been shown to form inclusions in HD and ALS-FTD [39, 40]. The clearance mechanisms for aberrant RNA and DNA species are comparatively less characterised. A recently discovered autophagy pathway, termed RNautophagy/DNautophagy is responsible for the specific degradation of RNA or DNA transcripts via lysosomes (**Figure 3**). This novel mechanism is governed by the lysosomal transmembrane protein SID1 transmembrane family member 2 (SIDT2) [41-44]. SIDT2 is a transmembrane protein that directly binds to DNA and RNA to facilitate their uptake into the lysosome. The discovery of this pathway is particularly significant for HD research, as SIDT2 has been shown to bind specifically to exon 1 of the HTT transcript via its arginine-rich motif in a CAG-dependent manner [45]. Furthermore, a study in postmortem PD cases demonstrated that SIDT2 formed inclusions and co-localised with phosphorylated α -synuclein protein [46]. Skeletal muscle-specific *Sidt2* knock-out in mice induces a muscular dystrophy-like phenotype[47], while the knock-out of *Sidt2* in mouse mesangial cells resulted in an increase in expression of pro-inflammatory cytokines [48]. The role of SIDT2 is an emerging field, given that HTT transcripts may be cleared through this SIDT2-mediated autophagy pathway, it may be crucial in understanding HD pathogenesis.

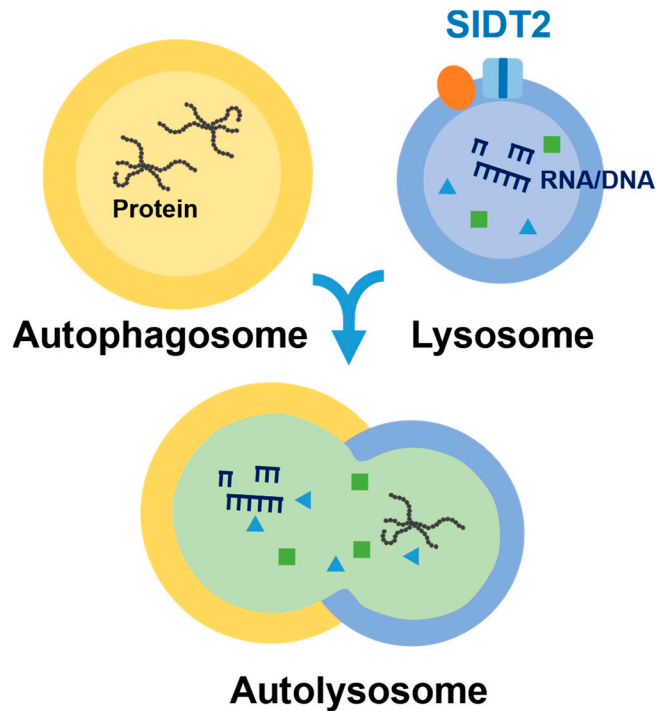


Figure 3. Cellular clearance via the autophagy pathway.

Macroautophagy clears misfolded or faulty proteins and damaged organelles. Tagged cargo is initially encapsulated within a double-membraned vesicle called an autophagosome. The autophagosome then fuses with a lysosome to form an autolysosome. The low-pH environment in the lysosome in combination with enzymes, breaks down the cargo for recycling. In RNautophagy/DNautophagy, SIDT2 directly transports DNA and RNA into the lysosome, resulting in degradation.

ALS-FTD disease spectrum

Clinical features of ALS

ALS is a rapidly progressive ND characterised by the selective degeneration of both upper motor neurons and lower motor neurons (**Figure 4**). Loss of upper motor neurons, involving neurons connecting the brain and spinal cord, typically presents as muscle stiffness (spasticity) and hyperreflexia, while loss of lower motor neurons, connecting the spinal cord to skeletal muscles, leads to muscle weakness and atrophy. ALS affects individuals around the mean age of 55 and typically results in death within 2-5 years of symptom onset [49, 50].

Crucially, the disease extends beyond the classic motor features, frequently affecting cortical and subcortical regions. The resulting non-motor features include metabolic dysregulation with substantial weight loss [51-53] as well as psychiatric

and cognitive impairments sometimes preceding the onset of motor disturbances. Patients commonly exhibit emotional dysfunction like depression and apathy, as well as deficits in social cognition and sleep impairments [51, 54-56].

ALS is overwhelmingly sporadic in etiology and the major pathological protein is TDP-43, usually present in the cytoplasm of affected motor neurons in the spinal cord and brain stem [50]. TDP-43 inclusions are also present in the frontal and temporal cortices as well as in the hypothalamus [57, 58]. Genetic mutations have also been associated with ALS such as *SOD1*, *C9ORF72* and the TDP-43 encoding gene *TARDBP* [50].

Clinical features of FTD

Frontotemporal lobar degeneration comprises a group of NDs characterised by the degeneration of the frontal and temporal lobes of the brain resulting in progressive behavioural and executive dysfunction (**Figure 4**). FTD is the second most common form of early-onset dementia after AD [59]. FTD is typically subcategorised into three main clinical syndromes: bvFTD is the most prevalent and usually presents around age 50 [60], and two language variants, semantic dementia and progressive non-fluent aphasia [60]. Core features of bvFTD include apathy and behavioural disinhibition. FTD patients also exhibit psychiatric and metabolic features, including anxiety, depression, changes in eating behaviour (hyperorality) and weight gain [61-63]. The clinical presentation of bvFTD can be diagnostically challenging as approximately half of all patients are initially misdiagnosed with a primary psychiatric disorder, such as bipolar disorder or major depressive disorder, due to the symptom overlap [64].

FTD is a highly heterogeneous disease, with different underlying protein pathologies. However, the majority of patients with FTD exhibit the similar TDP-43 pathology seen in ALS. These aggregates are mainly concentrated in the frontal and temporal cortices, but are also present in the hypothalamus [65, 66]. While less common, some FTD cases are linked to tau and FUS protein aggregation instead of TDP-43 [67].

ALS-FTD disease spectrum

ALS and FTD, particularly bvFTD, are widely regarded to be on a disease spectrum, due to clinical, genetic and neurological overlap [68], meaning that a proportion of patients present with features of both conditions. The percentages of symptom overlap can vary slightly across studies, but the relationship is well-established. At least 50% of patients with ALS develop cognitive and behavioural features, and result in a full FTD diagnosis up to 25% of the time [69, 70].

This spectrum was further reinforced by the discovery of the C9ORF72 hexanucleotide repeat expansion, which is the most common genetic cause for both familial ALS and FTD [71]. At the neuropathological level, both conditions are characterised by ubiquitin-immunoreactive TDP-43 inclusions in the cytoplasm in neurons and glial cells [57, 72].

Other neurodegenerative disorders

AD is the most common cause of dementia and defined by its progressive episodic memory loss and cognitive decline [73]. AD patients also exhibit early psychiatric features, like apathy, anxiety, depression and aggression [74, 75]. In addition, persons with AD also have sleep and metabolic disturbances, including sleep-wake disturbances, changes in appetite and eating. AD is neuropathologically defined by extracellular deposits of amyloid- β protein that form amyloid plaques, which have been observed within the hypothalamus [74]. TDP-43 pathology is also observed in a majority of AD cases [76].

PD is classically defined by its motor disturbances, including tremors, rigidity and bradykinesia [77]. However, PD is also a multisystem disorder due to the prevalence of non-motor features which frequently precede motor features. Key non-motor features involves sleep disturbances like REM sleep behaviour disorder [78], where patients act out their dreams, psychiatric features like anxiety and depression and mild cognitive impairment often progressing into dementia [79]. The pathological signature of PD is the accumulation of the α -synuclein protein which makes up Lewy bodies, which are preferentially observed in the limbic system including the hypothalamus [80]. TDP-43 is a secondary pathological protein seen in a subset of PD cases [81, 82].

Amyotrophic lateral sclerosis

Motor

- Progressive UMN & LMN loss
- Muscle weakness and atrophy
- Dysarthria/Dysphagia (Bulbar symptoms)

Cognitive

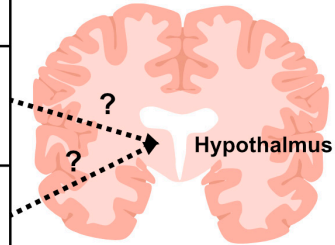
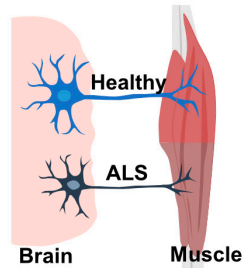
- Executive dysfunction
- social cognitive impairment
- Up to 15% develop FTD

Psychiatric

- Apathy
- Depression

Metabolic/Sleep

- Weight loss
- Sleep disturbances



Frontotemporal dementia

Motor

- Parkinsonism or ALS features in some variants

Cognitive

- Atrophy of frontal and temporal lobes
- Executive dysfunction, personality changes, social cognitive impairment
- Language inability (Aphasia)

Psychiatric

- Disinhibition/Inappropriate behavior.
- Apathy

Metabolic/Sleep

- Weight gain (hyperorality)
- Sleep-wake cycle disruption, insomnia

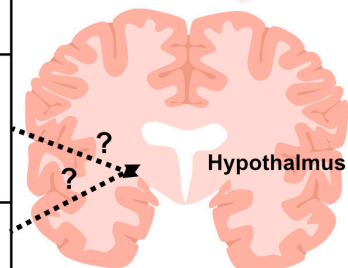
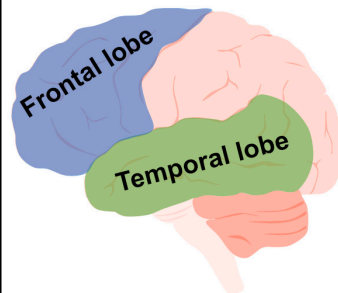


Figure 4. Neuropathology and clinical features of amyotrophic lateral sclerosis and frontotemporal dementia.

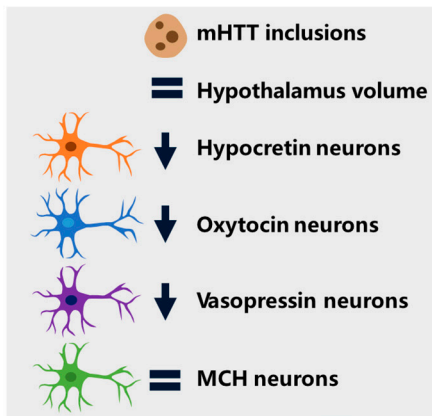
Hypothalamus

The hypothalamus is a brain region that makes up approximately 1% of the total brain weight. This small but critical region regulates autonomic functions and links the nervous system to the endocrine (hormone) system. The hypothalamus is the main control centre for body temperature, appetite, sleep-wake cycle and mood. Dysfunction of the hypothalamus is linked to several psychiatric disorders, including depression, bipolar disorder, schizophrenia and post-traumatic stress disorder, establishing its role in regulation of affective function [83-85]. Furthermore, pathology of the hypothalamus is observed in several NDs and may explain some of the psychiatric and metabolic features described in NDs.

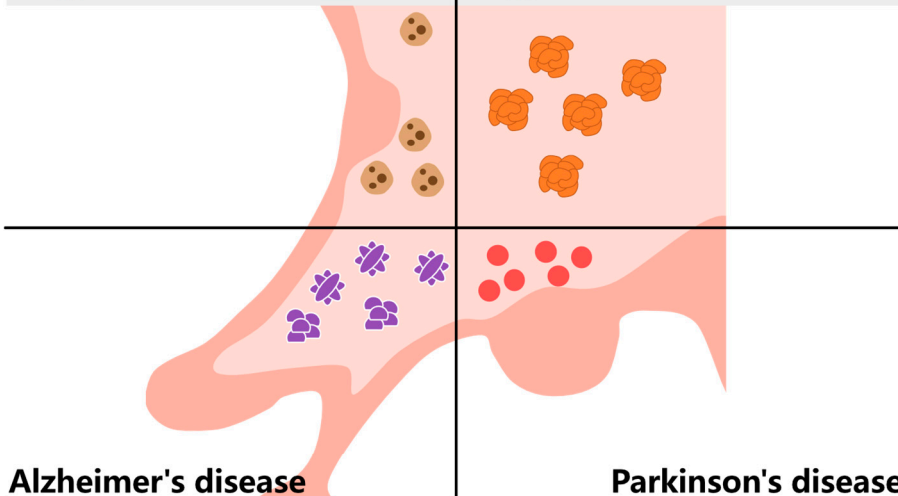
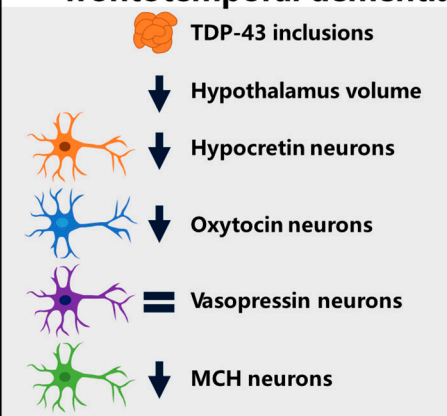
HD, ALS, FTD, AD and PD all exhibit similar hypothalamic pathology (**Figure 5**). Atrophy of hypothalamus is seen in early clinical ALS, FTD and AD [66, 86-89]. In HD, the volume of the lateral tuberal nucleus volume is reduced [90]. The distinct pathological signatures of these disorders have been observed in the hypothalamus, mHTT inclusions in HD [91], TDP-43 inclusions in both ALS and FTD[58, 66], amyloid plaques and neurofibrillary tangles in AD [74] and Lewy bodies in PD[92].

There is loss of key hypothalamic neuronal populations regulating emotion and metabolism across these disorders. In all of these NDs, neurons expressing hypocretin (orexin) are reduced [87, 91, 93-95]. Hypocretin is a neuropeptide involved in alertness, appetite and motivation. Neurons expressing melanin-concentrating hormone (MCH), involved in appetite and induction of REM sleep, are reduced in ALS and PD, but not HD and AD [94, 96, 97]. Both hypocretin and MCH are expressed in the lateral hypothalamic area. Furthermore, loss of neurons expressing oxytocin has been shown in HD, ALS and PD [87, 91, 93, 98] as well as vasopressin in HD [93]. Oxytocin and vasopressin both have an important role in social cognition, depression and stress [99].

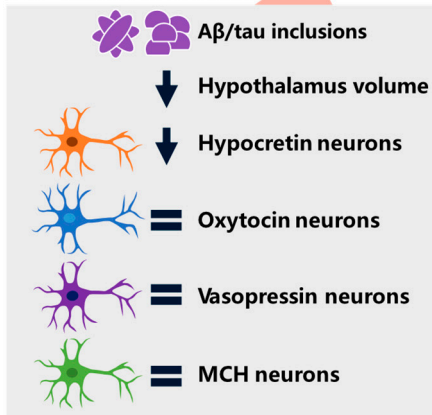
Huntington's disease



Amyotrophic lateral sclerosis frontotemporal dementia



Alzheimer's disease



Parkinson's disease

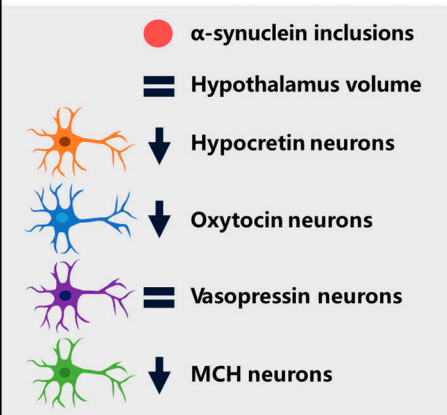


Figure 5. Hypothalamic pathology in neurodegenerative disorders.

Oxytocin

The oxytocin system constitutes a central focus of this thesis. Oxytocin is a neuropeptide synthesised primarily within the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus [100]. Oxytocin has been identified to play an important function in social behaviour as well as metabolism and food intake [101, 102]. Furthermore, oxytocin has a role in anxiety, depression and stress responses [103]. Given that non-motor features of HD and ALS-FTD include social cognition impairment and increased anxiety and depression, oxytocin may be involved in their pathophysiology. This is underscored by the correlation of oxytocin levels in the cerebrospinal fluid and social cognition in HD [104]. Furthermore, neuropathological data show loss of oxytocin-expressing neurons in persons with HD and ALS [87, 91, 93]. However, whether loss of oxytocin neurons occurs in FTD remains to be established.

TDP-43

Discovered over three decades ago as a novel transcriptional repressor of the HIV-1 virus [105], the TDP-43 protein has quickly emerged as having central role in a growing number of disorders. While it is the major pathological protein in ALS-FTD, TDP-43 pathology has been increasingly linked to other NDs, including AD, PD, HD and limbic-predominant age-related TDP-43 encephalopathy [76, 81, 106, 107]. More recently, TDP-43 has also been implicated in cancer and immunological disorders [108, 109]. This widespread involvement in NDs suggests a potential unifying pathological mechanism that may bridge these seemingly distinct conditions.

The TDP-43 protein is encoded by the *TARDBP* gene and is a highly conserved, multifunctional protein essential for overall cellular health. It is expressed ubiquitously throughout the body, including in neurons and glial cells in the central nervous system. TDP-43 primarily functions as an RNA binding protein to regulate gene expression. Its central role involves RNA processing and splicing [110, 111]. TDP-43 is also increasingly recognised for its importance in normal synaptic function and its involvement in the innate and adaptive immune system [112].

TDP-43 pathology

The term “TDP-43 pathology” is used throughout this thesis to refer to the defining mechanistic changes observed in TDP-43 proteinopathies (**Figure 6**). These changes include the mislocalisation of the protein from the nucleus to the cytoplasm, its subsequent biochemical modification via phosphorylation and/or ubiquitination

and its aggregation into inclusions within the cytoplasm or nucleus of neurons and glial cells [57, 72, 113]. These alterations are believed to severely disrupt normal cellular function and contribute to neurodegeneration.

TDP-43 pathology is the defining neuropathological hallmark of ALS-FTD. It is observed in brains from virtually all patients diagnosed with both conditions, including familial cases where the underlying genetic cause is a mutation in *C9ORF72* [114]. Separately, TDP-43 pathology is present in approximately 97% of ALS cases and 50% of all FTD cases.

The pathology is also prevalent in other NDs. In HD, TDP-43 is reduced in the nucleus, becomes phosphorylated and forms cytoplasmic inclusions that co-localise with mHTT [107]. Moreover, plasma TDP-43 levels have been shown to correlate with the severity of apathy in HD patients [115]. In AD, TDP-43 pathology is observed in the majority of cases (approximately 57%), where it is believed to be phosphorylated and interact with amyloid plaques and tau tangles [106]. TDP-43 pathology is also observed in a small subset of PD patients, typically characterised by cytoplasmic aggregation, phosphorylation and ubiquitination within the temporal lobe [82, 116].

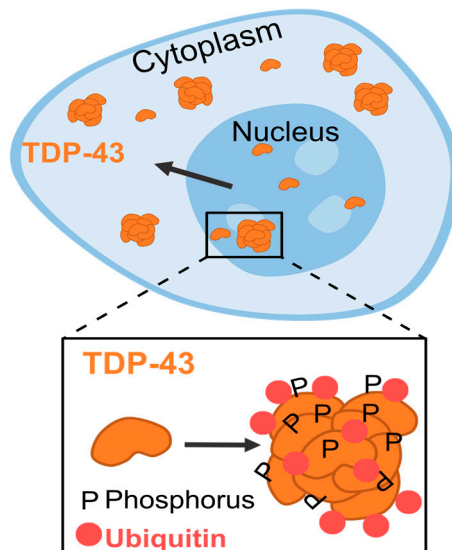


Figure 6. Pathological hallmarks of TDP-43 proteinopathies.

In affected neurons, the TDP-43 protein mislocalises from the nucleus into the cytoplasm. Once in the cytoplasm, the protein aggregates to form inclusions that are often hyperphosphorylated and ubiquitinated. These cytoplasmic inclusions are the most common pathological hallmark, though nuclear inclusions also form.

Aims

The overarching aim of this thesis was to identify the structure-to-function relationship between the hypothalamus and its circuitry and the development of psychiatric and metabolic features of HD and ALS-FTD. Our hypothesis was that the hypothalamus is especially vulnerable to the disease-related proteins, mHTT and TDP-43 and that this pathology contributes to the development of metabolic and psychiatric features of HD and ALS-FTD, respectively.

Aims and rationale

Paper I

TDP-43 is the major pathological protein in ALS-FTD and has been linked to an increasing number of NDs including HD. Although TDP-43-immunopositive inclusions are present in the hypothalamus in ALS-FTD, the role of hypothalamic TDP-43 for disease pathogenesis remains unclear. We hypothesize that TDP-43 leads to hypothalamic dysfunction, resulting in the development of psychiatric and metabolic features observed in patients with TDP-43 proteinopathies. In **Paper I**, we therefore aimed to investigate the causative effects of TDP-43 in the hypothalamus and its role in the development of features related to both ALS and FTD in mice.

Paper II

SIDT2 is a transmembrane protein mediating DNA/RNA autophagy via lysosome uptake and has been found to bind to mHTT, but its role in HD and the hypothalamus is unknown. We hypothesize that SIDT2 is involved in the clearance of mHTT protein via autophagy-mediated pathways and that the SIDT2 system is compromised in HD accelerating disease progression. Therefore, the aim of **Paper II** was to investigate the role of SIDT2 in HD.

Paper III and IV

The hypothalamic neuronal population that expresses oxytocin is known to be reduced in HD and ALS, but its role in these disorders remains unclear. We hypothesize that pathology within the oxytocin system contributes to specific psychiatric, social and metabolic impairments seen in patients with HD and ALS-FTD. The aims of these studies were to determine the effects of mHTT (**Paper III**) and TDP-43 (**Paper IV**) on the oxytocin system and its contribution to the development of non-motor features and neuropathology in mice. Furthermore, this thesis includes a meta-analysis investigating the comparative effects of TDP-43 and mHTT toxicity specifically within oxytocin neurons.

Summary of main results

Paper I

In **Paper I**, we investigated the role of wild-type TDP-43 specifically within the hypothalamus to investigate the development of neuropathology and the dysfunction of metabolism and emotion commonly observed in ALS-FTD (**Figure 7A**, main results in **Table 1**). To investigate this, we generated an adeno-associated viral (AAV) vector designed to express wild-type human TDP-43 (AAV-TDP43). The AAV vector utilised the same design and the same neuronal-specific synapsin promoter as previously reported in Hult et al. [117]. The vectors were administered via bilateral injections into the hypothalamus of adult female FVB/N mice.

AAV-vector mediated expression of human wild-type TDP-43 in the hypothalamus leads to neuropathology in a dose-dependent fashion

We first wanted to determine the neuropathological effects across different viral vector titres. TDP-43 overexpression across injected doses (5.0×10^{13} , 1.0×10^{14} , 2.5×10^{14} genome copies (gc)/ml) resulted in widespread TDP-43 immunoreactivity. This pathology was dose-dependent, with higher AAV-vector titres correlating with increased cellular loss and the presence of ubiquitin-immunoreactivity.

TDP-43 has previously been reported to result in neuroinflammation [118]. The AAV vector used in our study was designed to express TDP-43 exclusively within neurons, but we observed secondary effects on surrounding glial cells. Specifically, we observed increased glial fibrillary acidic protein (GFAP) immunoreactivity (suggesting astrogliosis) and distinct morphological changes in ionised calcium-binding adaptor molecule 1 (Iba1)-immunoreactive cells (suggesting microglial activation) in AAV-TDP43 mice compared to vehicle-injected controls. These findings suggest that neuronal TDP-43 overexpression triggers an inflammatory response.

TDP-43 overexpression causes hypothalamic atrophy with selective loss of neurons expressing hypocretin, oxytocin and MCH

To further investigate neuropathological effects of TDP-43 overexpression, we performed quantitative analysis that showed that AAV-TDP43 mice developed hypothalamic atrophy, exhibiting a 38% reduction in volume compared to control mice. This was accompanied by severe selective loss of neuropeptide-expressing populations: 92% loss of hypocretin-, 90% loss of MCH- and 42% loss of oxytocin-expressing neurons. In contrast, vasopressin-expressing neurons remained unaffected (**Figure 7B-F**).

This pattern of hypothalamic atrophy with loss of neurons expressing hypocretin, MCH and oxytocin while sparing vasopressin-expressing neurons in AAV-TDP43 mice replicates neuropathological findings reported in human ALS cases [87, 96] and is a common feature across multiple TDP-43 proteinopathies. Consistent with the neuropathological results in this study, bulk RNA sequencing revealed that mRNA levels of both the neuropeptide precursors and their corresponding receptors were reduced in the AAV-TDP43-injected mice. Taken together, our data suggest that TDP-43 overexpression in the hypothalamus results in pathology of MCH, hypocretin and oxytocin systems as seen in TDP-43 proteinopathies.

TDP-43 overexpression in the hypothalamus induces obesity and hyperglycaemia in mice

Metabolic dysfunction is a feature of ALS-FTD that might be caused by pathology of the hypothalamus. Given the selective loss of neurons expressing the neuropeptides hypocretin, MCH and oxytocin that regulate functions such as metabolism, we assessed metabolic function in another cohort of mice (**Figure 8A**). AAV-TDP43 mice displayed a 92% increase in body weight gain by 20 weeks post-injection (compared to 14% in vehicle-injected controls) and a doubling of body fat percentage (**Figure 8B-D**). Furthermore, mice exhibited a 20% increase in serum glucose, indicating the development of hyperglycaemia. Notably, the increase in body weight and fat was independent of food intake in a pair-housed cohort. Our data showed a profound disruption of metabolic homeostasis as a result of TDP-43 overexpression in the hypothalamus.

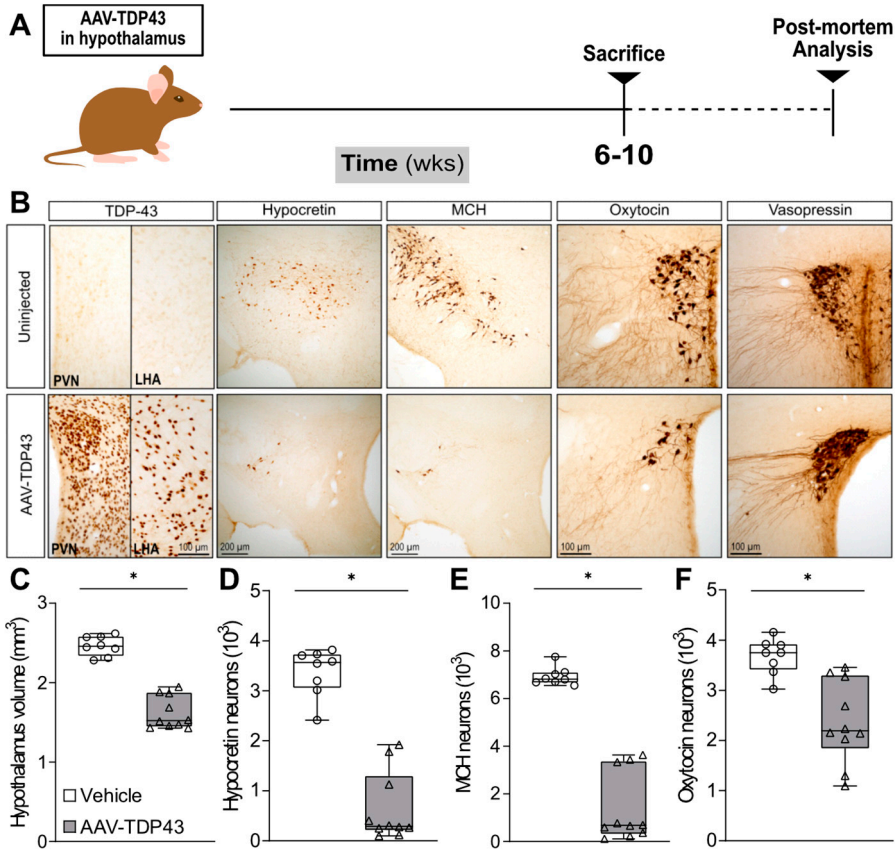


Figure 7. TDP-43 overexpression causes hypothalamic atrophy with selective loss of neurons expressing hypocretin, oxytocin and MCH.

(A) Schematic overview of experiment. (B) TDP-43 overexpression resulted in selective vulnerability to hypothalamic neuronal populations. AAV-TDP43 mice exhibited hypothalamic atrophy (C) and loss of neurons expressing hypocretin (D), MCH (E) and oxytocin (F).

Nuclear and cytoplasmic localisation of TDP-43 with inclusions following AAV-mediated TDP-43 expression in the hypothalamus

Wild-type TDP-43 is predominantly located in the nucleus, but a hallmark of TDP-43 pathology is the nuclear-to-cytoplasmic mislocalisation as well as nuclear and cytoplasmic inclusion formation [57, 72, 113]. In AAV-TDP43 mice, TDP-43 immunoreactivity was detected predominantly within the nucleus but was also notably present in the cytoplasm. Furthermore, TDP-43-immunopositive inclusions were identified in both the nucleus and the cytoplasm, although they were more prevalent in the cytoplasm (Figure 8E-H). These results demonstrate that AAV-mediated TDP-43 overexpression in the mouse hypothalamus successfully recapitulates key molecular hallmarks observed in ALS-FTD patients [57, 72, 113].

Mice overexpressing TDP-43 in the hypothalamus develop apathy-like behaviour

Apathy, anxiety and depression are features of ALS-FTD [54, 55, 60]. First, we used the open field test and rotarod to measure motor activity and motivation. AAV-TDP43 mice travelled a 67% shorter distance in the open field and showed an 82% decrease in latency to fall on the rotarod compared to vehicle-injected controls, showing a profound decrease in activity levels (**Figure 8I-J**). To assess anxiety-like behaviour we used the elevated plus maze, AAV-TDP43 mice reduced distance travelled by 93%, compared to vehicle-injected control mice, showing an even greater reduction in activity compared to the open field and rotarod test. The mice spent more time in the centre of the arena, where the trial began (**Figure 8K**). Specifically, the median time spent on the closed arms was 0% for AAV-TDP43 (versus 55% for vehicle-injected controls) and 0% on the open arms (versus 20% for controls). These results primarily suggest a reduction in exploratory behaviour and general motor activity, and thereby limiting the interpretation of an anxiety-like phenotype.

Crucially, the forced swim test, typically used to assess depression-like behaviour, revealed no difference in mobility, indicating that the mice are not depressed and that the reduction in activity was not due to an inability to move but was rather due to a lack of motivation, such as apathy (**Figure 8L**). This is further supported by reduced nesting behaviour observed in a separate cohort at 8 weeks post-injection (**Figure 9B-D**).

Mice overexpressing TDP-43 in the hypothalamus exhibited significant increased skipping of constitutive exons

Given that TDP-43 is an RNA-binding protein with a key role in splicing and RNA processing, we performed bulk RNA sequencing to further determine the molecular consequences of TDP-43 overexpression. A key feature of TDP-43 proteinopathies is a resulting increase in cryptic exons and the repression of constitutive exons [119, 120]. AAV-TDP43 mice displayed increased exon skipping of constitutive exons, such as *Ddi2* exon 9, *Psmc14* exon 5 and *Mrl45* exon 7 (**Figure 9E**), similar to what has been reported in the murine spinal cord overexpressing TDP-43 [121]. However, we found no significant expression of cryptic exons, including those previously described in *Ap3b2* and *Camk1g* in mice after TDP-43 deletion [122], or in *Stmn2*, *Sncb* and *Unc13a*. These results indicate that TDP-43 overexpression in the hypothalamus results in disruption in splicing.

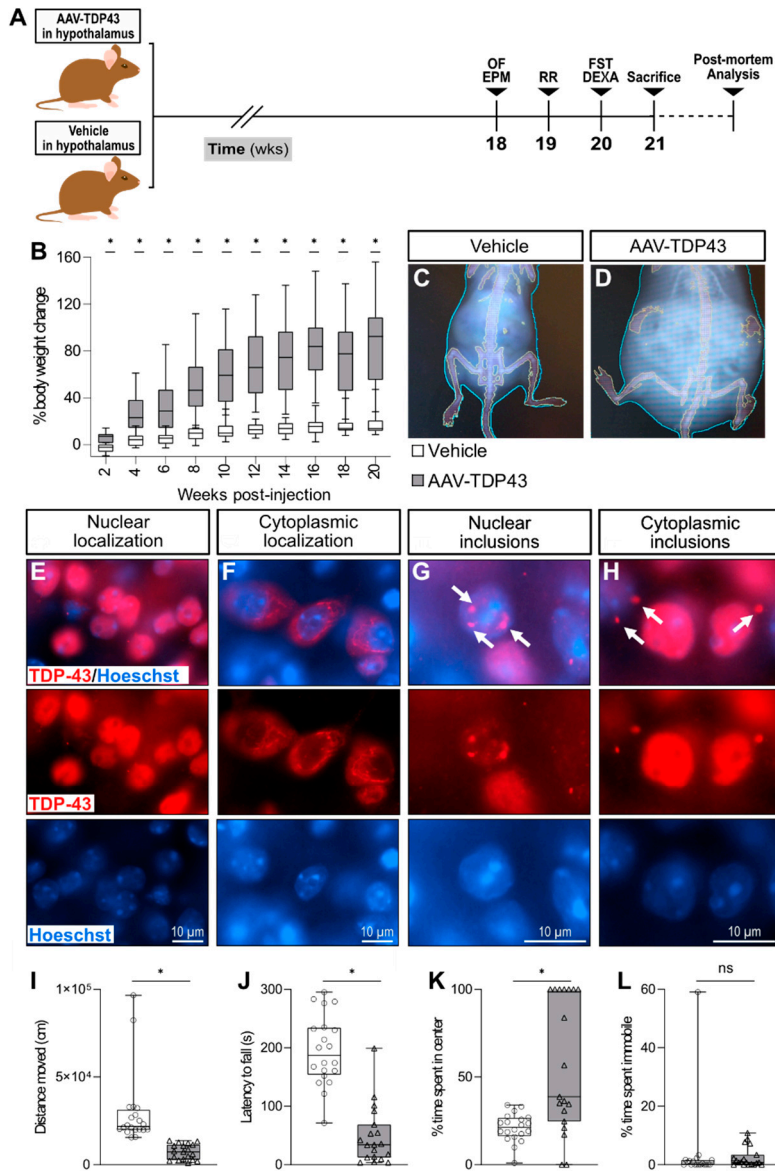


Figure 8. TDP-43 mislocalises with cytoplasmic inclusion formation, metabolic impairment, and reduced motor activity following hypothalamic TDP-43 overexpression.

(A) Experimental schematic. **(B)** AAV-TDP43 mice showed significant weight gain starting at two weeks post-injection, resulting in nearly double the total weight gain compared to vehicle-injected controls by 20 weeks. **(C-D)** dual-energy X-ray absorptiometry (DEXA) scanning showed a doubling in body fat percentage at 20 weeks post-injection. **(I-J)** TDP-43 immunoreactivity was predominantly nuclear **(I)** but also notably present in the cytoplasm **(J)**. **(K-L)** TDP-43-immunopositive inclusions were identified in both the nucleus **(K)** and the cytoplasm **(L)**. **(E-F)** AAV-TDP43 mice exhibited reduced distance travelled in the open field test and decreased latency to fall in the rotarod test. **(G)** In the elevated plus maze, AAV-TDP43 mice spent more time in the centre of the arena, where mice were placed at the beginning of the test. **(H)** The forced swim test revealed no difference in mobility.

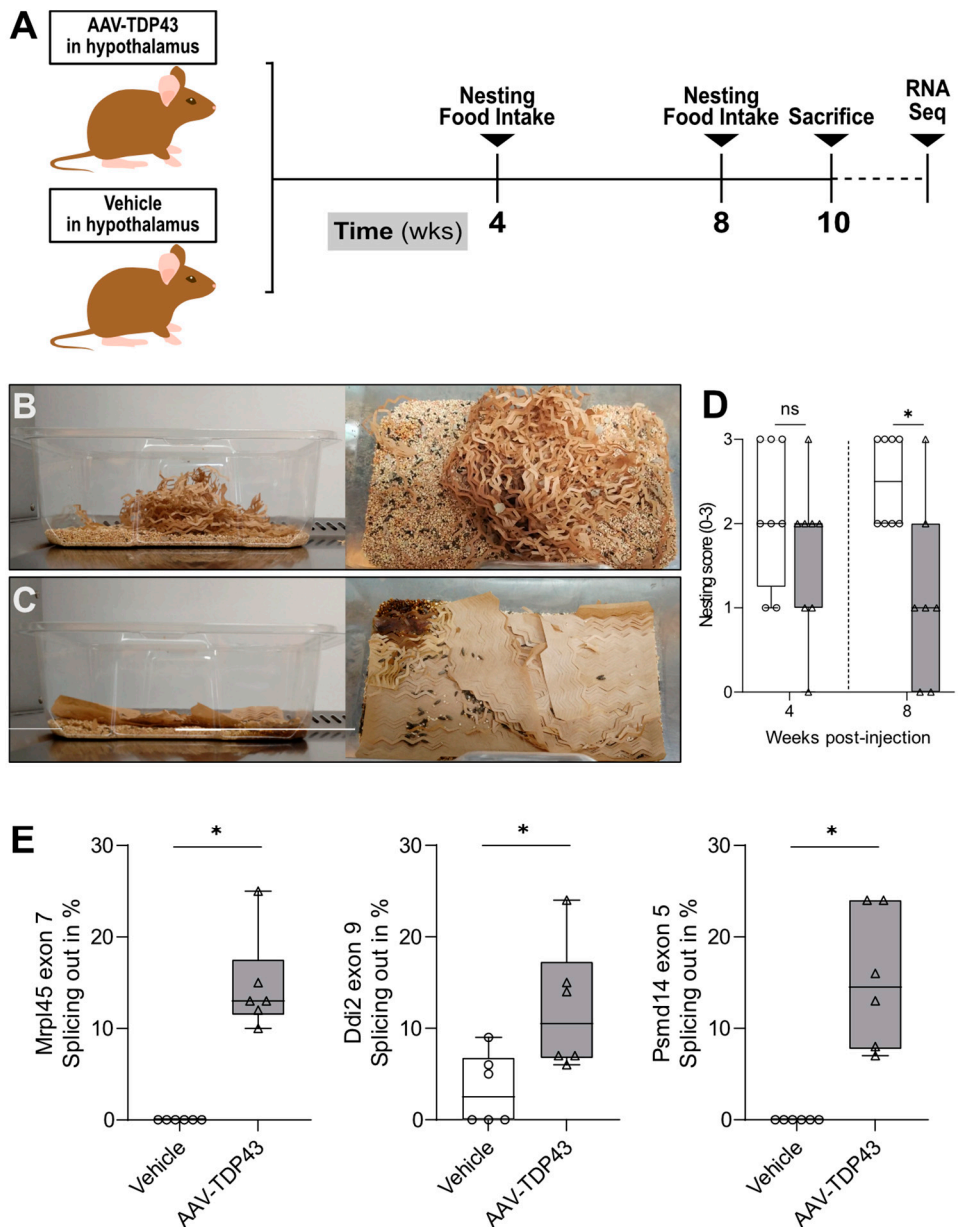


Figure 9. Hypothalamic TDP-43 overexpression induces apathy-like behaviour and alters RNA splicing.

(A) Experimental schematic. **(B-C)** Representative images of nesting behaviour scored on a 0-3 scale, illustrating a dome-like nest **(B)** and poor nesting behaviour **(C)**. **(D)** AAV-TDP43 mice exhibited significantly reduced nesting behaviour, indicating an apathy-like phenotype, at 8 weeks post-injection. **(E)** AAV-TDP43 mice displayed increased exon skipping on constitutive exons, including *Ddi2* exon 9, *Psm14* exon 5 and *Mrpl45* exon 7.

Table 1. Main results from Paper I.

	Analysis	Treatment		Results
Neuropathology	IHC, stereology	AAV-TDP43 vs uninjected	↓	MCH, Hypocretin (orexin), oxytocin neurons
			=	Vasopressin neurons
			↓	Hypothalamus volume
				TDP-43 cytoplasmic localisation
				TDP-43 nuclear and cytoplasmic inclusions
	RNA seq	AAV-TDP43 vs vehicle		Ubiquitin-immunoreactivity
			↑	GFAP-immunoreactivity
			↑	Microglia activation
			↓	MCH-, Hypocretin (orexin)-, oxytocin-, AGRP- related transcripts
			↓	Ddi2 exon 9, Psmc14 exon 5 and Mrl45 exon 7
			=	Ap3b2, Camk1g, Stmn2, Snca and Unc13a CE
Metabolism	Glucose oxidase assay		↑	Serum glucose concentration
	DEXA		↑	Body fat
	Weighing		↑	Body weight gain
Behaviour	Nesting	AAV-TDP43 vs vehicle	↓	Nesting behaviour
	FST		=	Depressive-like behaviour
	RR		↓	Motor activity, motivation
	EPM			Inconclusive
	OF		↓	Motor activity

Abbreviations IHC: immunohistochemistry, MCH: melanin-concentrating hormone, GFAP: glial fibrillary acidic protein, Iba1: ionised calcium-binding adaptor molecule 1, AGRP: agouti-related peptide, CE: cryptic exons, DEXA: dual-energy X-ray absorptiometry, FST: forced swim test, RR: rotarod, EPM: elevated plus maze, OF: open field.

Paper II

In **Paper II**, we examined the involvement of the SIDT2 system in post-mortem HD brains (with Vonsattel grades 0-4), in three mouse models (R6/2, BACHD and AAV-mediated mHTT expressing mice) and in one *in vitro* system for HD (main results in **Table 2**).

SIDT2 levels are reduced in striatum and lateral hypothalamus in human HD cases

SIDT2 is involved in the process of DNA/RNA mediated autophagy and may represent a critical protein in understanding the clearance of mHTT transcripts. We first investigated the SIDT2 protein levels in HD. Western blot analysis of human post-mortem tissue showed a significant depletion of SIDT2 protein in affected regions, with a 68-93% reduction detected in the caudate nucleus of the striatum and a 67% reduction in the lateral hypothalamic area of HD cases compared to controls (**Figure 10a-c**). No significant differences were observed in the frontal cortex protein levels or in SIDT2 mRNA levels in the caudate nucleus or frontal cortex (**Figure 10d-f**). Our data reveal a dysregulation of the SIDT2 system, with depletion of the SIDT2 protein in the most vulnerable brain regions in HD, which seems to occur independently of SIDT2 transcription.

CAG-dependent frequency of SIDT2-immunoreactive inclusions in the HD anterior cingulate cortex

The anterior cingulate cortex (ACC) of the cerebral cortex is a vulnerable brain area that exhibits frequent mHTT inclusions. Immunohistochemical analysis revealed SIDT2 immunoreactivity in the nucleus, cytoplasm and the dendritic processes of neurons in HD. The frequency of SIDT2 inclusions was dependent on CAG repeat length, being rare in cases with CAG 42-45 but frequent in those with CAG 50-66 (**Figure 11a, c, e, g, i**). HTT was detected and visualised using the EM48 antibody that recognizes exon 1 within the N-terminal and is conformation-specific [123]. The density of mHTT inclusions similarly increased with CAG length, as detected with the EM48 antibody confirming prior observations in the HD cerebral cortex [124] (**Figure 11b, d, f, h, j**). Furthermore, HD cases exhibited SIDT2-immunoreactive intranuclear inclusions, primarily localised to layers IV and V of the ACC (**Figure 11k**). SIDT2 and mHTT inclusions rarely co-localised (**Figure 11l**). Our data further suggests dysregulation of the SIDT2 system and forms inclusions in HD brains in a CAG repeat-dependent fashion.

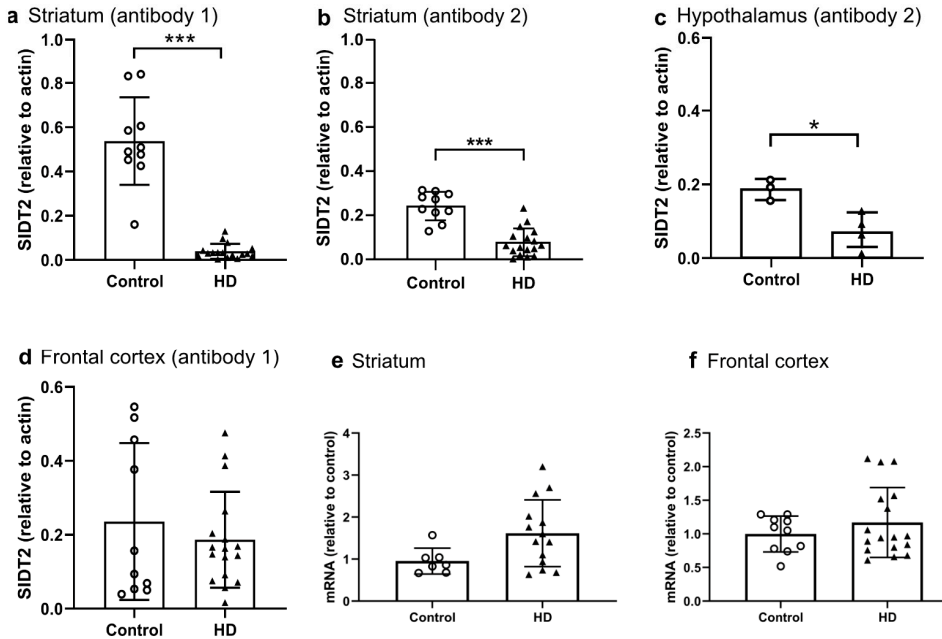
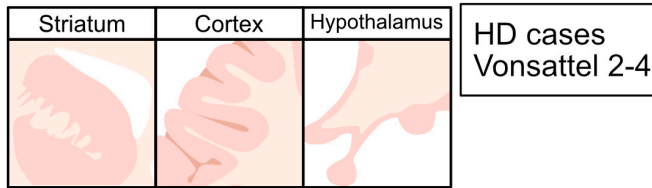


Figure 10. SIDT2 protein levels are reduced in affected brain regions of HD cases.

(a-c) Western blot analysis of post-mortem human tissue revealed a significant reduction of SIDT2 protein in the caudate nucleus of the striatum (a-b) and the lateral hypothalamic area (c) in HD cases (Vonsattel Grade 2-4) compared to controls. (d) No significant difference was detected in SIDT2 protein levels in the frontal cortex. (e) SIDT2 mRNA levels in the caudate nucleus and frontal cortex were unchanged, indicating the depletion is post-transcriptional.

SIDT2-immunoreactive inclusions are present in an HD case with Vonsattel grade 0

To investigate the early neuropathological profile of SIDT2 in HD before the onset of motor disturbances, we analysed a Vonsattel grade 0 HD case (43 CAG repeats) [93]. Inclusions of mHTT were frequent in the frontal cortex, caudate nucleus of the striatum and lateral hypothalamic area as detected using the EM48 antibody and SIDT2-immunoreactive inclusions were detected in the same regions (Figure 11o-p). SIDT2 inclusions were mainly located in layers I and II in the frontal cortex. Immunofluorescent analysis confirmed that most SIDT2 inclusions do not co-

localise with mHTT inclusions (**Figure 11m-n**). The present study reveals that SIDA2-immunoreactive inclusions constitute an additional neuropathological hallmark. Since SIDA2-immunoreactive inclusions are rarely co-localised with mHTT inclusions, the formation of these inclusions may occur independently.

SIDA2-immunoreactive inclusions appear later than the formation of mHTT inclusions in the R6/2 HD mouse model

We observed clear changes in the SIDA2 system in cases with HD. In order to further investigate the effects on the SIDA2 system in HD, we assessed whether SIDA2 immunoreactive inclusions would be present in animal models of HD that express different variants of mHTT. In this study, we used an R6/2 mouse model that expresses a 276-296 CAG repeat tract within the N-terminal fragment of HTT. This model characterised by its rapid onset and an aggressive disease phenotype [125, 126]. The R6/2 mouse model showed that while mHTT inclusions were present by 6 weeks of age, SIDA2-immunoreactive inclusions became detectable at 16 weeks of age in the striatum, hypothalamus and cortex (**Figure 12**). We investigated the effects of SIDA2 inclusion formation in another HD model with AAV-mediated expression of a large HTT fragment with 78Q in the hypothalamus. We observed a similar delayed appearance of SIDA2 inclusion formation (**Figure 13a-c**) following mHTT inclusion formation (**Figure 13d-f**). Mutant HTT was detected with the SC-8767 antibody which binds upstream to the polyglutamine tract. In contrast, the BACHD mouse model, which expresses full-length HTT with approximately 97 polyglutamine is more stable than the R6/2 mouse [127]. In this model we found no mHTT or SIDA2 inclusions. Collectively, these neuropathological findings suggest a pathological sequence where SIDA2 inclusions form after the mHTT inclusions.

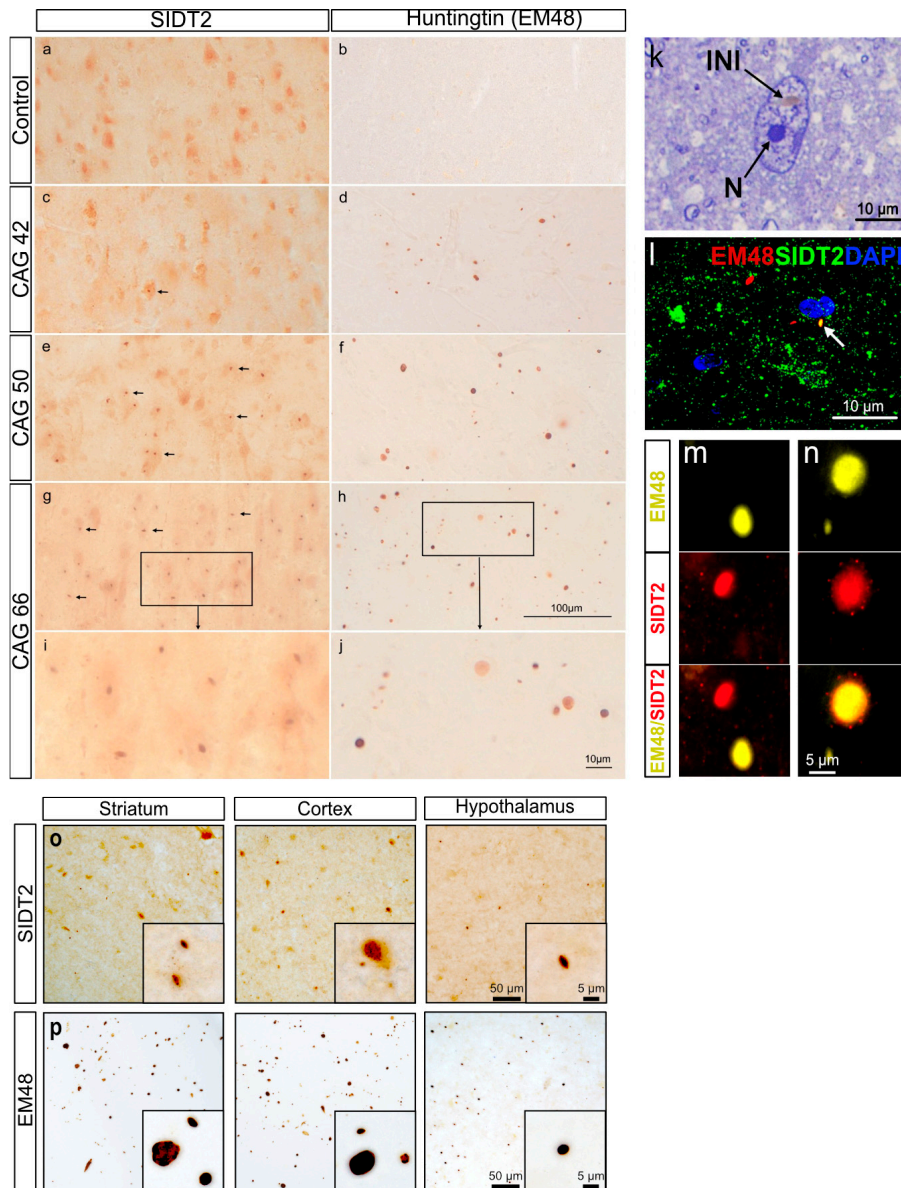


Figure 11. SIDT2-immunoreactive inclusions exhibit CAG-dependent frequency and are present in an HD Vonsattel 0 case.

(a, c, e, g, i) The frequency of SIDT2-immunoreactive inclusions in the anterior cingulate cortex was dependent on CAG repeat length, being rare in cases with CAG 42-45 but frequent in those with CAG 50-66. (b, d, f, h, j) The density of mHTT inclusions similarly increased with CAG length. (k) HD cases exhibited SIDT2-immunoreactive intranuclear inclusions. (l) Immunofluorescent analysis showed that SIDT2 and mHTT inclusions rarely co-localised. (m-p) In a Vonsattel grade 0 HD case (43 CAG repeats), inclusions of SIDT2 (o) and mHTT (p) were both detected in the frontal cortex, caudate nucleus of the striatum and lateral hypothalamic area, but rarely co-localised (m, n).

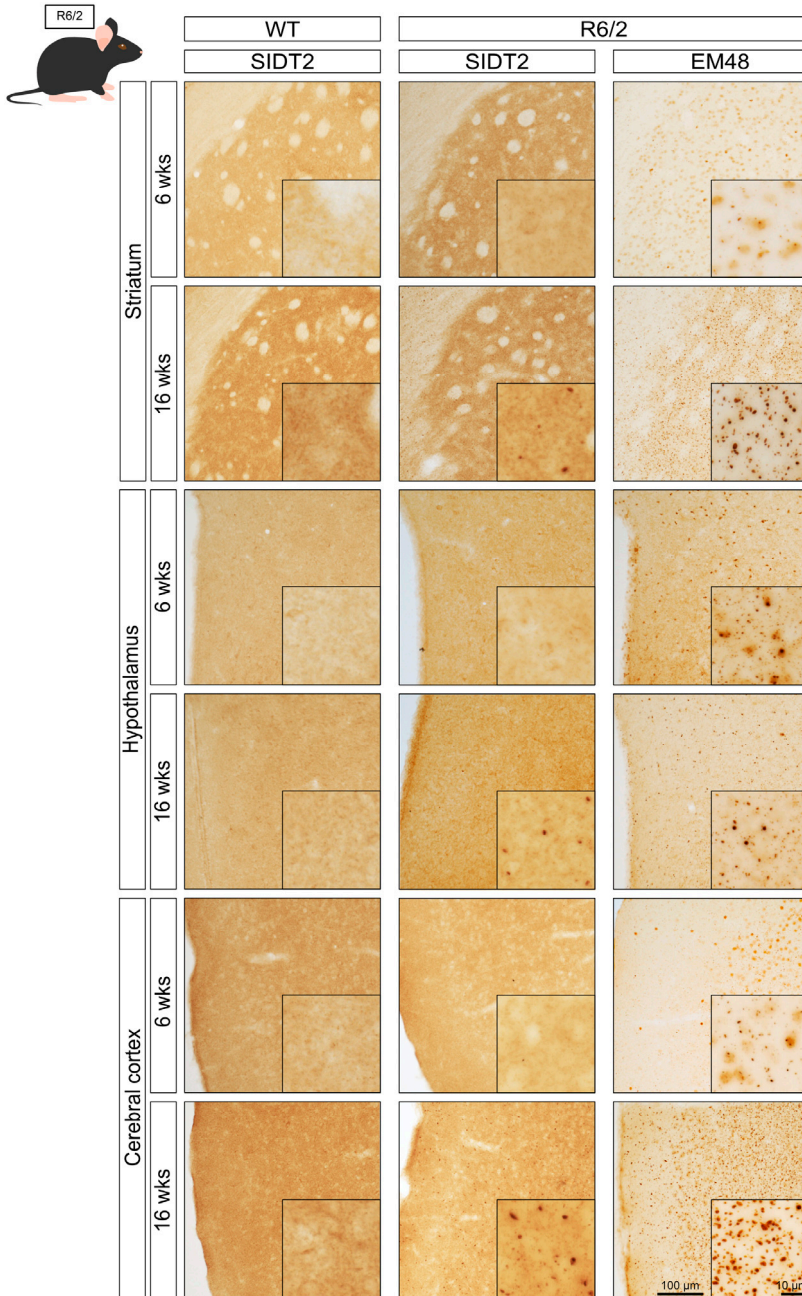


Figure 12. SIDT2-immunoreactive inclusions appear subsequent to mHTT inclusion formation in R6/2 mice.

In the R6/2 mouse model, mHTT inclusions were present by 6 weeks of age, but SIDT2-immunoreactive inclusions only became detectable at 18 weeks of age across the striatum, hypothalamus and cerebral cortex.

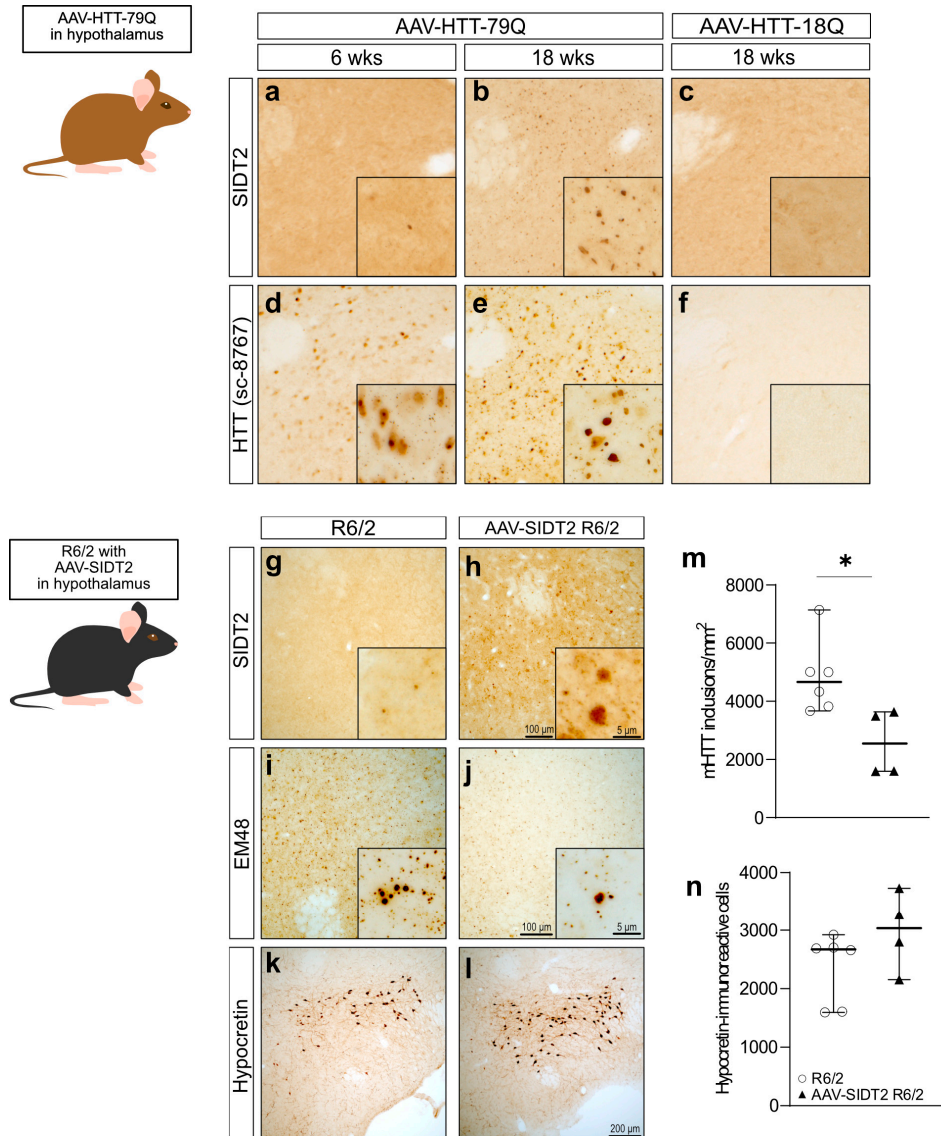


Figure 13. SIDT2 inclusions follow mHTT formation in AAV-HTT-79Q mice, and SIDT2 overexpression reduces mHTT pathology *in vivo*.

(a,d) In the AAV-HTT-79Q mouse model overexpressing mHTT in the hypothalamus, individual SIDT2 inclusions were present at six weeks post-injection (a), while mHTT inclusions were already abundant (d). (b,e) at 18 weeks post-injection, SIDT2 inclusions were abundant (b) as well mHTT inclusions (e). (c, f) No SIDT2 or HTT inclusions were present in mice injected with AAV-HTT-18Q, overexpressing wild-type HTT in the hypothalamus, at 18 weeks post-injection. (g-j, m) In R6/2 mice, AAV-vector mediated SIDT2 overexpression in the lateral hypothalamic area resulted in a significant reduction in the density of mHTT inclusions at 10 weeks post-injection compared to uninjected R6/2 mice. (k, l, n) Furthermore, SIDT2 overexpression showed a trend toward increased preservation of vulnerable hypocretin neurons compared to R6/2 controls.

Overexpression of SIDT2 using AAV-vectors reduces the number of mHTT inclusions in the lateral hypothalamic area in R6/2 mice

Considering the role of SIDT2 in autophagy, we wanted to investigate potential therapeutic potential of SIDT2. In an *in vivo* proof-of-principle experiment, we overexpressed human SIDT2 via an AAV vector injected into the lateral hypothalamic area of R6/2 mice (**Figure 13g, h**). At 10 weeks post-injection, SIDT2 overexpression resulted in a significant 45% reduction in the density of mHTT inclusions (**Figure 13i, j, m**). Furthermore, we observed a trend toward increased preservation of vulnerable hypocretin neurons (16% increase) (**Figure 13k, l, n**). Previous studies report loss of hypocretin neurons in HD cases as well as in R6/2 mice [91, 128]. This is the first demonstration that SIDT2 overexpression can reduce mHTT pathology *in vivo*, supporting the therapeutic potential of modulating this pathway.

Overexpression of SIDT2 reduces soluble and insoluble mHTT protein in SH-SY5Y cells without affecting major proteolytic degradation pathways

To validate these findings in a human cell model and investigate the mechanism of SIDT2, we overexpressed mouse *Sidt2* in SH-SY5Y neuroblastoma cells that co-expressed HTT exon 1 (72Q or 16Q) (**Figure 14a**). SIDT2 overexpression significantly reduced both soluble and insoluble mHTT exon 1 72Q levels (**Figure 14b-c**), confirming findings from a murine cell model [45]. Analysis of protein degradation markers revealed no significant changes in K48-polyubiquitin, p62, or the LC3B-II to LC3B-I ratio. These data confirm the mHTT-lowering potential of SIDT2 and suggests that this effect is independent of the canonical proteasomal UPS and baseline macroautophagy pathways. Furthermore, SIDT2 overexpression induced inclusion-like structures that rarely co-localised with mHTT inclusions (**Figure 14d-e**). These findings mirror our findings in HD cases and further support the therapeutic potential of SIDT2 with HTT protein lowering potential independently of the two major protein degradation pathways.

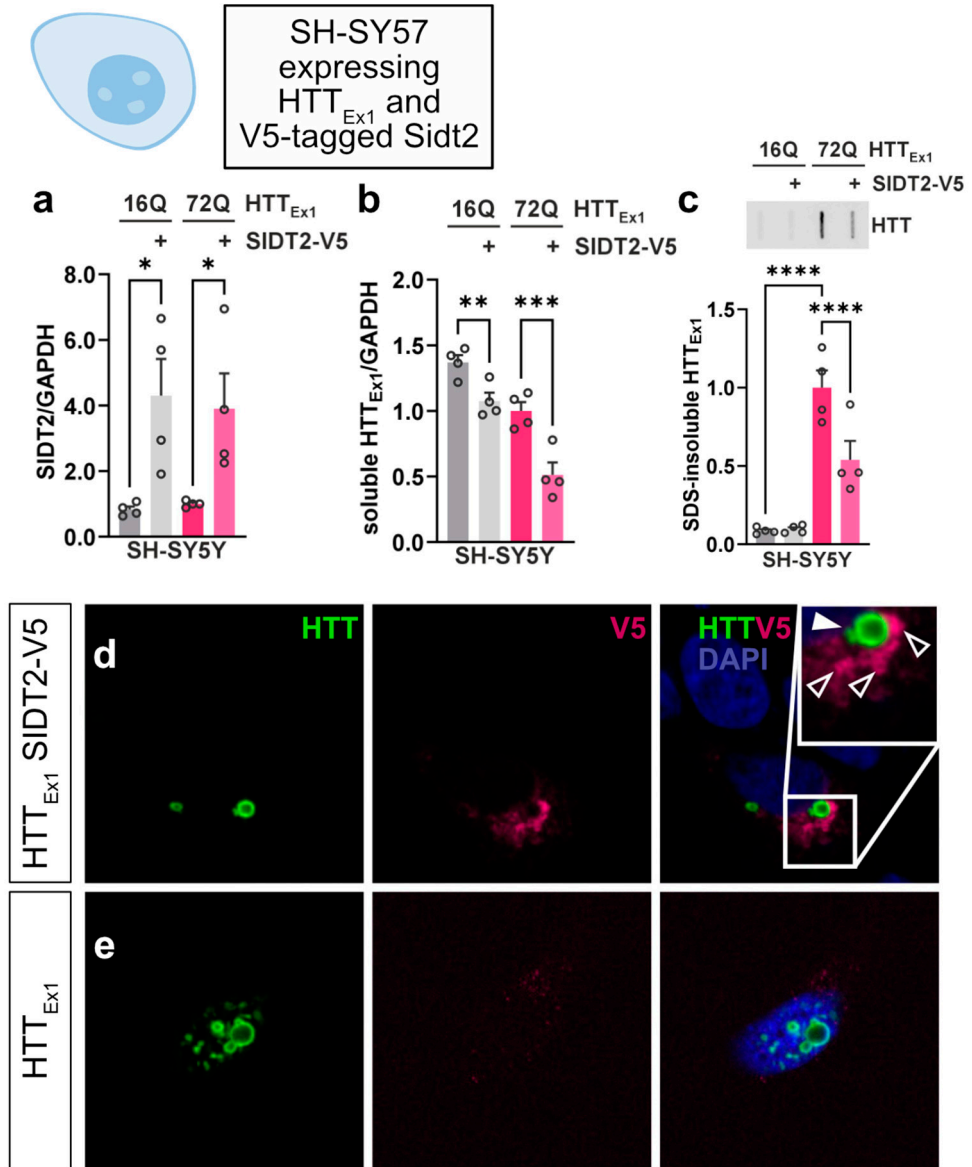


Figure 14. SIDT2 overexpression reduces soluble and insoluble mHTT protein in SH-SY5Y cells. (a) SIDT2 was overexpressed in SH-SY5Y neuroblastoma cells co-expressing HTT exon 1 (72Q or 16Q). (b-c) SIDT2 overexpression significantly reduced both soluble (b) and insoluble (c) mHTT exon 1 72Q protein levels. (d-e) Immunofluorescent analysis showed that SIDT2 overexpression induced inclusion-like structures that rarely co-localised with mHTT inclusions.

Table 2. Main results from Paper II

Organism	Tissue	Analysis	Groups	Results
Human	Striatum, LHA, F. cortex, ACC	IHC	Grade 0-4	↑ SIDT2 and HTT inclusions
	F. cortex, ACC		Grade 0-4	SIDT2 and HTT inclusions can co-localise
	ACC		Grade 2-4	CAG-dependent increase in SIDT2 inclusions
	Striatum, LHA	Western blot		↓ SIDT2 protein levels
	Striatum, F. cortex	qRT-PCR		= SIDT2 mRNA
Mouse (R6/2)	Striatum, hypothalamus, cortex	IHC	R6/2 vs WT	↑ SIDT2 and HTT inclusions
				↑ SIDT2 inclusions over time SIDT2 inclusions form after mHTT inclusions
	LHA	IHC	AAV-SIDT2 R6/2 vs R6/2	↑ SIDT2 expression
				↓ HTT inclusions
Mouse (AAV)	Hypothalamus	IHC	HTT-79Q vs HTT-18Q	↑ SIDT2 and HTT inclusions
				↑ SIDT2 inclusions over time SIDT2 inclusion form after HTT inclusions
Mouse (BACHD)	Striatum, hypothalamus, cortex	IHC	BACHD vs WT	No SIDT2 or HTT inclusion
Cell (SH-SY5Y)	Neuro-blastoma	Western blot	72Q-SIDT2 vs 72Q, 16Q-SIDT2 vs 16Q	↑ SIDT2 expression
		Western blot	72Q-SIDT2 vs 72Q, 16Q-SIDT2 vs 16Q	↓ Soluble HTT exon 1
		Filter retardation	72Q-SIDT2 vs 72Q	↓ Insoluble HTT exon 1
		Western blot	72Q-SIDT2 vs 72Q, 16Q-SIDT2 vs 16Q	= K48+ proteins, P62, LC3B-II/LC3B-I
		ICC	72Q-SIDT2, 16Q-SIDT2	SIDT2 and HTT inclusions can co-localise

Abbreviations, LHA: lateral hypothalamic area, IHC: immunohistochemistry, F: frontal, ACC: anterior cingulate cortex, WT: wild-type, ICC: immunocytochemistry.

Paper III

Paper III aimed to investigate the role of mHTT in oxytocin neurons. The paper was composed of two distinct experiments (main results in **Table 3**) designed to investigate the causative role of mHTT specifically in oxytocin neurons on the development of neuropathology and non-motor features of HD.

In the first experiment, to ensure mHTT expression only in oxytocin neurons, we used a flex-switch AAV vector to selectively express mHTT with 100Q (AAV-N248-100Q) in the presence of Cre-recombinase (Cre) (**Figure 15A**). The vector was bilaterally injected into the PVN of a mouse model expressing Cre under the control of the neuronal synapsin-1 promoter (oxytocin-Cre). Control groups consisted of uninjected mice or mice expressing GFP (flex-switch AAV-mCherry/GFP) or wild-type HTT(AAV-N166-18Q).

In the second experiment, we deleted mHTT in oxytocin neurons within the BACHD mouse model. The BACHD mouse, which ubiquitously expresses full-length mHTT with approximately 97 CAG repeats [127], was crossed with the oxytocin-Cre line, resulting in BACHD/oxytocin-Cre mice that have ubiquitous mHTT expression except in oxytocin neurons (**Figure 15B**).

Loss of oxytocin-expressing neurons in mice with intranuclear mHTT inclusions in oxytocin neurons

Oxytocin neurons have been reported to be decreased in numbers in advanced and pre-symptomatic HD cases [91, 93]. We first investigated the neuropathological profile following selective mHTT expression in oxytocin neurons. AAV-N248-100Q injection produced two inclusion subtypes. One subset formed larger, predominantly intranuclear inclusions (IN), while the other formed smaller more clustered and predominantly extranuclear inclusions (EN) (**Figure 15C**). Both subtypes developed mHTT inclusions within the PVN. The subset forming IN mHTT inclusions showed a significant 22-26% reduction in oxytocin-expressing neurons compared to control groups and the EN subset (**Figure 15D**). This finding aligns with the 45% loss of oxytocin neurons reported in human HD cases [91, 93]. This suggests a potential direct link between IN formation and neurotoxicity in the oxytocin system.

Increased body weight gain in female mice that formed intranuclear mHTT inclusions

We observed a loss of oxytocin-expressing neurons following formation of mHTT IN inclusions. Increased body weight gain has previously been linked to expression of mHTT in the entire hypothalamus [129], contrary to observations in persons with

HD. We investigated whether selective mHTT expression in oxytocin neurons would affect body weight. Selective mHTT expression produced a significant sex-dependent effect. Female mice with IN mHTT inclusions exhibited increased body weight gain at 12 weeks post-injection compared to EN mHTT inclusion mice (**Figure 15E-F**). However, mHTT deletion in the BACHD model offered no rescue effect on weight gain. The development of increased body weight gain has also been reported in BACHD mice [130, 131], an effect that has been attributed to mHTT expression selectively in the hypothalamus, leading to increased food intake [129]. Therefore, while we investigated whether the weight gain observed following mHTT expression could be specifically linked to the oxytocin system, the data from the present study found no definitive support for this notion.

Increased anxiety-like behaviour in mice that formed intranuclear inclusions compared with uninjected mice

Oxytocin has been linked to a range of non-motor features of HD, including anxiety and depression, and social behaviour [132]. Next, we wanted to investigate if mHTT in oxytocin neurons affects anxiety-like behaviour. The elevated plus maze showed an increase in anxiety-like behaviour in N248-100Q IN and N166-18Q mice compared to uninjected mice, but not with other groups (**Figure 15G**). Depletion of mHTT expression in oxytocin neurons in the BACHD model showed no rescue of the anxiety-like phenotype observed in BACHD mice. Overall, we found no major effect on anxiety-like behaviour in the mHTT overexpression or deletion experiments. This suggests that mHTT pathology specific to oxytocin neurons may not be the primary driver of the anxiety phenotype seen in HD. Expression nor deletion of mHTT in oxytocin neurons affected depressive-like behaviour (assessed using forced swim test) or social behaviour (assessed with the social interaction test) at the measured time points. The lack of effect suggests that mHTT in oxytocin neurons is insufficient to drive these phenotypes at this time point.

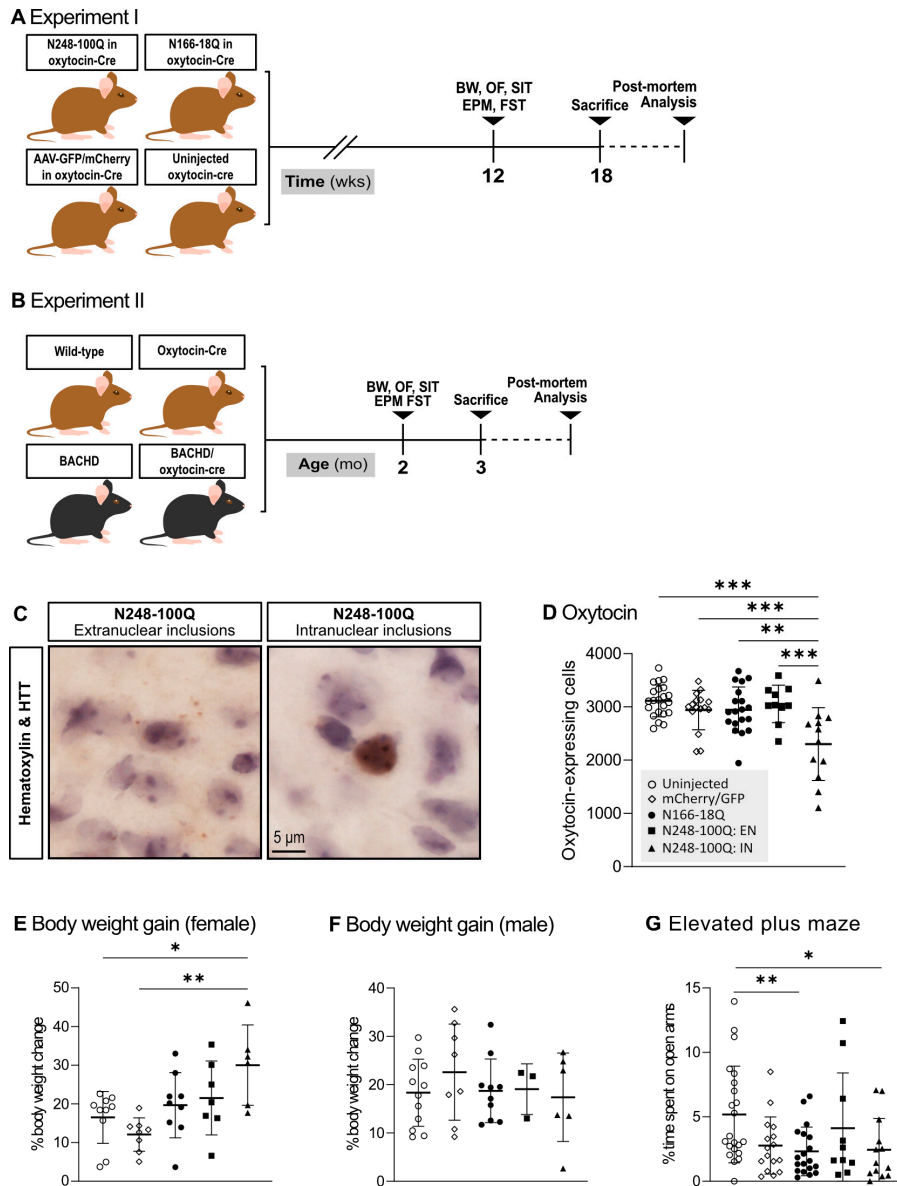


Figure 15. Intranuclear mHTT inclusions in oxytocin neurons drives loss of these neurons, increase both body weight and anxiety-like behaviour in female mice.

(A-B) Schematic of Experiment I (A) and II (B). (C) AAV-N248-100Q injection produced two distinct inclusion phenotypes: a subset with intranuclear (IN) inclusions and a subset with extranuclear (EN) inclusions. (D) The IN subset showed a significant reduction in oxytocin-expressing neurons compared to control groups and the EN subset. (E-F) Female mice developing IN mHTT inclusions exhibited increased body weight at 12 weeks post-injection (E), no body weight change was observed in male mice (F). Both N248-100Q IN and N166-18Q mice displayed increased anxiety-like behaviour in the elevated plus maze compared to uninjected controls (G).

Paper IV

Selective TDP-43 overexpression in oxytocin neurons results in loss of oxytocin-expressing neurons compared to uninjected mice with no effect on behaviour and metabolism

In **Paper IV**, we investigated the role of TDP-43 in oxytocin neurons. We investigated the direct causal effects of selective wild-type TDP-43 overexpression in oxytocin neurons on emotional and behavioural features of ALS-FTD (**Figure 16A**, main results in **Table 3**) similar to the first experiment in **Paper III**. Here we used a flex-switch vector designed to express TDP-43 in oxytocin neurons. This vector was bilaterally injected into the PVN of oxytocin-Cre mice.

Oxytocin neurons have been reported to be decreased by 33% and phosphorylated TDP-43 has been observed in oxytocin neurons in ALS cases [87]. TDP-43 overexpression in oxytocin neurons resulted in a 19% reduction in oxytocin-expressing neurons compared to uninjected controls. However, no significant difference was observed relative to mice expressing GFP in oxytocin neurons (AAV-mCherry/GFP) (**Figure 16B, D**). Furthermore, TDP-43 overexpression in oxytocin neurons had no major effects on metabolism or behaviour at this timepoint. Male mice showed a strong negative correlation between TDP-43 targeting efficacy and body weight gain, whereas female mice exhibited a non-significant trend toward a positive correlation (**Figure 16E-K**). An interesting exception was the rotarod test, where AAV-TDP43 mice showed an unexpected rescue effect compared to AAV-GFP controls, which may point to a beneficial impact on motor activity. We found no evidence of pathogenic molecular properties such as TDP-43 cytoplasmic mislocalisation or inclusion formation (**Figure 16C**).

These results stand in contrast to **Paper I**, where widespread TDP-43 overexpression across the entire hypothalamus led to a pronounced 42% loss of oxytocin-expressing neurons, downregulation of oxytocin-related transcripts, and clear metabolic and behavioural impairment. The significant difference in oxytocin system pathology between these two models suggests that the effects of TDP-43 are not solely cell-autonomous. Our data also indicate that the metabolic and behavioural phenotype observed in clinical ALS-FTD may not be attributed solely to the effects of TDP-43 within oxytocin neurons.

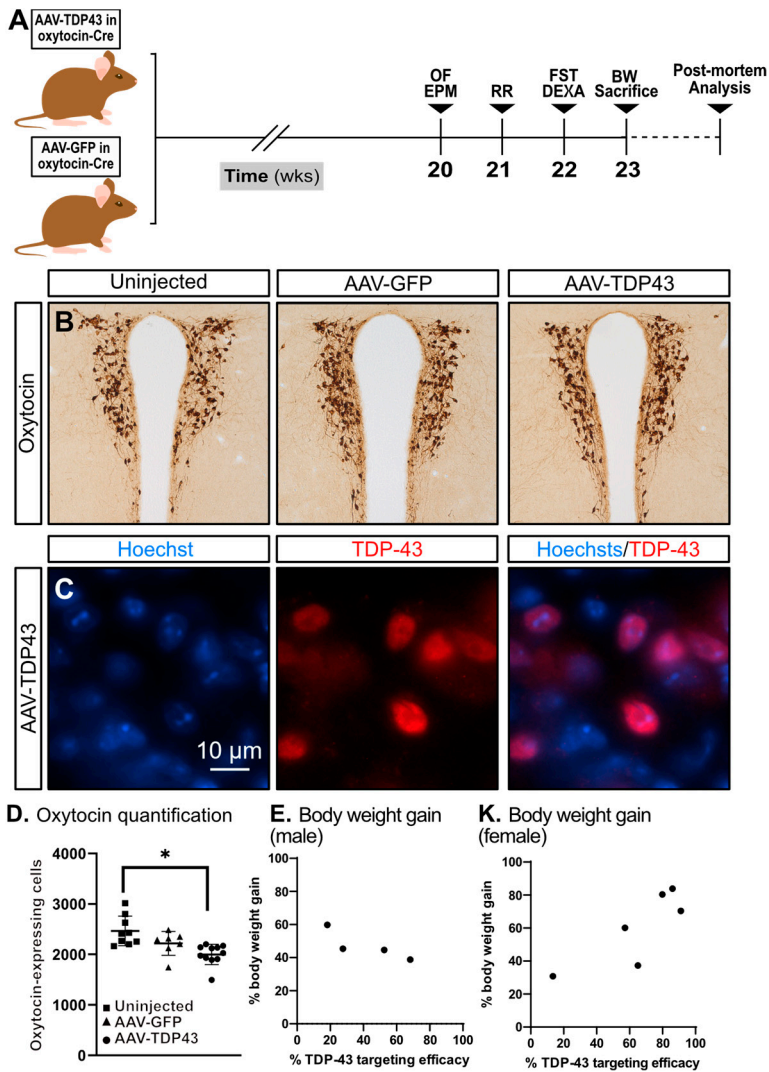


Figure 16. Selective TDP-43 overexpression in oxytocin neurons results in loss of oxytocin-expressing neurons compared to uninjected mice with no effect on behaviour and metabolism. (A) Experimental schematic. (B, D) TDP-43 overexpression in oxytocin neurons resulted in a reduction in oxytocin-expressing neurons compared to uninjected controls, but no significant difference was observed relative to GFP-expressing controls. (C) We found no evidence of pathogenic molecular properties such as TDP-43 mislocalisation or inclusion formation. (E-F) TDP-43 overexpression in oxytocin neurons had no major effects on metabolism or behaviour at this timepoint. A strong negative correlation was found in male mice between the TDP-43 targeting efficacy and body weight gain (E), while female mice displayed a trend toward a positive correlation (F).

Table 3. Main results from Paper III and Paper IV.

Experiment	Analysis	Groups	Results
AAV-N248-100Q oxytocin-Cre	IHC, stereology	N248-100Q IN vs all groups	↓ Oxytocin neurons
	Weighing	N248-100Q IN vs N248-100Q EN	↑ Body weight gain (female)
	EPM	N248-100Q IN, N166-18Q vs uninjected	↑ Anxiety-like behaviour
	SIT		= Social behaviour
	FST		= Depressive-like behaviour
	OF		= Motor activity
BACHD/oxytocin-Cre	Weighing	BACHD/oxytocin-Cre vs BACHD	= Body weight gain
	EPM		= Anxiety-like behaviour
	SIT		= Social behaviour
	FST		= Depressive-like behaviour
	OF		= Motor activity
AAV-TDP43 oxytocin-Cre	IHC, stereology	AAV-TDP43 vs uninjected	↓ Oxytocin neurons
	Weighing	AAV-TDP43 vs AAV-GFP/mCherry	= Body weight gain
	DEXA		= Body fat
	EPM		= Anxiety-like behaviour
	SIT		= Social behaviour
	FST		= Depressive-like behaviour
	RR		↑ Motor coordination, motivation

Abbreviations, IHC: immunohistochemistry, EPM: elevated plus maze, SIT: social interaction test, FST: forced swim test, OF: open field, DEXA: dual-energy X-ray absorptiometry, RR: rotarod.

Comparative effects of mHTT and TDP-43 overexpression in oxytocin neurons

Cell-autonomous neurotoxic effects were observed in oxytocin neurons in both TDP-43 overexpressing mice (AAV-TDP43, **Paper IV**) and mice forming mutant IN mHTT inclusions (AAV-N248-100Q IN, **Paper III**), relative to uninjected controls. Both papers used a similar experimental design and viral vector construct. Mice expressing Cre under the oxytocin promoter were injected at six weeks of age with AAV vectors designed to express either mHTT with 100 polyglutamine or wild-type TDP-43 (1.9×10^{14} GC/ml AAV-N248-100Q and 2.0×10^{14} GC/ml AAV-TDP43). By contrasting the data from Experiment I in **Paper III** with **Paper IV**, the relative toxicities of mHTT and TDP-43 can be compared more directly in a meta-analysis. To account for potential variation between the different experiments, the treatment groups were normalised to either uninjected or AAV-GFP-injected controls from their respective studies.

Post-mortem tissue was collected at 18 weeks post-injection in AAV-N248-100Q mice and 24 weeks post-injection in AAV-TDP43 mice. Following normalisation to uninjected controls, both mice with TDP-43 overexpression and mHTT IN inclusions showed a significantly more pronounced loss of oxytocin-expressing neurons compared to the EN mHTT inclusion group (AAV-N248-100Q IN vs. EN $p = 0.003$; TDP-43 vs. AAV-N248-100Q EN $p = 0.044$). No significant difference in toxicity was observed between the TDP-43 and mHTT IN groups ($p = 0.506$, **Figure 17A**). These results indicate that IN inclusion formation and wild-type TDP-43 overexpression are significantly more toxic than mHTT expression with only EN inclusions present.

The elevated plus maze (**Figure 17B**) and forced swim test (**Figure 17C**) revealed no significant differences in anxiety-like or depressive-like behaviours between the AAV-TDP43 and AAV-N248-100Q treatment groups when normalised to their respective AAV-GFP controls. Behavioural analyses were conducted at 12 weeks of age in AAV-N248-100Q IN and EN treatment groups and at 20-22 weeks in AAV-TDP43 mice.

In the first phase of the social interaction test, where the subject can choose to interact with either a stranger mouse or an empty cup, AAV-N248-100Q IN mice had a higher preference for the stranger mouse compared to AAV-TDP43 mice when normalised to respective AAV-GFP controls ($p = 0.032$), and a non-significant trend toward a higher preference for the stranger mouse compared to AAV-N248-100Q EN mice ($p = 0.089$) (**Figure 17D**), suggesting that mice that form IN mHTT inclusions have a differential effect on social behaviour. In the second phase of the social interaction test, where the subject chooses between a familiar mouse and a novel mouse, there was no difference between treatment groups (**Figure 17E**).

Lastly, analysis of body weight gain from 2 to 12 weeks post-injection in AAV-TDP43 and AAV-N248 mice revealed significant effects for both group ($p = 0.022$) and sex ($p = 0.024$). However, after splitting the data according to sex the effects on body weight gain between treatment groups were non-significant in males ($p = 0.184$, **Figure 17F**) and females ($p = 0.068$, **Figure 17G**).

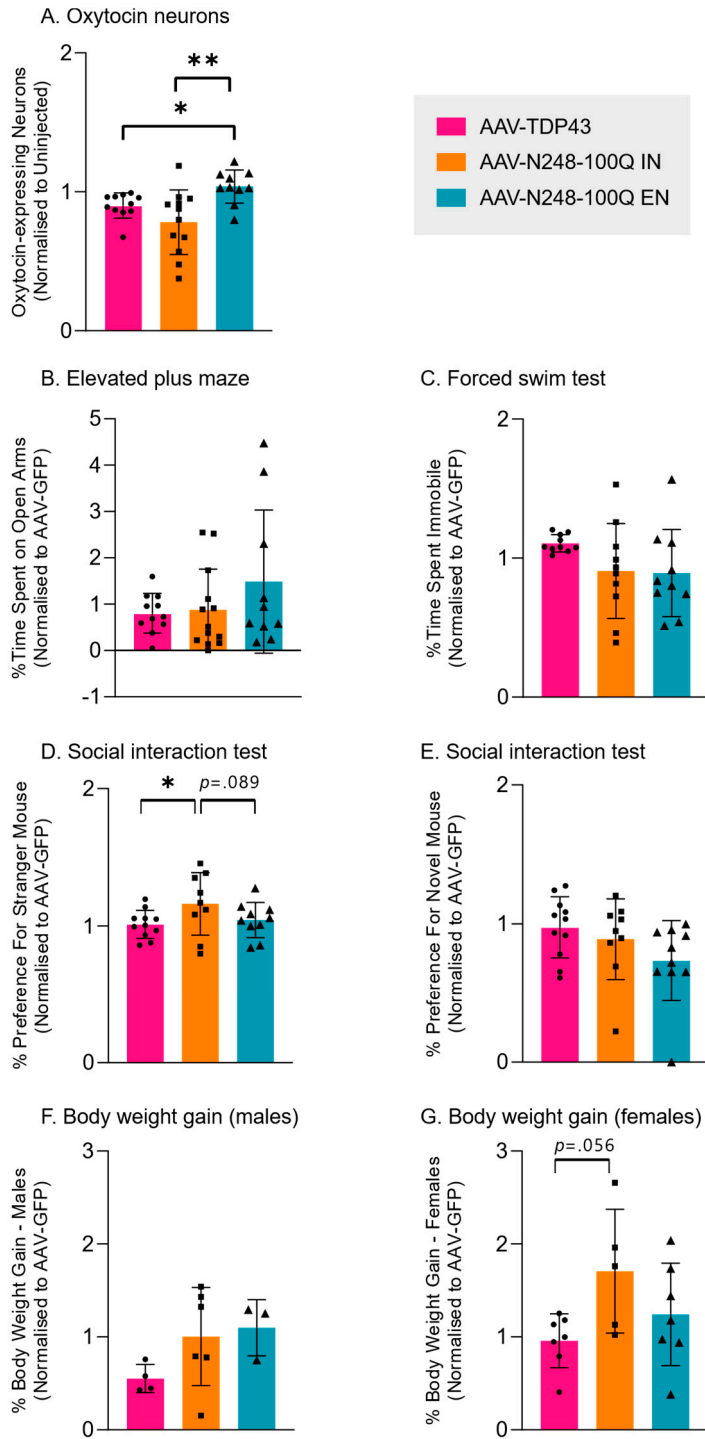


Figure 17. Comparative effects of TDP-43 overexpression as well as mHTT intranuclear (IN) and extranuclear (EN) inclusions in oxytocin neurons normalised to respective controls.

Data from mice injected with AAV-N248-100Q, forming IN or EN mHTT inclusions, were normalised to uninjected or AAV-GFP mice from Experiment I in **Paper III** and AAV-TDP43 mice were normalised to respective controls from **Paper IV**. Mice were injected at six weeks of age with similar viral titres (1.9×10^{14} GC/ml AAV-N248-100Q and 2.0×10^{14} GC/ml AAV-TDP43). For the AAV-N248-100Q groups, behavioral analysis was conducted at 12 weeks post-injection, and postmortem tissue was collected at 18 weeks post-injection. For the AAV-TDP43 group, behavioural assessment was performed between 20–22 weeks post-injection, with postmortem tissue collection at 24 weeks post-injection. **(A)** AAV-TDP43 mice and AAV-N248-100Q IN mice exhibited fewer oxytocin-expressing neurons normalised to uninjected controls compared to AAV-N248-100Q EN mice. **(B–C)** No significant differences were observed in the development of anxiety-like or depressive-like behaviors, as assessed by the elevated plus maze **(B)** and forced swim test **(C)** relative to AAV-GFP controls. **(D–E)** In the first phase of the social interaction test, AAV-N248-100Q IN mice had an increased preference for the stranger mouse compared to AAV-TDP43 mice, with a non-significant trend toward a higher interaction compared to AAV-N248-100Q EN mice **(D)**. No significant effects were observed during the introduction of the novel mouse in the second phase of the test **(E)**. Data are presented as mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test **(A–E)** or two-way ANOVA to assess the influence of sex and treatment followed by Tukey's post-hoc analysis **(F–G)**.

Discussion and future directions

Paper I

TDP-43 pathology is the major pathological protein in ALS-FTD and has also been associated with an increasing number of NDs [76, 81, 106, 107]. The results presented in **Paper I** demonstrated clear hypothalamic dysfunction in mice overexpressing wild-type TDP-43 in the hypothalamus, replicating ALS-FTD-like neuropathological hallmarks and displayed emotional and metabolic impairment. Specifically, we observed a significant loss of MCH-, hypocretin- and oxytocin-expressing neurons, as well as a reduction in hypothalamic volume. AAV-TDP43 mice displayed apathy-like behaviour, evidenced by a drastic reduction in motor activity in the open field and rotarod tests, despite displaying normal swimming ability in the forced swim test. This motor phenotype, in combination with reduced nesting behaviour, suggests a deficit in motivation rather than physical impairment. The lateral hypothalamic area has been linked to a number of vital behaviours, including locomotion. A previous study demonstrated that damage to the lateral hypothalamic area in rats resulted in a similar phenotype to that observed in **Paper I**, where the rats exhibited movement when the stimulus was sufficiently intense [133]. Both hypocretin and MCH are expressed in neuronal populations within the lateral hypothalamic area and notably, a reduction in hypocretin has been shown to decrease locomotion [134].

Furthermore, the AAV-TDP43 mice became obese and hyperglycaemic. These results align with prior findings in mice overexpressing mHTT in the hypothalamus [117, 135]. However, it is crucial to note that this finding contrasts with clinical observations in human ALS and HD patients, who frequently exhibit low BMI [27, 136], but may align more closely with the increased BMI sometimes reported in FTD [63, 137].

We found that AAV-mediated TDP-43 overexpression resulted in typical TDP-43 pathology seen in ALS-FTD including TDP-43 mislocalisation and the subsequent formation of both nuclear and cytoplasmic inclusions [57, 72, 113]. Our study proposes a mechanism of action where wild-type TDP-43 homeostasis becomes disrupted and results in disease onset. Even though TwDP-43 levels are not increased in TDP-43 proteinopathies, it might be caused by a dysregulation of the proteins or RNA that TDP-43 interacts with that could be replicated with TDP-43 overexpression.

Paper II

SIDT2 is a lysosomal protein that has been identified in the novel autophagy pathway known as RNA/DNAutophagy. To date, only one prior published study has linked SIDT2 to HD [45], showing that SIDT2 binds to HTT mRNA in a CAG dependent fashion. In addition to the already established intranuclear and extranuclear mHTT inclusions, **Paper II** reveals SIDT2-immunoreactive inclusions as an additional neuropathological hallmark. In advanced cases of HD, SIDT2 inclusions appeared in a CAG dependent fashion and rarely co-localised with mHTT inclusions. Furthermore, in vivo evidence suggests that SIDT2 inclusions form chronologically after the appearance of mHTT inclusions.

The role of SIDT2 in the direct uptake of mHTT transcripts, rather than the mHTT protein, may explain why SIDT2 and mHTT inclusions rarely co-localise. If the HTT protein is expressed, either full-length or exon 1 fragments, aggregates can form as typically seen in HD. Conversely, SIDT2 inclusions may result from the impaired uptake of HTT transcripts into the lysosome, potentially driven by conformational changes in mHTT DNA and RNA, such as the hairpin loops characteristic of CAG repeat tracts. If uptake is partially inhibited while SIDT2 remains bound to the *HTT* transcript, exposed sections of the CAG repeat tract may allow SIDT2 molecules from other lysosomes to bind to the same transcript. This process could lead to the sequestration of SIDT2 and subsequent inclusion formation. A longer CAG repeat tract likely facilitates increased SIDT2 binding, thereby resulting in the more rapid formation of larger SIDT2 inclusions.

We also observed that SIDT2 protein levels are depleted in the striatum and hypothalamus of advanced HD cases (Vonsattel grades 2–4), while *SIDT2* mRNA levels remain unaffected. This depletion may result from the sequestration of SIDT2 into insoluble inclusions, which are more difficult to detect using Western blot. Reduced levels of SIDT2 might impair the clearance of *mHTT* transcripts, thereby accelerating disease progression. We demonstrated both in vivo and in vitro that SIDT2 overexpression reduces the mHTT load. These data suggest that a potential therapeutic approach could involve AAV vectors or other gene therapies to substitute or enhance SIDT2 expression, potentially mitigating mHTT toxicity.

The frequency of SIDT2 inclusions correlates positively with CAG length in HD patients. Hase et al. [45] showed that SIDT2 binds to *HTT* in a CAG-dependent fashion, highlighting that SIDT2 might be even more critical at later stages of HD, where CAG repeat lengths increase as a result of somatic expansion [138]. Furthermore, somatic expansion could potentially worsen the dysfunction of the SIDT2 system and result in further depletion of the protein. Recent studies suggest a pathogenic threshold, where the CAG repeat becomes toxic after a specific repeat length is reached [138, 139], this toxicity may be a result of SIDT2 depletion.

Targeting SIDT2 might offer enhanced neuroprotective benefits in advanced HD by facilitating the clearance of mHTT or other transcripts cleared via RNA/DNAutophagy.

Paper III and IV

Oxytocin neurons have been shown to be reduced in HD and ALS-FTD [58, 91, 93]. In **Paper III** and **IV**, we observed that mHTT and TDP-43 overexpression specifically within oxytocin neurons resulted in cell-autonomous effects. These findings align with the loss of oxytocin-expressing neurons in human HD cases [91, 93] and ALS cases [58] compared to control cases. In **Paper I** we observed a greater reduction in oxytocin-expressing neurons at an earlier time point (19% at 24 weeks versus 42% at 10 weeks post-injection) using slightly different viral constructs but at similar viral titres (2.0×10^{14} versus 2.5×10^{14}). The variation in oxytocin-expression cells between **Paper I** and **IV** might indicate that oxytocin pathology is not solely cell-autonomous.

The meta-analysis results provided insights into relative toxicity between TDP-43 and mHTT. We observed that both TDP-43 overexpression and intranuclear mHTT inclusions were significantly more toxic to oxytocin neurons compared to mHTT extranuclear inclusions. However, no significant differences were observed between TDP-43 overexpression and IN mHTT inclusions, suggesting that they have similar neurotoxicity in oxytocin-expressing neurons.

Oxytocin is a neuropeptide involved in depression, anxiety and metabolism [101-103], these features are altered in HD and ALS-FTD [25, 27, 53, 54, 61, 62]. In **Paper III** we observed an increase in anxiety-like behaviour in N248-100Q IN mice compared to uninjected mice, but not other control groups. In addition, the meta-analysis revealed no difference between treatment groups, suggesting no major effects on anxiety-like features. Similarly, there was no major effects on depressive-like or metabolic features in **Paper III** and **IV**, or when comparing treatment groups in the meta-analysis. However, we observed a positive correlation in the number of TDP43 overexpressing cells and body weight. It is possible that the vector limited vector targeted might have masked an effect on body weight.

Clinical features of ALS, FTD and HD report social impairments with effects on empathy processing [24, 140-142]. The effects on oxytocin system in TDP-43 models have not been previously investigated, and we observed no clear effect on social interaction in AAV-TDP43 mice in **Paper IV**. In **Paper III** we observed no effects on social cognition in AAV-N248-100Q mice or BACHD/OXT-Cre mice. However, previous reports have observed social impairments in BACHD mice after six months, suggesting that oxytocin-related deficits may require a later time-point to fully manifest [143]. However, the meta-analysis showed an increased preference

to interact with a stranger mouse in mice that form intranuclear inclusions compared to AAV-TDP43 mice and a non-significant trend toward the same behaviour compared to mice that form extranuclear mHTT inclusions. While a willingness to interact with strangers is typically a sign of social cognitive health and anxiolytic effects [144], higher levels of interaction are also present in certain autism models [145]. This ambiguity makes it difficult to determine which phenotype represents social impairment. Further investigation of the oxytocin system in mHTT and TDP-43 models may be warranted to fully evaluate the effects on the development of altered social cognition.

Limitations

Throughout this thesis, several disease models were generated by overexpressing disease-related proteins in the hypothalamus. While this methodology allows us to emphasise the role of specific transcripts and proteins in the pathogenesis and study localised effects, it has inherent limitations. One limitation is the use of overexpression itself, since the underlying pathology of NDs like ALS-FTD and HD is typically not driven by elevated protein levels of TDP-43 or mHTT. However, in the case of mHTT, overexpression may emphasise toxic gain-of-function properties, and for TDP-43 overexpression it can disrupt protein homeostasis and still provide valuable data. Although cre-dependent knock-out or knock-down approaches using viral vectors is an alternative, it still does not represent protein levels in patients.

A further limitation within these models involves the selection of appropriate controls. We have used several control groups throughout this thesis, and none are without drawbacks. Uninjected controls do not account for the potential complications associated with stereotaxic surgery. Vehicle-only injections do not control for overexpression of a protein and cannot be verified to target the hypothalamus. Furthermore, while AAV vectors overexpressing GFP is a standard control, it has been reported to have neurotoxic effects [146], and in **Paper III** it was shown to increase body weight in male mice compared to other groups. Wild-type HTT overexpression is an alternative but still might have confounding results. We also showed in **Paper III** that wild-type HTT results in increased anxiety-like behaviour compared to uninjected controls. While using multiple control groups (as seen in **Paper III**) provides the most robust experimental design it reduces the sample size per group and compromises statistical power.

In **Paper I** we showed that mice with pan-hypothalamic TDP-43 overexpression exhibit apathy-like behaviour based on a combination of reduced nesting ability and lack of movement not attributed to inability to move. However, it should be acknowledged that there are no definite behavioural tests to directly quantify apathy in rodents. Translating results from behavioural tests in mice to clinical features in

patients remains a challenge, and it is difficult to determine the extent to which these behavioural phenotypes mimic human psychiatric features.

Finally, visualisation and quantification of neuropathology in this thesis relied mainly on immunohistochemistry and western blotting. The accuracy of these findings is dependent on antibody specificity. Potential cross-reactivity and low signal-to-noise ratios introduce a degree of variability. Testing multiple different antibodies and optimising protocols can help mitigate this, but relying on antibody-based detection alone is a notable limitation when quantification and identification of neuropathology.

Future directions

Paper I

We demonstrated that wild-type TDP-43 overexpression in the hypothalamus is sufficient to mimic some ALS-FTD-like phenotypes in mice. A future direction is to determine how normal TDP-43 function is comprised and results in the development of TDP-43 pathology. It might be an indirect effect of other disease processes, resulting in disruption of TDP-43 homeostasis similar to what is seen with overexpression.

In **Paper I**, we demonstrated widespread TDP-43 immunoreactivity throughout the hypothalamus, with only a small subset of neurons exhibiting cytoplasmic mislocalisation while the remainder had nuclear localisation. This suggests that nuclear wild-type TDP-43 is sufficient to drive a disease phenotype. However, it remains unclear whether mislocalisation is actually a prerequisite for the loss of the specific neuropeptide-expressing neuronal populations observed in **Paper I**. The observed nuclear localisation might indicate that these specific neurons are less vulnerable to TDP-43 pathology. It is possible that once mislocalisation occurs, it rapidly leads to neuronal death. Our observations may be limited to surviving cells where TDP-43 is still detectable. To address this, an important future direction would be to utilize AAV vectors designed to express TDP-43 lacking a nuclear localization signal. Comparing the neuropathological effects and resulting phenotypes of cytoplasmic TDP-43 against wild-type nuclear TDP-43 would help delineate the respective contributions of nuclear loss-of-function versus cytoplasmic gain-of-toxicity.

Paper II

We observed that the SIDT2 system is disrupted in HD, SIDT2 has been shown to bind to mHTT and reduce its load, but it may also be involved in other proteinopathies. Dysfunction in the SIDT2 pathway might hinder the clearance of other disease-related RNA and DNA, thereby facilitating disease progression. Interestingly, SIDT2 dysfunction has already been identified in PD [46]. Future research should investigate whether SIDT2 is affected in other NDs, but particularly other CAG repeats disorders such as spinocerebellar ataxias caused by CAG repeat expansions, as SIDT2 binds to the CAG repeat tract itself.

Additionally, an interesting future direction would be to explore SIDT2 knock-out models. Silencing SIDT2 expression specifically in the striatum or hypothalamus of wild-type mice could determine if its loss alone is sufficient to induce motor or behavioural phenotypes similar to what is observed in HD. Furthermore, silencing SIDT2 in an HD mouse model would allow us to investigate whether impaired SIDT2-mediated clearance exacerbates disease-related features.

Lastly, we observed depleted SIDT2 protein levels using Western blot in the striatum and hypothalamus, without affecting *SIDT2* mRNA levels. This methodology primarily measures soluble proteins. A next step would be to test the hypothesis that this loss is due to the sequestration of SIDT2 into insoluble aggregates.

Paper III and IV

The results from **Papers III and IV** suggest that oxytocin pathology may not be the primary driver of the behavioural and metabolic features observed in mHTT and TDP-43 proteinopathies. Consequently, future studies should target alternative neuronal populations. For instance, hypocretin levels are significantly reduced in HD, PD, AD, and ALS [87, 91, 93-95]. Pathology in this neuronal population may represent a common denominator across these disorders. This could be investigated using a similar experimental setup to the one used in this thesis, overexpression of disease-related proteins only in hypocretin neurons, and could provide insights on the causal effect of hypocretin pathology on the development of non-motor features of NDs.

Conclusions

Paper I showed that wild-type human TDP-43 overexpression in the hypothalamus is sufficient to induce key ALS-FTD-like molecular and neuropathological hallmarks, including TDP-43 mislocalisation and the formation of nuclear and cytoplasmic inclusions. Wild-type TDP-43 overexpression resulted in gain-of-toxic functions within the hypothalamus, resulting in atrophy, neuroinflammation and loss of neurons expressing hypocretin, MCH and oxytocin. Furthermore, TDP-43 overexpression led to the development of metabolic and emotional features. This paper gave insights to the molecular and cellular mechanisms of TDP-43 in the hypothalamus, identifying a critical brain region for developing novel therapeutic targets for TDP-43 proteinopathies.

Paper II identified SIRT2-immunopositive inclusions as a novel neuropathological hallmark in HD. Our results suggest that targeting SIRT2-mediated DNA/RNA autophagy could be a beneficial strategy for reducing mHTT load, independently of the major protein degradation pathways.

Intranuclear mHTT inclusions (**Paper III**) and overexpression of wild-type TDP-43 (**Paper IV**) results in a cell-autonomous neurotoxic effect in oxytocin neurons compared to uninjected controls. However, selective mHTT or TDP-43 overexpression in oxytocin neurons alone is not sufficient to cause emotional and metabolic features in mice. These papers suggest that oxytocin pathology, while present, is not the major driver of non-motor features of HD or TDP-43 proteinopathies.

The meta-analysis demonstrates comparable toxicity between wild-type TDP-43 and mHTT in oxytocin neurons. TDP-43 and intranuclear mHTT inclusions appear to exert more severe cell-autonomous effects on oxytocin neurons than extranuclear inclusion formation alone. Furthermore, mice that form mHTT IN inclusions showed distinct alterations in social interaction compared to TDP-43 overexpressing mice.

Concluding remarks

This thesis establishes the hypothalamus as a key mediator in TDP-43 proteinopathies, demonstrating that it serves as a focal point for the cellular toxicity that drives non-motor dysfunction. The work also identifies SIRT2 inclusions as a novel pathological hallmark in HD, present not only in the hypothalamus but also in other vulnerable brain regions. Furthermore, while the hypothalamic neuronal population expressing oxytocin is vulnerable to mHTT and TDP-43 toxicity, it does not appear to be the main driver of non-motor features. This suggests that the clinical features of NDs may emerge from a widespread pathology of hypothalamic circuits or the loss of other specific cell types. In summary, this work demonstrates the hypothalamus as a critical convergence point in both TDP-43 and mHTT proteinopathies, and the identification of SIRT2 dysfunction throughout the HD brain provides a new therapeutic avenue for intervening in pathogenesis.

Methods

Ethical permits

All experimental procedures involving animals were approved by the Regional Ethical Committee in Lund, Sweden under permit number 17113/2022 for (**Paper I, II, IV**), as well as M65-13 and 15499/2017 (**Paper III**).

Human post-mortem tissues used in **Paper II** were obtained and research approved by the Victorian Brain Bank and the Sydney Brain Bank at Neuroscience Research Australia (PID073, PID0111, PID167) as well as the Ethic Committee of the Medical Faculty of the Ruhr-University Bochum, Germany (17-5939) and the Swedish Ethical Review Authority and the regional ethical review board at Lund University (2022-05348-01 and 2014/466). All donors provided informed consent prior to brain donation.

Animals

Mice were housed in groups of 2–6 animals under a 12-hour light/dark cycle with ad libitum access to food and water. Humane endpoints, including complete immobility, eye squinting, and raggedness of fur, were consistently monitored across all experiments to ensure animal welfare. Mice were anaesthetised using a mixture of 2% isoflurane and nitrous oxide for all surgical procedures. All surgical procedures were performed at 6–8 weeks of age.

Study I

Wild-type female FVB/N mice were used to study the effects of TDP-43 overexpression in the hypothalamus. An AAV vector designed to express wild-type human TDP-43 (AAV-TDP43) was injected bilaterally into the hypothalamus.

Experiment I was used for initial neuropathological assessment of wild-type TDP-43 overexpression. Mice were injected with three different doses of AAV-TDP43: 5.0×10^{13} , 1.0×10^{14} or 2.5×10^{14} gc/ml ($n = 6$ per dose) and perfused nine weeks post-injection for neuropathological examination.

Experiment II served to further assess neuropathology of hypothalamic overexpression, mice were injected with AAV-TDP43, AAV-GFP or AAV-mHTT. AAV-TDP43-injected mice were euthanised at six- or ten-weeks post-injection (n = 5 per timepoint) based on humane endpoints.

Experiment III determined the molecular properties of TDP-43 and the development of behavioural and metabolic features. Mice were injected with AAV-TDP43 (1.0×10^{14} gc/ml, n = 19) or vehicle (n = 20). Behavioural testing (open field, elevated plus maze, rotarod, forced swim test) and dual-energy X-ray absorptiometry (DEXA) scanning were performed between 18- and 20-weeks post-injection. Mice were perfused at 21 weeks post-injection, and blood was collected for serum glucose measurements.

Experiment IV focused on investigating the effects on apathy-like behaviour and for RNA sequencing. Mice were injected with AAV-TDP43 (1.0×10^{14} gc/ml, n = 16) or vehicle (n = 16). Food intake and nesting behaviour were monitored, and hypothalami were collected ten weeks post-injection for qRT-PCR and RNA sequencing.

Study II

To investigate the neuropathological characteristics of S1D2 in HD we used three different mouse models. The R6/2 mice with approximately 150Q in *HTT*, were analysed at six and 16 weeks of age (n = 4 per genotype at 6 weeks; n = 6 R6/2 and n = 2 wild-type at 16 weeks) and the BACHD mice expressing full-length mHTT with 97Q were analysed at six months of age (n = 6 BACHD and n = 3 wild-type). In addition, mice bilaterally injected into the hypothalamus with an AAV vector designed to express a large fragment of HTT (79Q or 18Q in HTT) was analysed at six- and 18-weeks post-injection (n=1/group).

To test the potential therapeutic effect of S1D2 in HD, R6/2 mice were bilaterally injected into the hypothalamus with an AAV vector designed to overexpress S1D2 (n = 4). Neuropathological analyses were performed 10 weeks post-injection (at 16 weeks of age) and compared to uninjected R6/2 littermates.

Study III

To study the effects of mHTT in oxytocin neurons we used the oxytocin-Cre mouse model or the BACHD mouse model.

Experiment I was designed to investigate the effects of mHTT expression in oxytocin neurons. To ensure selective mHTT expression in oxytocin neurons in wild-type mice, flex-switch AAV vectors were bilaterally injected into the PVN of oxytocin-Cre mice at two months of age. We designed three different flex-switch AAV vectors that resulted in transgene expression of GFP, wild-type HTT (N-terminal fragment of 166 amino acids with 18Q) and mHTT (N-terminal fragment

with 248 amino acids of HTT with 100Q). The mice underwent behavioural analyses and body weight measurements at three months post-injection and were sacrificed at four months post-injection.

Experiment II was conducted to investigate the effects of deletion of mHTT expression in oxytocin neurons in BACHD mice, a mutant line was generated by crossing BACHD mice with oxytocin-Cre mice, creating a line that ubiquitously expresses mHTT except in oxytocin neurons (BACHD/oxytocin-Cre). When Cre is present in the loxP-flanked BACHD mice, a fragment of exon 1 of the mHTT gene is deleted. This mutant line, along with littermate controls (BACHD, wild-type and oxytocin-Cre), was subjected to behavioural analyses and body weight measurements at two months of age and sacrificed at three months of age.

Study IV

To study the effects of wild-type TDP-43 overexpression in oxytocin neurons we used the same oxytocin-Cre model as in **Study III**. A flex-switch AAV vector was used to express either wild-type human TDP-43 (AAV-TDP43) or GFP (AAV-GFP) in oxytocin neurons. The vectors were bilaterally injected into the PVN.

Human post-mortem tissue

Human post-mortem brain tissue was sourced from three locations to investigate the role of SIDT2 in HD in **Paper II**. To study the effects of SIDT2 protein and mRNA levels, blocks of frozen tissue of the caudate nucleus of the striatum and frontal cortex (n = 17 HD with Vonsattel grades 2-4 and n = 10 control cases) as well as the lateral hypothalamic area (n = 4 HD with Vonsattel grades 2-4 and n = 2 control cases) were obtained from the Victorian and Sydney Brain Banks, Australia and used for western blot and qRT-PCR. To determine neuropathological effects in the ACC, paraformaldehyde (PFA)-fixed ACC (n = 4 HD and n = 2 control cases) was provided by the Huntington brain collection of Bochum, Germany. Formalin-fixed tissue from the frontal cortex, the striatum and the hypothalamus of an HD case with Vonsattel grade 0 was obtained from the Department of Neuropathology in Lund, Sweden.

Cell cultures

To investigate molecular properties of SIDT2 overexpression in cell culture in **Paper II**, pcDNA3.1 vectors were used to co-transfect SH-SY5Y neuroblastoma cells with either HTT exon 1 (16Q or 72Q) and Sidt2 C-terminally tagged with V5 or with an empty vector. Cells were transiently transfected for 72 hours using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific).

Behavioural tests

In **Paper I, III and IV** we performed a battery of behavioural analyses to investigate the effects of TDP-43 and mHTT toxicity on the development of features related to ALS-FTD and HD.

Elevated plus maze

Anxiety-like behaviour was quantified using the elevated plus maze (**Figure 18A**). The maze featured two opposing open arms and two opposing closed arms, connected by a centre platform. Mice were placed in the centre and tracked automatically using the Ethovision software for 5 min.

Forced swim test

Depressive-like behaviour and motivation was assessed with the forced swim test (**Figure 18B**). This assay involves placing a mouse in a cylinder of water and their movements were recorded (4 min swimming time in **Paper I** and 5 min in **Paper III and IV**). After the acclimation phase the total immobility time was scored manually by a blinded experimenter. Immobility is defined as the mouse making only the minimal movements necessary to keep its head above the water surface.

Social interaction test

Paper III-IV investigated social interaction because of oxytocin's role in social cognition. The social interaction test assessed social affiliation and novelty preference in a three-chamber acrylic box (**Figure 18C**). Following a 5 min habituation period, two 10 min phases were conducted. The first phase, social affiliation compared interactions (cup touches) with an empty cup versus a stranger mouse. The second phase, social novelty compared interactions with the now-familiar mouse versus a novel mouse. The interactions were scored manually by a blinded experimenter.

Open field test

General activity level was measured in an activity box (**Figure 18D**). Mice were placed in the centre, and the total distance travelled (1h) was automatically recorded and tracked in the Ethovision software.

Rotarod

Motor activity and balance were assessed in **Paper I** and **III** using an accelerating rotarod (**Figure 18E**). Mice were trained for three sessions (2 min at 5 rpm, 2h intervals) over three days. The formal test consisted of three repetitions (2h intervals) of a 5-minute acceleration from five to 40 rpm. The latency to fall was recorded automatically on the rotarod device.

Nesting

Nesting behaviour was assessed over a 4-day period in **Paper I**. Mice were pair-housed (treatment-matched) with a 2-day acclimatisation period. Mice started with intact nesting material in each cage. Nesting ability was scored blindly using a modified Gaskill et al. scale (0-3) [147], where three is a fully cupped dome and zero is untouched material.

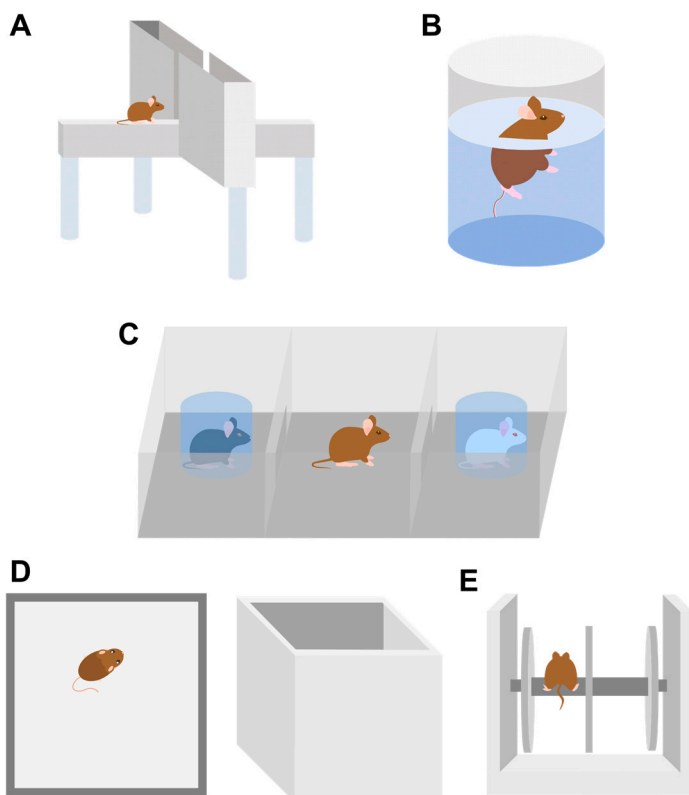


Figure 18. Schematic of behavioural tests used to assess non-motor and motor features.

(A) The behavioural battery included the elevated plus maze to assess anxiety-like features, (B) the forced swim test to assess for depressive-like features, (C) the social interaction test to assess social behaviour, (D) the open field test to assess general activity and (E) the rotarod to assess motor activity and balance.

Metabolic tests

Given that the hypothalamus has a role in metabolism, metabolic analyses were conducted in **Paper I, III** and **IV**.

Food intake

Food intake was analysed over a 4-day period in **Paper I**. Mice were pair-housed (treatment-matched) with a 2-day acclimatisation period.

DEXA scan

Body fat percentage was determined in **Paper I** and **IV**. Mice were anaesthetised (2% isoflurane/nitrous oxide) and scanned individually using a DEXA Lunar PIXImus2 scanner. Calculations were performed using PIXImus 2.10 software.

Serum glucose concentration

In **Paper I** serum glucose concentration was quantified using the glucose oxidase method, which uses a peroxidase reaction for the colorimetric detection of hydrogen peroxide formation.

qRT-PCR

To quantify the mRNA expression levels of TDP-43 (**Paper I**), SIDT2 (**Paper II**) and HTT (**Paper III**), quantitative reverse transcription polymerase chain reaction (qRT-PCR) was employed. Total RNA was isolated from the tissue samples and was treated with DNase digestion. The RNA was mixed with a reverse transcriptase and random hexamer primers to generate cDNA. The cDNA was combined with forward and reverse primers (**Table 4**), DNA polymerase and a SYBR green fluorescent dye. The qRT-PCR was run on a thermocycler. After amplification, a melting curve analysis was performed to confirm that only the intended single product was amplified. The data were quantified using the $\Delta\Delta CT$ method and normalised to the expression house-keeping genes expression.

Table 4. Primers used for qRT-PCR.

Gene	Paper	Application	Primer	Sequence (5' to 3')
TARDBP/Tardbp	I	Target Gene	Forward	AAGGAATTCTGCATGCCCCA
			Reverse	TGTTTTCTGGACTGCTCTTTTCAC
SIDT2	II	Target Gene	Forward	TGAACGTCCTGAACAAGCAG
			Reverse	CACAGGGTTCGTTCCACTTT
HTT	III	Target Gene	Forward	GCACCGACCAAAGAAAGA
			Reverse	ACCATCCTGACATCTGAC
Gapdh	I	Normalisation	Forward	TGTCCGTCGTGGATCTGAC
			Reverse	CCTGCTTCACCACCTTTTG
Pdhb	I	Normalisation	Forward	GTAGAGGACACGGGCAAGAT
			Reverse	TGAAAACGCCTCTTCAGCA
ACTB	II	Normalisation	Forward	ATATGAGATGCGTTGTTA
			Reverse	AAGTATTAAGGCCAAGAT
GAPDH	II	Normalisation	Forward	CTCTGGTAAAGTGGATATTGT
			Reverse	GGTGGAAATCATATTGGAACA
Hprt	III	Normalisation	Forward	ACCTTCTATGAATGTTACTG
			Reverse	GATAAGCGACAATCTACC
Actb	III	Normalisation	Forward	GCTGTGCTATGTTGCTCTA
			Reverse	TCGTTGCCAATAGTGATGA
Gapdh	III	Normalisation	Forward	AACCTGCCAAGTATGATG
			Reverse	GGAGTTGCTGTTGAAGTC

Immunoblotting

In **Paper II**, protein levels of SIDT2 and HTT was obtained via immunoblotting techniques Primary antibodies used can be found in **Table 5**.

Western blot of human brain tissue

Human post-mortem tissue samples, including frontal cortex, caudate nucleus and lateral hypothalamic area, were lysed (1:10 weight/volume ratio) and supplemented with protease and phosphatase inhibitors. Tissue was homogenised by sonication, incubated on ice (15 min) and centrifuged and total protein concentration in the lysate was determined using the DC Protein Assay Kit.

Before loading on the SDS-PAGE, the samples were heat-denatured (95 °C, 5 min) in SDS sample buffer. Protein (40µg) was loaded on each well on a 4%-15% gradient polyacrylamide gel. The samples were electrophoretically separated (90V for 30 min followed by 120V for 1h). Proteins were then transferred onto a PVDF membrane (25 V, 7 min). The membranes were blocked (5% skimmed milk in TBS-T, 1h) and incubated overnight with the primary antibody followed by horseradish

peroxidase conjugated secondary antibody (1h). The membranes were developed using enhanced chemiluminescence. The images were acquired with the VersaDoc instrument. Band intensities were quantified using the ImageLab software and expressed as a ratio relative to β -actin.

Western blot of cell cultures

For protein extracts obtained from cell culture experiments, protein extract (25 μ g) from cell homogenates was mixed in LDS sample buffer and heat-denatured (70 °C, 10 min). The product was then electrophoretically separated on 10% Bis-Tris gels. Separated proteins were transferred onto nitrocellulose membrane (80 V, 2h).

Filter retardation assay of cell cultures

Detection of HTT SDS-insoluble proteins was performed by heat-denaturing (95 °C, 5 min) protein extract (3 μ g) from cell homogenates and was loaded onto a nitrocellulose membrane and samples were subsequently filtered through applying a vacuum.

Visualisation of filter retardation assay of cell cultures

Membranes obtained from western blots or filter retardation assays from cell culture samples were blocked (5% skim milk powder in TBS) and incubated with primary antibodies overnight. Afterwards, membranes were probed for fluorescent-labelled secondary IRDye® antibodies (1:5000, 1h). Fluorescence signals were detected and quantified using the LI-COR ODYSSEY® FC and the Image Studio software.

Table 5. Primary antibodies for western blot and filter retardation assay.

Tissue	Antibody	Host	Dilution	Company	Catalogue No.
Human	SIDT2	Rabbit	1:1000	Abnova	PAB27211
	SIDT2	Rabbit	1:1000	Abcam	ab85847
	β -actin	mouse	1:10000	Sigma-Aldrich	A5441
Cell	SIDT2	Rabbit	1:500	Abnova	PAB27211
	Huntingtin	Rabbit	1:5000	Abcam	ab109115
	P62	Rabbit	1:500	Cell Signalling	5114
	Ubiquitin	Rabbit	1:11000	Cell Signalling	8081
	LC3B	Rabbit	1:500	Cell Signalling	2775
	GAPDH	Mouse	1:2000	Santa Cruz	sc-47724
	GAPDH	Rabbit	1:5000	Proteintech	10494-1-AP

Viral vectors

Production

Viral vectors were central in all papers of this thesis (**Figure 19**). Recombinant AAV2/5 vectors were produced via a double-transfection method. All transgenes were regulated by the human Synapsin-1 promoter and packaged in an AAV5 capsid, flanked by AAV2 inverted terminal repeats and a SV40 polyadenylation signal. Vectors were titered by TaqMan qPCR and formulated in PBS with 10 ppm Poloxamer-188.

Neuronal-specific AAV vectors

Several neuronal-specific vectors were employed in **Paper I-II**. In **Paper I**, the AAV-TDP43 vector was designed to express the coding sequence of wild-type human *TARDP* gene and a control vector was designed to express GFP (AAV-GFP). Both **Paper I** (AAV-mHTT) and **II** (AAV-HTT-79Q) used the same vector expressing the first 853 amino acids of the N-terminal fragment of HTT with 79Q. In addition, **Paper II** included a control vector AAV-HTT-18Q (expressing 792 amino acids of the N-terminal HTT fragment with 18Q) and a vector expressing the coding sequence of the human *SIDT2* (AAV-SIDT2).

Flex-switch AAV vectors

Flex-switch vectors were designed to specifically express the transgene only in the presence of Cre. **Paper III** and **IV** used AAV-GFP/mCherry as a control vector. In the absence of Cre, the sense transgene mCherry is expressed and the antisense transgene GFP is silenced. The presence of Cre flips the sequence, resulting in the expression of GFP and silencing of mCherry. **Paper III** additionally used two other vectors; one expressing 166 amino acids of the N-terminal exon 1 fragment of HTT with 16Q (N166-18Q) and another expressing 248 amino acids of the N-terminal fragment with 100Q (N248-100Q). **Paper IV** used a flex-switch vector designed to express full-length wild-type TDP-43.

Vector delivery

Mice were anesthetised using a mixture of isoflurane and nitrogen oxide before being placed into a stereotactic frame for bilateral viral vector or vehicle injection. For **Paper I-II**, the entire hypothalamus was targeted and in **Paper III** and **IV** specifically the PVN within the hypothalamus was targeted (**Table 6**). In all procedures, a volume of 0.5 μ L of vector or vehicle solution was delivered per side using a Hamilton syringe attached to a pulled glass capillary, the needle was left in place for an additional five minutes before slow retraction.

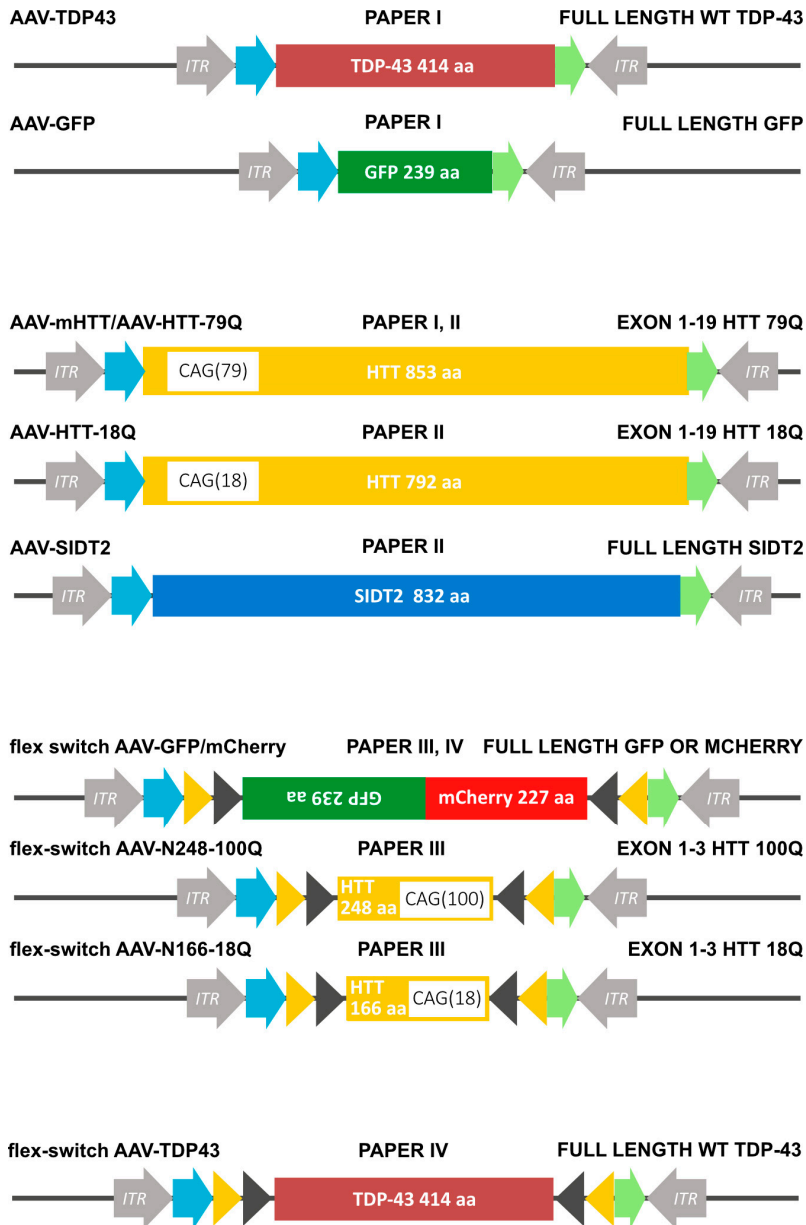


Figure 19. Schematic overview of recombinant AAV vectors.

All vector constructs are designed for specific expression in neuronal populations regulated under the Synapsin-1 promoter (represented by blue arrow). The expression cassette is flanked by AAV2 inverted terminal repeats (ITR, represented by grey arrows). Termination of transcription is ensured by the SV40 polyadenylation (polyA) signal (represented by the green arrow). The Cre-dependent flex switch vectors contain additional orthogonal recombination sites: Lox2272 (represented by the yellow triangle) and LoxP (represented by the grey triangle), allowing for the conditional, Cre-dependent inversion and expression of the transgene.

Table 6. Stereotactic injection coordinates for viral vector delivery.

All coordinates are reported in millimetres (mm) relative to bregma (anterior/posterior and medial/lateral) and dura (ventral/dorsal).

Target	Paper	Plane	Coordinate (mm)
Hypothalamus	I, II	Anterior/posterior	-0.7
		Medial/lateral	± 0.55
		Ventral/dorsal	-5.2
PVN	III, IV	Anterior/posterior	-0.6
		Medial/lateral	± 0.45
		Ventral/dorsal	-4.9

Histological analyses

For neuropathological visualisation of proteins and cell structures, immunohistochemistry was a central method in this thesis. All primary antibodies used for histological analysis can be found in **Table 7**.

Immunohistochemistry

Mouse brain tissue

Peroxidase-based immunohistochemistry was performed on mouse brains tissue fixed in 4% PFA, cryoprotected in 25% sucrose and coronally sectioned (30 µm thickness in six series). Free-floating sections were incubated overnight with a primary antibody and were labelled with suitable secondary antibody (1:200, 1h) and visualised with the 3,3'-diaminobenzidine (DAB) chromogen using hydrogen peroxidase. Sections were then mounted, dehydrated through an ethanol-xylene series, and coverslipped with distyrene plasticizer xylene (DPX). Certain mounted sections were also counterstained with either cresyl violet (**Paper I**) or haematoxylin (**Paper III**) to investigate cell morphology or the inclusion intracellular location. Images were acquired using a Zeiss Axio Imager M2 microscope.

Human brain tissue

Immunohistochemical analysis was conducted on post-mortem human tissue (**Paper II**). First, 4% PFA-fixed free-floating vibratome sections (80 µm) of the ACC were treated with 1% NaBH₄ before a blocking step (10% normal serum, 30 min), followed by 72h incubation with antibodies against SIDT2 and the N-terminus of HTT (EM48) followed by incubating with the appropriate biotinylated secondary antibody (3h), visualisation was performed with DAB. Finally, sections were mounted on Superfrost Plus slides, dehydrated and coverslipped. Photodocumentation was conducted using an Olympus BH-2 Microscope.

For immunohistochemistry of the HD Vonsattel grade 0 case in **Paper II**, formalin-fixed 50 μm sections cryoprotected in 30 % sucrose and cut into 15 series. Antigen retrieval was performed with Tris-EDTA buffer (80°C, 1h). The sections were then quenched and blocked (5% normal serum, 1h). Subsequently, the sections were incubated with a primary antibody (overnight) and biotin-conjugated secondary antibody (1:200, 2h) and were visualised using DAB development. Finally, the sections were mounted on chrome alum coated glass slides and coverslipped with DPX after a series of dehydration steps in ascending concentrations of ethanol and xylene. Image acquisitions were performed using a Zeiss Axio Imager M2 microscope.

For immune electron microscopic analysis of ACC tissue in **Paper II**, sections were postfixed with 1% osmium tetroxide, dehydrated, and flat-embedded in Araldite. Alternating semithin (0.75 μm) and ultrathin (100 nm) sections were cut using a Leica Ultracut R microtome. Semithin sections were counterstained with Toluidine Blue O (1%), while ultrathin sections were contrasted with uranyl acetate (5%, 10 min) and lead citrate (2%, 5 min) prior to being viewed by the LVEM25 microscope.

Immunofluorescence

Mouse brain tissue

Fluorescence immunostaining in mouse was primarily used to confirm viral vector targeting and analyse the intracellular localisation of TDP-43. To confirm vector targeting in **Paper I**, sections were incubated overnight with the primary antibodies and labelled with an Alexa Fluor 647-conjugated secondary antibody, followed by an additional blocking step before adding biotin-conjugated TDP-43 primary antibody followed by Alexa Fluor 488-conjugated streptavidin. For TDP-43 intracellular localisation, the samples were incubated with a primary antibody against TDP-43 followed by Alexa Fluor 647-conjugated secondary antibody. All samples were counterstained with Hoechst 33342 to visualise nuclei and coverslipped. All images were captured with a Zeiss Axio Imager M2 microscope.

Human brain tissue

Immunofluorescence microscopy was performed on 14 μm ACC cryosections in **Paper II** (30% sucrose). Sections were mounted and dried on Superfrost Plus slides (40°C, 1.5h). The sections were simultaneously incubated overnight with primary antibodies targeting SIDT2 and HTT (EM48), followed by secondary antibodies conjugated with Alexa Fluor 594 and Alexa Fluor 488 (1:500, 2h). To minimise non-specific background signal, True Black autofluorescence quencher (1:20, 1 min) was applied briefly. All samples were counterstained with DAPI. All sections were documented in a confocal fluorescence microscope (Nikon Spinning Disk).

For immunofluorescent staining of the HD case with Vonsattel grade 0 in **Paper II**, we performed antigen retrieval (Tris-EDTA, 80°C, 1h), blocking (5% normal serum) and incubation of primary (overnight) and biotin-conjugated secondary (1:200, 2h) antibodies followed by Alexa Fluor 647-conjugated streptavidin and Alexa Fluor 488-conjugated secondary antibody (1:200, 2h). Sections were mounted on chrome alum coated glass slides, coverslipped and visualised on the Zeiss Axio Imager M2 microscope.

Immunocytochemistry

For immunocytochemistry in **Paper II**, SH-SY5Y cells were cultured on poly-L-lysine-coated chamber slides and subsequently transfected (96 h). Cells were fixed with 4% PFA and blocked (10% bovine serum albumin, 1h) before overnight incubation with primary antibodies targeting the V5 tag and HTT (**Table 7**). Cells were incubated with Alexa Fluor 594 and Alexa Fluor 488 conjugated secondary antibodies (1h). Samples were then counterstained with DAPI to visualise nuclei. Images were acquired with an Axioplan 2 imaging system equipped with an ApoTome.

Table 7: Primary antibodies for immunohistochemistry.

Tissue	Antibody	Host	IHC dilution	IF dilution	Company	Catalogue No.
Mouse	Oxytocin	Rabbit	1:4000	1:2000	Phoenix Pharmaceuticals, Inc.	H-051-01
	Huntingtin (sc-8767)	Goat	1:500	—	Santa Cruz, Biotechnology, Inc.	SC-8167
	Huntingtin (EM48)	Mouse	1:500	—	Merck	MAB5374
	GFP	Chicken	1:20000	1:10000	Abcam	ab13970
	Ubiquitin	Rabbit	1:2000	—	DakoCytomation	Z0458
	DARPP-32	Rabbit	1:20000	—	Sigma-Aldrich	ZRB2260
	MCH	Rabbit	1:20000	1:2000	Phoenix Pharmaceuticals, Inc.	H-070-47
	Vasopressin	Rabbit	1:20000	1:2000	Merck	AB1565
	Orexin A	Rabbit	1:4000	1:2000	Phoenix Pharmaceuticals, Inc.	H-003-30
	SIDT2	Rabbit	1:2000	—	Abnova	PAB27211
	P62	Guineapig	1:10000	—	Progen	GP62-C
	TDP-43	Rabbit	1:100000	1:1000	Proteintech	10782-2-AP
	TDP-43	Rabbit	—	1:200	Proteintech	Biotin-10782
	Huntingtin (EM48)	Mouse	1:200/ 1:1000	1:100/ 1:300	Merck	MAB5374
Human	SIDT2	Rabbit	1:2500	1:300	Abnova	PAB27211
	pTDP-43	Mouse	1:2000	—	Comso Bio Co	TID-PTD-M01
	V5	Mouse	—	1:500	Thermo Fisher Scientific	R960-25
Cell		Rabbit	—	1:500	Abcam	ab109115

Quantification and volume measurements

To investigate the effects of overexpression of TDP-43 (**Paper I, IV**), SIRT2 (**Paper II**) or mHTT (**Paper III**) quantification was performed to determine the effect on cell count and inclusions. All quantifications and volume measurements were performed by an experimenter blinded to the experimental groups and sex.

Cell measurements

Tissue sections were processed for hypocretin, MCH, oxytocin or vasopressin immunohistochemistry. Hypocretin and MCH-expressing neurons were counted bilaterally in the lateral hypothalamic area (bregma -1.06 to -2.30 mm), and oxytocin and vasopressin-expressing neurons were counted in or bordering the PVN (bregma -0.46 to -1.22 mm). Cells in **Paper I** and **III** were analysed using a Nikon Eclipse 80i microscope with a 60x Plan-Apochromat (1.4 N.A. oil objective) and counted manually using the VIS software tool. In **Paper II** and **IV** images were captured with Zeiss PrimoStar microscope (10x/0.25 N.A., 4x/0.1 N.A.) and quantified in ImageJ. Oxytocin-expressing neurons in **Paper IV** were quantified manually with the ImageJ's multipoint tool. In **Paper II**, hypocretin-expressing neurons were quantified with an automated ImageJ script. In all other total cell counts, the total number of neurons was estimated by multiplying the total observed cell count by the number of series, and were corrected for both hemispheres.

Quantification of inclusions and vector expression

Automated quantification methods were used for mHTT inclusions and TDP-43 expressing cells. Images were acquired bilaterally using a Zeiss PrimoStar microscope (40x/0.6 NA and 10x/0.25 NA objectives). For mHTT inclusion counting (**Paper II**), EM48-immunoreactive inclusions were acquired in the superior lateral part of the fornix at bregma -1.70 mm. The mHTT inclusions were quantified using an automated ImageJ script with thresholding parameters set for pixel intensity (0-100), pixel size (0-130), and circularity (0.20-1.00). mHTT inclusion density were calculated as the total number of inclusions per square millimetre.

For quantifying TDP-43 expressing cells in **Paper IV**, images were acquired at 10x/0.25 NA in the PVN sections spanning bregma -0.46 to -1.22 mm. This quantification was also performed using an automated ImageJ script, applying thresholding for pixel intensity (0-100) and circularity (0.30-1.00). Any missing sections were addressed by imputing an average value from the same group and bregma level.

Volume measurements

Hypothalamic volume (**Paper I**) was measured in sections spanning bregma -0.35 mm to -2.18 mm. The hypothalamus was outlined along the third ventricle to the optic tract. Images were captured on a Nikon Eclipse 80i (4x/0.1 NA) and measured bilaterally using VIS software or with Zeiss PrimoStar (4x/0.1 NA) and measured unilaterally using ImageJ 1.53t. Volume was calculated by multiplying the outlined hypothalamic area by the section thickness (30 μ m), the number of series (6), and, where applicable, correcting for both hemispheres.

RNA sequencing and exon skipping quantification

To determine the effects of TDP-43 overexpression in the hypothalamus on constitutive and cryptic exons, bulk RNA sequencing and subsequent exon skipping quantification was performed. Total RNA was isolated from frozen hypothalamic tissue in **Paper I** using the RNeasy Mini Kit. RNA concentration and integrity (RIN > 8.8) were verified using Qubit Fluorometric Quantitation and the Fragment Analyser 5300. For library preparation, the mRNA fraction was enriched from total RNA (100 ng) using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB). Libraries were prepared using the NEB Next Ultra II Directional RNA Library Prep Kit and sequenced as 100 bp paired-end reads on an Illumina NovaSeq 6000, aiming for greater than 25 million clusters per sample. A Biomek i7 Liquid Handler was used during library preparation to minimise technical batch effects.

Bioinformatic analysis began with read quality assessment (ngs-bits), followed by alignment to the GRCh39 reference genome using STAR. Normalised read counts were obtained via Subread and edgeR. The raw data was filtered to include genes with a minimum expression of 1 count per million in at least two samples (16 189 genes). Differential gene expression was computed on this filtered set using the edgeR generalised linear model and quasi-likelihood method. Exon skipping was quantified using Integrative Genome Viewer (IGV) and sashimi plots, calculated as the ratio of reads bypassing the exon of interest to the mean of the reads from the preceding and following exon junctions. Junctions with fewer than five reads were excluded from presentation.

Statistics

Statistical analyses were performed using IBM SPSS software. Data were first assessed for normal distribution, and if necessary, log₁₀ or square root transformation was applied for parametric testing. Parametric data were analysed

using Student's *t*-test or one-way or two-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis. Non-parametric data were analysed using the independent samples Mann-Whitney U test. Spearman's rank test was used for correlation analyses. P-values were corrected for multiple comparisons using the Bonferroni method where applicable. Statistical significance was accepted at a *p*-value of less than 0.05. Data are expressed as mean plus or minus standard deviation, median with range, or mean plus or minus standard error of the mean (s.e.m.) for cell culture experiments.

Acknowledgements:

I start with the most obvious and well-deserved acknowledgement, which is of course **Åsa**. Although I love to write, I am struggling to find the words to express my gratitude for everything you have done for me both in my Ph.D. and outside of it. I think you must be the best supervisor in the world. It meant so much to me when someone I look up to believes in me, and as a result I started doing the same.

I am deeply grateful to my co-supervisor, **Maria**, whose guidance throughout my Ph.D. has been like a beacon. You're kind, unstoppable and a true inspiration.

It is rare to meet someone who is so busy and yet still finds the time for you. Thank you **Sanaz** for providing your invaluable insights and for seeing a potential in me.

Thank you to the other Ph.D. students in the lab. To **Jen**, for your companionship and the fun stories that made the long days shorter. To **Karin**, for your wonderful conversations and company.

I am also deeply grateful to the technical staff—**Björn, Ulrika, Anna** (who has now found new work) and **Anneli** (who is now happily retired). Thank you for your hard work, without you this thesis would not possibly be so long. A special thanks to **Courtney** and **Deniz** who know everything and can answer all my (sometimes relevant) questions.

Thank you to all the collaborators and co-authors for making these studies possible, **Hoa, Nicolas, Jonasz, Glenda, João, Chrisovalantou, Simone, Rachel, Barbara, Paul, Marie-Louisa Schoebel, Linda, Elisabet, Elisabeth, Catriona** and **Carsten**.

Thank you to **TNU** and **BRAINS** for all wonderful lunches and fikas together. Thank you to the rest of **Brain Disease Biomarker Unit**, to **Alina, Marie, Shriya** and **Elna** for great company and for our intriguing discussions, journal clubs and conferences. A special thanks to **Mats** and your amazing printed notes on the lunch table. I am grateful for your constant excitement about my projects (and fika!).

It's amazing how some people can make you excited and motivated to come to work every. single. day. For that, I want to thank **Oskar, Elif, Ronja, Elena, Rose, Taygun**, and **Agnes**. Outside of work you fill my days with trips to the beach and nights with movies. The only difficult part was when you had to leave the lab, breaking my heart on seven different occasions. The board game crowd at D11 will also have a special place in my heart and especially the organiser **Sanja**, I just wish

you all would have moved here sooner. Only special persons can make a house like BMC feel like my second home.

I want to thank all my friends — I love you. To my best PhD friends, **Antton** and **Vendela**, thank you for our wonderful lunches filled with warm and at times delightfully disturbing conversations. To **Sherry** and **Emma K**, thank you for being with me since the very first day of our studies. Thank you, **Emma N**, for just being lovely and joining me to every MultiPark event. I'm also so grateful for all my friends outside of academia, to **Johanna**, for being amazing despite being too far away (Helsingborg). To **Alex** and **Veronica** for all the arts and craft inspiration and lovely company. To my besties **Vicky** (Blomman) and **Sara** (Bubblan) for all the incredibly fun (and efficient!) time together. To **Julia** for just being wonderful and always making me laugh. Lastly, to **Alvar**, I seriously owe you for every achievement I reach, and I want to remind you of this every chance I get.

I am also so grateful for all the friends I've made at conferences. Thank you to **Anupriya** from UCL, **Carlos** and **Sam** from McMaster and **Max**. Thank you **Mike** from the University of Pennsylvania for getting tattoos with me and giving me a great story to cherish forever. Finally, a huge thank you to my co-chair for the Gordon Research Seminar, **Joe**, who was amazing to work with building my favourite memory from my Ph.D. studies.

Thank you to the **Lund Doctoral Council (LDK)** and **medical doctoral research (MDR)** student council for helping me keep busy during my PhD and feel like I am making a difference. I am also grateful for the organisation committee at **MultiPark Café**, I have had a lot of fun attending, helping out and presenting at your events!

To my family, you will always in a way be the foundation of this work. To my grandmother, who has been so proud of my becoming a doctor (perhaps not in the way she originally thought). I also thank my dad **Kjell** and my step-mom **Gunilla**. Your long drive with me up to Kiruna for me to start Rymdgymnasiet (“space school”) taught me a vital lesson early on: physics isn’t for me, and in that realisation helped me find the right direction early on. To my uncle **Peter** and to **Tobias**, thank you for being the steadfast role models I have always needed.

Lastly, I want to acknowledge my brother, **Felix**, to whom this thesis is dedicated. Thank you for the supportive calls that give me the strength to do anything. I wish I had half of your generosity, genuineness and just sheer genius. It fills me with pride to be your sister and to know that, with you, I find the truest meaning of the word family.

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Paper I



RESEARCH

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TDP-43 overexpression in the hypothalamus drives neuropathology, dysregulates metabolism and impairs behavior in mice

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Abstract

TAR DNA-binding protein 43 (TDP-43) pathology is linked to the neurodegenerative disorders amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) and Huntington disease (HD). Dysregulation of metabolism and emotion is shared across these disorders and may be caused by hypothalamic pathology. Inclusions with TDP-43 are present in the hypothalamus in clinical ALS, as well as selective loss of hypothalamic neurons expressing the metabolism and emotion regulating neuropeptides hypocretin (orexin), melanin-concentrating hormone (MCH) and oxytocin. We aimed to investigate whether there is a causal link between the effects of TDP-43 in the hypothalamus and the development of neuropathology, as well as changes in metabolism and behavior. We generated an adeno-associated viral (AAV) vector expressing human TDP-43 under the neuronal-specific synapsin promoter, which was injected bilaterally into the hypothalamus of wild-type FVB/N mice. TDP-43 overexpression resulted in hypothalamic pathology in a dose-dependent fashion replicating clinical pathology with hypothalamic atrophy and loss of hypocretin-, MCH- and oxytocin-expressing neurons. Nuclear and cytoplasmic inclusions of TDP-43 were found in the hypothalamus. Mice overexpressing TDP-43 in the hypothalamus developed metabolic dysregulation with hyperglycaemia independent of food intake. Additionally, mice overexpressing TDP-43 in the hypothalamus exhibited reduced motor activity and nesting ability, suggesting the development of an apathy-like phenotype. Taken together, AAV-vector mediated TDP-43 overexpression in the hypothalamus leads to neuropathology with the development of metabolic dysfunction and apathy-like behavior. These results indicate that TDP-43 can exert direct pathological effects in the hypothalamus, which may contribute to the development of the non-motor phenotype in TDP-43 proteinopathies.

Keywords (4 to 6): TDP-43, Hypothalamus, Metabolism, Amyotrophic lateral sclerosis, Frontotemporal dementia

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Introduction

TAR DNA-binding protein of 43 kDa (TDP-43) proteinopathies including amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and Huntington disease (HD) are neurodegenerative disorders characterized by TDP-43 pathology [16]. The TDP-43 protein encoded by the *TARDBP* gene is a ubiquitously expressed DNA and RNA binding protein mainly localized in the cell nucleus [13, 14, 65]. TDP-43 forms inclusions in the brain of almost all individuals with ALS and in around half of individuals with FTD [5, 57, 59, 62, 63, 76]. These inclusions are both nuclear and cytoplasmic, and TDP-43 has been found to be ubiquitinated as well as to undergo nuclear-to-cytoplasmic mislocalization leading to both gain-of function and loss of function mechanisms [5, 37, 63]. Interestingly, TDP-43 also forms inclusions in HD and interacts with mutant huntingtin, the disease-causing protein of HD [33, 64, 72, 73, 78].

In addition to TDP-43 pathology, these neurodegenerative diseases share early manifestation of non-motor features including psychiatric and metabolic changes that may be caused by pathology of the hypothalamus, a brain region critical for regulating these functions [2, 3, 8, 17, 34, 38, 66, 69, 74, 92]. Hypothalamic atrophy is observed in these disorders, affecting the entire hypothalamus in ALS/FTD and the lateral tuberal and paraventricular nuclei in HD [21, 28, 30, 34, 47, 52, 69, 74, 82, 86]. Selective loss of hypothalamic neurons expressing the emotion and metabolism regulating neuropeptides oxytocin and hypocretin (orexin) has been identified in both ALS and HD [6, 28–30, 68]. Furthermore, TDP-43 inclusions have been found in hypothalamic nuclei in post-mortem tissue from individuals with ALS and FTD, including in hypocretin and oxytocin-expressing neurons [21]. The number of melanin-concentrating hormone (MCH) neurons has been shown to be reduced in ALS, but not in clinical HD [6, 8, 12]. Loss of MCH neurons have recently been linked to the early manifestation of sleep disturbances in presymptomatic ALS gene carriers [35]. Hence, this selective vulnerability of hypothalamic neuronal populations may contribute to the early onset of non-motor features in these disorders. However, direct causal relationships between hypothalamic pathology in these disorders and disease manifestations remain to be fully established. Hypothalamic atrophy has been shown to correlate to reduced body mass index (BMI), abnormal eating habits as well as behavioral changes such as apathy in cases with ALS and FTD using MRI analyses [34, 82].

Hypothalamic pathology may be an important part of the early development of neurodegenerative disorders. We hypothesize that TDP-43 exerts direct pathological effects in the hypothalamus leading to the development of behavioral and metabolic changes. In the present study, we investigated whether there is direct causality

between overexpression of TDP-43 in the hypothalamus and the development of neuropathology as well as metabolic and behavioral dysfunction. We generated an adeno-associated viral (AAV) vector expressing wild-type human TDP-43 under the synapsin promoter and delivered it bilaterally to the hypothalamus of FVB/N mice. We found that TDP-43 can directly elicit pathological effects in the hypothalamus, relevant to the early clinical phenotype of TDP-43 proteinopathies.

Materials and methods

Animals

Female FVB/N mice (Janvier Labs) were housed in cages of 2–5 animals and maintained in a 12 h light/dark cycle with *ad libitum* access to food and water. The experimental procedures were approved by the Malmö/Lund Regional Ethical Committee, Sweden (Permit: 17113/2022). In accordance with this ethical permit, mice needed to be sacrificed if a humane endpoint is reached. These humane endpoints were defined as manifestation of complete immobility, eye squinting and raggedness of fur. The requirements were consistently monitored across all experiments to ensure animal welfare. Only mice in Experiment II reached the humane endpoint prior to the scheduled experiment endpoint.

Experiment I

We constructed an AAV vector designed to express wild-type human TDP-43 under the human synapsin 1 promoter (AAV-TDP43). To verify overexpression of TDP-43 and to determine the dose-dependent effect of the AAV-TDP43 vector on hypothalamic neuropathology, mice were bilaterally injected with three doses of the AAV vector (5.0×10^{13} ($n=6$); 1.0×10^{14} ($n=6$); 2.5×10^{14} ($n=6$) genome copies (gc/ml)) in the hypothalamus at 6 weeks of age. Two uninjected mice were used as controls. Mice were perfused 9 weeks post-injection.

Experiment II

To further evaluate the effects on neuropathology following TDP-43 overexpression in the hypothalamus, 10 mice were bilaterally injected in the hypothalamus with the AAV-TDP43 vector (2.5×10^{14} gc/ml) at 6 weeks of age and 8 uninjected mice of the same age served as controls. Injected mice were required to be euthanized 6 or 10 weeks post-injection ($n=5$ /timepoint) according to humane endpoints defined in the ethical permit 17113/2022 including the manifestation of immobility, eye squinting and raggedness of fur. The uninjected controls were sacrificed at 18 weeks of age. Brains were neuropathologically examined. To confirm that the neuropathological effects following overexpression of TDP-43 was not due to simply overexpression of any protein, AAV vectors expressing green fluorescent protein (GFP)

(AAV-GFP) or a fragment of mutant huntingtin (mHTT) with a 79 polyglutamine tract (AAV-mHTT) were used at the same titer (2.5×10^{14} gc/ml). These vectors have been produced and described previously [42]. Mice were bilaterally injected with either AAV-GFP ($n=5$) or AAV-mHTT ($n=4$) in the hypothalamus at 6 weeks of age and sacrificed at 10 weeks post-injection.

Experiment III

To investigate effects on metabolism, behavior and molecular properties of TDP-43 following TDP-43 overexpression in the hypothalamus, 19 mice were bilaterally injected in the hypothalamus with the AAV-TDP43 vector (1.0×10^{14} gc/ml) and 20 mice with vehicle (10 ppm poloxamer-188 in phosphate-buffered saline (PBS)). Mice were weighed biweekly throughout the experiment. Mice underwent behavioural testing at 18 weeks post-injection using open field and elevated plus maze, at 19 weeks post-injection using the rotarod test and at 20 weeks post-injection using the forced swim test. Fat composition was determined with dual energy X-ray absorptiometry (DEXA) scan at 20 weeks post-injection. Mice were perfused at 21 weeks post-injection. Brains were immunohistochemically processed for TDP-43 and targeting of neuropeptide neuronal populations. Blood was collected from the right ventricle between 8:00 and 15:00 without prior fasting, and serum was saved for glucose measurements.

Experiment IV

To determine effects on food intake, nesting behavior, neuroinflammation and gene expression following TDP-43 overexpression in the hypothalamus, 16 mice were bilaterally injected in the hypothalamus with the AAV-TDP43 vector (1.0×10^{14} gc/ml) and 16 control mice were injected with vehicle. Mice were weighed biweekly throughout the experiment. Prior to the 8-week post-injection assessments, one AAV-TDP43-injected mouse unexpectedly died, leading to the exclusion of its cage from nesting and food intake measurements. Mice were sacrificed at 10 weeks post-injection and hypothalami were dissected for qRT-PCR and RNA sequencing analyses.

AAV vector production and stereotactic delivery

Recombinant AAV vector plasmids were derived from a previously described double-transfection method [84]. The AAV-TDP43 vector was designed to express the coding sequence of wild-type human *TARDP* gene. The design of the vector including the choice of AAV serotype as well as the delivery method was based on previous experiments expressing transgenes into the hypothalamus models for HD [42]. We also used two previously described AAV-vectors, the AAV-mHTT vector

that expresses the first 853 amino acids of the N-terminal fragment of huntingtin with 79 polyglutamines and the AAV-GFP vector expressing GFP [42]. In all vectors, the transgene sequences were regulated by the human synapsin 1 promoter and flanked with simian virus 40 polyadenylation signal sequence between the inverted terminal repeat from AAV2 genome and packaged in a capsid from AAV5 genome. All vectors were dissolved with 10 ppm poloxamer-188 in PBS and titers were estimated by TaqMan quantitative polymerase-chain reaction. The AAV-TDP43 titers were 5.0×10^{13} , 1.0×10^{14} and 2.5×10^{14} gc/ml. The AAV-mHTT and AAV-GFP titers were 2.5×10^{14} gc/ml. Stereotactic delivery of 0.5 μ l/side of the AAV vectors or vehicle was performed as previously described [10] at the following coordinates: anterior/posterior = -0.7 mm, medial/lateral: $\pm$ 0.55 mm, ventral/dorsal = -5.2 mm. The stereotactic coordinates were calculated relative to the bregma and dura.

Immunohistochemistry

Peroxidase-based immunohistochemistry

Mice were sacrificed using sodium pentobarbital (~150 mg/kg i.p.) and transcardially perfused with first saline and then pre-cooled 4% paraformaldehyde (PFA) solution. Brains were removed, and then post-fixed for 24 h in 4% PFA and placed in 25% sucrose for cryoprotection. Brains were sectioned frozen in the coronal plane at a thickness of 30 μ m in six series. The sections were processed as previously described [10]. Primary antibodies were raised against TDP-43 (1:100 000, made in rabbit, 10782-2-AP, ProteinTech), oxytocin (1:4000, made in rabbit, H-051-01, Phoenix Pharmaceuticals), hypocretin (1:4000, made in rabbit, H-003-30, Phoenix Pharmaceuticals), vasopressin (1:10 000, made in rabbit, AB1565, Merck Milipore), MCH (1:20 000, made in rabbit, H-070-47, Phoenix Pharmaceuticals), huntingtin (1:500, made in goat, SC-8767, Santa Cruz Biotechnology), GFP (1:20 000, made in chicken, ab13970, Abcam), ubiquitin (1:2000, made in rabbit, Z0458, DakoCytomation), GFAP (1:10 000, made in rabbit, Z0334, DakoCytomation) and Iba1 (1:1000, made in rabbit, 019-19741, FUJIFILM Wako Pure Chemical Corporation). Sections were labelled with suitable secondary antibody (1:200, anti-rabbit, anti-goat or anti-chicken) and visualised with 3,3'-diaminobenzidine (DAB) as chromogen using hydrogen peroxidase. Lastly, sections were mounted on chromatin-gelatin coated glass slides and dehydrated in an ethanol and xylene series and coverslipped with DPX (Sigma Aldrich).

For cresyl violet (CV) staining, slide-mounted sections were first dehydrated in an ethanol and xylene series. Sections were then briefly dipped in deionized water followed by lithium carbonate solution (0.5 g lithium carbonate in 1000 ml deionized water) for 30 s, 70% ethanol

for 30 s, deionized water for 30 s and 0.5% CV solution for 1 min with 20 drops of 10% acetic acid were added per 100 ml CV solution. Slides were again dehydrated through an ethanol series.

Fluorescence immunohistochemistry

To confirm targeting of AAV-mediated TDP-43 over-expression in hypocretin, MCH, oxytocin or vasopressin neurons, brain sections were blocked in 0.25% triton X-100 in potassium phosphate-buffered saline (KPBS-T) containing 5% donkey serum for 1 h. Subsequently, the sections were incubated overnight using antibodies against oxytocin (1:2000, made in rabbit, H-051-01, Phoenix Pharmaceuticals), hypocretin (1:2000, made in rabbit, H-003-30, Phoenix Pharmaceuticals), vasopressin (1:1000, made in rabbit, AB1565, Merck Milipore) and MCH (1:2000, made in rabbit, H-070-47, Phoenix Pharmaceuticals) in KPBS-T containing 5% donkey serum. Sections were labelled with Alexa Fluor 647-conjugated donkey anti-rabbit secondary antibody (1:200, Jackson Laboratories), followed by an additional blocking step of 5% rabbit serum in KPBS-T for 2 h. Samples were then incubated overnight with biotin-conjugated TDP-43 (1:200, made in rabbit, Biotin-10782, ProteinTech), followed by Alexa Fluor 488-conjugated streptavidin (1:200, Jackson Laboratories). For analyses of intracellular TDP-43 localisation, the samples were incubated with a primary antibody against TDP-43 (1:1000, made in rabbit, 10782-2-AP, Proteintech) followed by Alexa Fluor 647-conjugated secondary antibody (1:200, Jackson Laboratories). After secondary antibody incubation, sections were stained with Hoechst 33342 (1:1000, 62249, Thermo Fisher) for 5 min. All sections were mounted on chromatin-gelatin coated glass slides and coverslipped using Vectashield mounting medium (Vector Laboratories).

Image acquisition

Brightfield and fluorescence image acquisition was performed using a Zeiss Axio Imager M2 microscope (Zeiss, Göttingen, Germany) equipped with apochromatic objectives (5x/0.15 NA, 10x/0.3 NA, 20x/0.8 NA, and 40x/1.3 NA oil). Brightfield images were acquired with a Zeiss AxioCam 305 color camera, and fluorescent images with a Zeiss AxioCam 805 monochrome camera. For each figure, images were acquired with consistent settings, and contrast was optimized by adjusting the histogram using the Zeiss Zen 3.8 software. Image panels were created and edited using Affinity Designer 1.10.

Cell quantification and hypothalamic volume measurements

Brain sections immunohistochemically processed with antibodies against hypocretin, MCH, oxytocin, and vasopressin were analyzed on a Nikon Eclipse 80i microscope

using a 60x plan-apochromat 1.4 N.A. oil objective. The VIS software (Visiopharm) was used for all analyses. The experimenter was blinded to the experimental groups for all cell quantification and volume measurements. All hypocretin- and MCH-expressing neurons were counted bilaterally in the hypothalamus. Oxytocin- and vasopressin-expressing neurons in or bordering the paraventricular nucleus were counted bilaterally. Hypocretin- and MCH-expressing neurons were located around bregma -1.06 to -2.30 mm, and oxytocin- and vasopressin-expressing neurons at bregma -0.46 to -1.22 mm. The total number of neurons was estimated by total cell count multiplied by the number of series (6).

Hypothalamic volume was measured from sections spanning bregma -0.35 mm to -2.18 mm. The hypothalamus was outlined along the third ventricle to the optic tract. In experiment II, images were captured on a Nikon Eclipse 80i (4x/0.1 N.A.). The hypothalamus was measured bilaterally using VIS software. In experiment III, images were captured with the Zeiss PrimoStar microscope (4x/0.1 N.A.). The hypothalamus was measured unilaterally using the ImageJ 1.53t software. Volume was calculated by multiplying the outlined hypothalamic area by section thickness (30 μ m), the number of series (6), and, in experiment III, the number of hemispheres (2).

RNA sequencing

RNA isolation was performed on frozen hypothalami from mice 10 weeks post-injection in experiment IV using the RNeasy Mini Kit (Qiagen), according to the manufacturer's recommendations, except for the omission of β -mercaptoethanol in the buffers ($n=6$ /group). Disruption of samples was performed using the TissueLyser II (Qiagen) for 2 $\times$ 2 min at 30 Hz using 3 mm Tungsten Carbide Beads and RLT buffer, and the inclusion of the RNase-Free DNase Set. Upon completion of the protocol, the RNA was eluted in 35 μ l of RNase-free water. The concentration of RNA was measured using the Qubit Fluorometric Quantitation and RNA Broad-Range Assay (Thermo Fisher Scientific). RNA integrity number (RIN) was determined using the Fragment Analyzer 5300 and Fragment Analyzer RNA kit (Agilent Technologies) and showed good integrity (RIN > 8.8). For library preparation, the mRNA fraction was enriched using polyA capture from 100 ng of total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB). Subsequently, libraries were prepared using the NEB Next Ultra II Directional RNA Library Prep Kit for Illumina and NEBNext UDI UMI (NEB) following the manufacturer's instructions. To minimize technical batch effects, library preparations were performed using the Liquid Handler Biomek i7 (Beckman Coulter). The library molarity was determined by measuring the library size (approximately 400 bp) using the Fragment Analyzer

5300 and the Fragment Analyzer DNA HS NGS fragment kit (Agilent Technologies), and the library concentration (>2 ng/μl) using Qubit Fluorometric Quantitation and dsDNA High Sensitivity Assay (Thermo Fisher Scientific). The libraries were denatured according to the manufacturer's instructions, diluted to 150 pM, and sequenced as paired-end 100 bp reads on an Illumina NovaSeq 6000 (Illumina). Sequencing was aimed at achieving a depth of >25 million clusters per sample. The read quality of RNA-seq data in fastq files was assessed using ngs-bits (2023_11) to identify sequencing cycles with low average quality, adaptor contamination, or repetitive sequences from the PCR amplification. Reads were aligned using STAR 2.7.11b [23] to the GRCm39 and alignment quality was analyzed using ngs-bits and visually inspected in the Integrative Genome Viewer 2.15.4. Normalized read counts for all genes were obtained using Subread 2.0.6 and edgeR 3.42.4. Raw expression values were available for 55 416 genes in 12 samples. Raw gene expression was filtered by a minimum expression value of 1 count per million (cpm) in at least two samples. The filtered data contained the expression values for 16 189 genes. The distribution of the logarithmized cpm-normalized expression values showed similar characteristics for all samples. Based on the filtered dataset, samples were investigated with respect to their pairwise similarity. Differential gene expression analysis was conducted based on the filtered gene expression dataset. For each gene, gene expression log2 fold changes were computed, and was statistically tested for significance using edgeR generalized linear model and the quasi-likelihood method.

Quantification of exon skipping

Exon skipping was quantified using Integrative Genome Viewer 2.15.4, with sashimi plots of exon junctions displayed for each sample. The number of reads that bypassed the exon of interest was divided by the mean of the reads for exon junctions preceding and following the exon of interest. Junctions with fewer than five reads were excluded from data presentation.

qRT-PCR

To confirm hypothalamic TDP-43 overexpression, mRNA levels of *TARDBP/Tardbp* were analyzed by quantitative real-time PCR. Reverse transcription was conducted using a QuantiTect Reverse Transcription Kit (Qiagen) with 250 ng of RNA input and gDNA digestion. Gene-specific primers were designed to amplify both human *TARDBP* (NM_007375.4) and mouse *Tardbp* (NM_145556.4) using Primer-BLAST [91]. with an amplicon size of 75–150 bp and melting at 60 °C. An exon junction was incorporated to mitigate potential signals related to genomic DNA, resulting in the forward primer AAGGAATTCTGCATGCCCA and reverse

primer TGTTTTCTGGACTGCTCTTTTCAC. For reference genes, primers targeting the mouse *Gapdh* forward primer TGTCCGTCGTGGATCTGAC and reverse primer CCTGCTTACCACCTTCTTG, as well as the mouse *Pdhh* forward primer GTAGAGGACACGGGC AAGAT and reverse primer TGAAAACGCCTCTTCA GCA were employed. Quantitative PCR was performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) with a master mix of 10 μl and including 1 μl of the resulting cDNA samples diluted to 1:20. All real-time PCR were conducted using a LightCycler 480 Instrument II (Roche). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 45 cycles of 94 °C for 15s, 55 °C for 30s and 72 °C for 30s. A melting curve was obtained from 65 °C to 95 °C with a Ramp Rate of 0.04 °C/s and 15 acquisitions per °C. A standard curve of all mixed cDNA was generated using a raw dilution, and the cDNA was diluted to 1:5, 1:20, 1:80, and 1:320 to correct for qPCR efficiency. Experiments were performed in duplicate for each data point, and the relative quantification of gene expression was determined using the efficiency-corrected 2-ΔΔCt method [58].

Body fat and serum glucose concentration

To measure body fat percentage at 20 weeks post-injection, mice were anaesthetized with a 2% isoflurane/N₂O mixture and individually scanned using the DEXA Lunar PIXImus2 scanner (Lunar Corporation). Body fat percentage was calculated using PIXImus2 2.10 software. Serum glucose concentration was determined by the glucose oxidase method coupled to a peroxidase reaction to colorimetrically detect H₂O₂ formation [24].

Behavioural procedures

Open field and elevated plus maze

The open field test was used to assess motor activity and elevated plus maze to assess anxiety-like behaviour of mice bilaterally injected with AAV-TDP43 or vehicle at 18 weeks post-injection as previously described [10, 41, 67]. Mice were recorded for 1 h in the open field test and 5 min in elevated plus maze using the Ethovision XT 13 software. Mice were randomly assigned to a trial using a random number generator. Mice were moved into testing area 1 h before testing and the experiments were performed between 8:00–13:00.

Rotarod

An accelerating rotarod device (Ugo Basile S.R.L.) was used to evaluate motor activity and balance in mice at 19 weeks post-injection. Prior to the rotarod test, mice underwent three 2-minute-long training sessions at a constant speed of 5 revolutions per minute (rpm) over 3 consecutive days, with 2-hour inter-session intervals. If the mice fell, they were returned to the rod. The

rotarod test consisted of accelerating rotation from 5 rpm to 40 rpm over 5 min. The test ended if the mouse fell off the rod or if 5 min had passed. The test was repeated three times with 2-hour intersession intervals. The order in which the mice were tested was randomized and the mice were transferred to the testing area 1 h before testing. The tests were performed between 8:00–13:00.

Forced swim test

The forced swim test was used to assess depressive-like behaviour as immobility in mice at 20-weeks post-injection as described previously [20, 41]. Mice were recorded for 5 min and the time spent immobile during the last 3 min was manually scored by an experimenter blinded to the treatment groups. The order in which the mice were tested was randomized and the mice were transferred to the testing area 1 h before testing. The tests were performed between 8:00–13:00.

Nesting and food intake

Nesting and food intake were assessed over a 4-day period (with measurements at day 0 and 4) at 4- and 8-weeks post-injection of AAV-TDP43 or vehicle at the same time of the day. Mice of the same treatment group were pair-housed with a 2-day acclimatizing period with *ad libitum* access to food and water. Food intake per cage over four days was determined by calculating the difference in total food weight between day 0 and day 4. At the start of the nesting behavior analysis, 7–8 g of intact nesting material was provided in each cage. The experimenter was blinded to the experimental groups during nesting behavior assessment at both time points. Nesting ability was assessed based on a modified scoring system from Gaskill et al. [32]. Briefly, a score of 0 was assigned if the material appeared untouched, a score of 1 was given for manipulated material without starting to cup or form a dome, a score of 2 indicated manipulated material and a partially formed nest with a developing cup, and a score of 3 indicated complete manipulation of material with a fully cupped dome.

Statistics

Data was analysed using the independent samples Mann-Whitney U test in SPSS 18.0, and p-values were corrected for multiple comparisons using Bonferroni when applicable. Spearman's rank test was used for correlation analyses. Statistical significance was set at $p < 0.05$ (exact, 2-tailed). Graphs were generated using GraphPad Prism 10.2. Data are presented as median with range.

Results

AAV-vector mediated expression of human wild-type TDP-43 in the hypothalamus leads to neuropathology in a dose-dependent fashion

We generated an AAV vector to express wild-type human TDP-43 using the same AAV-vector design and the same neuronal specific synapsin promotor used in Hult et al. [42]. To confirm overexpression of TDP-43 and to begin investigating the potential neuropathological effects, we first performed stereotaxic bilateral injections of AAV-TDP43 at three different titers: 5.0×10^{13} gc/ml ($n=6$), 1.0×10^{14} gc/ml, ($n=6$), or 2.5×10^{14} gc/ml ($n=6$) into the hypothalamus of adult female FVB/N mice. TDP-43 overexpression in the hypothalamus led to widespread TDP-43 immunoreactivity throughout the hypothalamus across all groups, including in the paraventricular nucleus and the lateral hypothalamic area, which contain hypocretin- and oxytocin-expressing neurons that are significantly affected in postmortem ALS cases (Fig. 1a) [28]. TDP-43 immunoreactivity was concentrated to the hypothalamus after the AAV-delivery, but single cells overexpressing TDP-43 could be observed in other brain regions, including the cerebral cortex, striatum, and hippocampus (Supplementary Fig. 1). Qualitative analysis showed a decrease in the number of hypocretin- and oxytocin-expressing neurons in a dose-dependent fashion, with the most pronounced loss in highest titer group (Fig. 1b-c). Neuronal loss was confirmed by analysis of CV-stained sections (Fig. 1d). Additionally, ubiquitin-immunoreactivity was increased in a dose-dependent fashion with ubiquitin-immunoreactive inclusions present in mice overexpressing TDP-43 (Fig. 1e). Consistent with previous findings using the same AAV-vector design [42], we confirmed that AAV-mediated TDP-43 overexpression targets hypothalamic neurons expressing hypocretin, MCH, oxytocin, and vasopressin (Supplementary Fig. 2).

As TDP-43 overexpression in neurons could lead to non-cell autonomous effects on glial cells such as astrocytes and microglia triggering neuroinflammation [39] we investigated effects on glial neurofibrillary protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) immunoreactivity in mice overexpressing TDP-43 ($n=3$) and vehicle-injected mice ($n=3$). We observed increased GFAP immunoreactivity in mice overexpressing TDP-43 compared to vehicle-injected control mice. Furthermore, Iba1-immunoreactive cells in mice overexpressing TDP-43 showed distinct morphological changes, including increased and thickening of branches. These findings suggest astrogliosis and microglia activation indicative of an inflammatory response to TDP-43 overexpression (Supplementary Fig. 3).

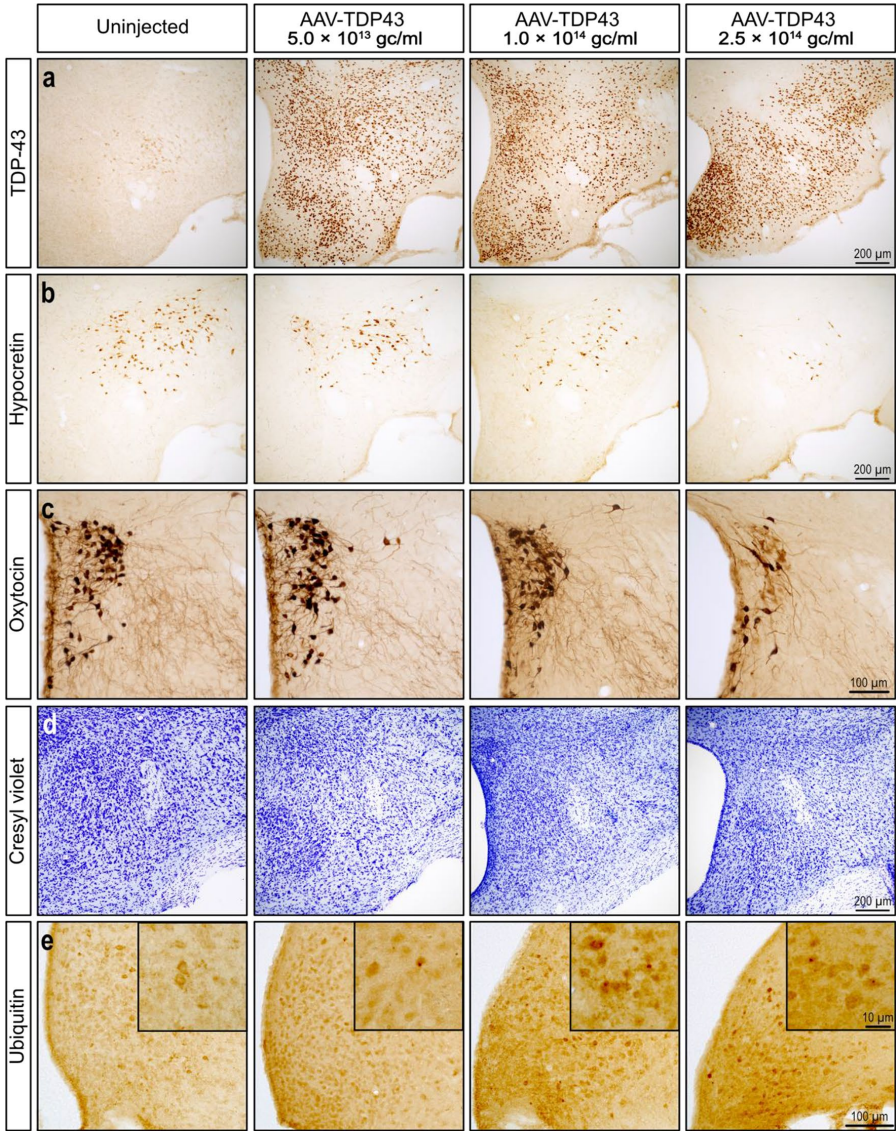


Fig. 1 Overexpression of TDP-43 induces neuropathology in the hypothalamus in a dose-dependent fashion. Mice were injected with 5.0×10^{13} genome copies (gc)/ml, 1.0×10^{14} gc/ml, or 2.5×10^{14} gc/ml titers of the AAV-TDP43 vector. **a.** Immunoreactivity for TDP-43 was comparable in the hypothalamus across all injected groups. The number of neurons immunoreactive for hypocretin (**b**) and oxytocin (**c**) appeared to decrease in a dose-dependent fashion. **d.** Neuronal loss was observed in the lateral hypothalamic area only in mice injected with the highest dose. **e.** Mice injected with the AAV-TDP43 vectors showed increased ubiquitin immunoreactivity in a dose-dependent fashion with ubiquitin-immunopositive inclusions

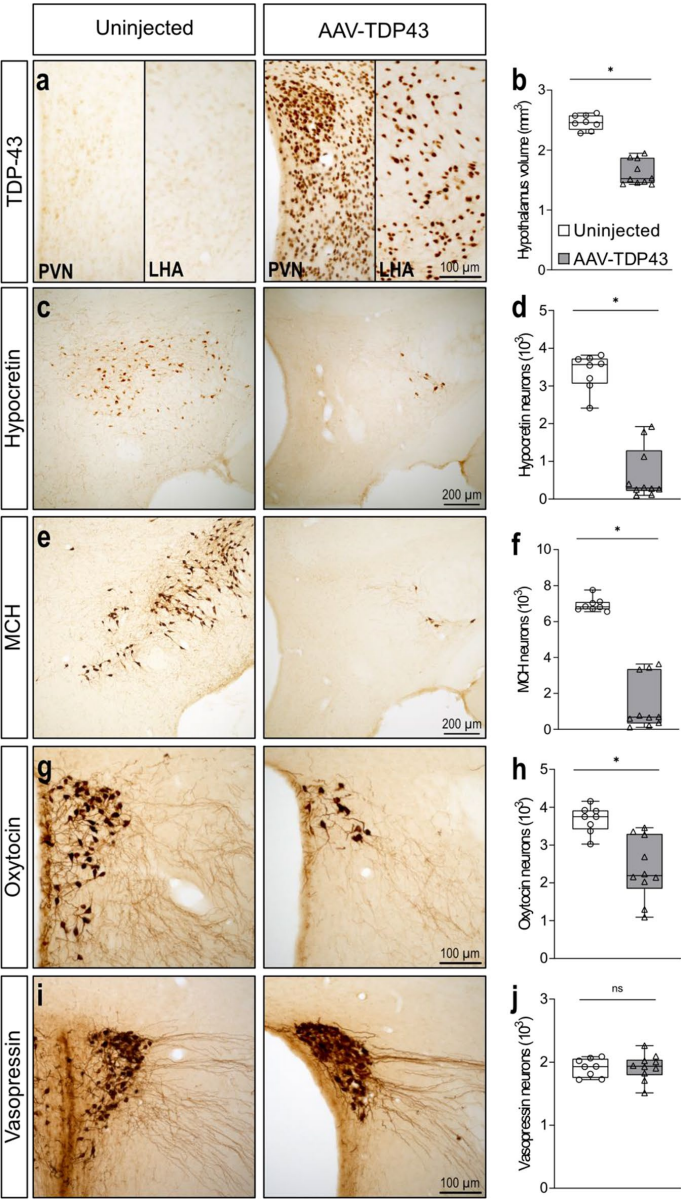


Fig. 2 (See legend on next page.)

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Fig. 2 TDP-43 overexpression causes hypothalamic atrophy with selective loss of hypocretin-, oxytocin- and melanin concentrating hormone (MCH)-expressing neurons. **a**. TDP-43 immunoreactivity was detected in the hypothalamus in mice injected with AAV-TDP43, including in the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA), which contain neurons expressing oxytocin and vasopressin as well as hypocretin and MCH, respectively. **b**. Mice overexpressing TDP-43 in the hypothalamus displayed reduced hypothalamic volume compared to uninjected control mice ($p < 0.001$). **c-d**. Loss of hypocretin-expressing neurons was present in mice overexpressing TDP-43 in the hypothalamus compared to control mice ($p < 0.001$). **e-f**. A similar pattern was observed in MCH-expressing neurons with significant reductions in mice overexpressing TDP-43 ($p < 0.001$). **g-h**. The number of oxytocin-expressing neurons was reduced in mice overexpressing TDP-43 compared to control mice ($p = 0.001$). **i-j**. We observed no loss of vasopressin-expressing neurons. The data are presented as box-and-whisker plots with whiskers representing minimum and maximum values, * = $p < 0.01$ (2-tailed). Statistical analyses were performed using a Mann-Whitney U test

Overexpression of TDP-43 causes hypothalamic atrophy with selective loss of hypocretin-, oxytocin- and melanin-concentrating hormone-expressing neurons

To further investigate the effects on neuropathology following TDP-43 overexpression in the hypothalamus, quantitative analyses of the hypothalamic volume and number of neuropeptide expressing neurons were performed in another cohort of mice bilaterally injected in the hypothalamus with the AAV-TDP43 vector (2.5×10^{14} gc/ml) and 8 uninjected mice of the same age that served as controls (Fig. 2a). The hypothalamic volume was reduced by 38% (1.5 mm^3 , $1.4\text{--}1.9 \text{ mm}^3$, $p < 0.001$, $n = 10$) in mice overexpressing TDP-43 compared to uninjected control mice (2.5 mm^3 , $2.3\text{--}2.6 \text{ mm}^3$, $n = 8$) (Fig. 2b). Mice overexpressing TDP-43 exhibited a 92% loss of hypocretin-expressing neurons (291, $96\text{--}1920$, $p < 0.001$, $n = 10$) compared to control mice (3567, $2412\text{--}3816$, $n = 8$, Fig. 2c-d). Similarly, mice overexpressing TDP-43 exhibited a 90% loss of MCH-expressing neurons (681, $114\text{--}3636$, $p < 0.001$, $n = 10$) compared to control mice (6819, $6552\text{--}7752$, $n = 8$) (Fig. 2e-f). Mice overexpressing TDP-43 also exhibited a 42% loss of oxytocin-expressing neurons (2193, $1092\text{--}3456$, $p = 0.001$, $n = 10$) compared to control mice (3750, $3024\text{--}4158$, $n = 8$) (Fig. 2g-h). In contrast, vasopressin-expressing neurons were not affected in mice overexpressing TDP-43 ($p = 1.000$, $n = 10$) compared to control mice ($n = 8$, Fig. 2i-j). Interestingly, hypothalamic atrophy, selective loss of neurons expressing hypocretin, MCH and oxytocin neurons and sparing of vasopressin-expressing neurons as observed in mice overexpressing TDP-43 are consistent with previous studies in postmortem human ALS cases [12, 28].

In order to confirm that the effects on the hypothalamus were not due to overexpression of any protein, a similar experiment was performed where mice received hypothalamic injections of AAV vectors expressing either GFP (AAV-GFP) or a large fragment of mutant huntingtin (AAV-mHTT) at the same titer used in experiment II (2.5×10^{14} gc/ml). At 10 weeks post-injection, mice overexpressing GFP showed no overt neuropathology of hypocretin- MCH-, or oxytocin-expressing neurons (Supplementary Fig. 4). As expected, mutant huntingtin overexpression resulted in decreased numbers of hypocretin-expressing neurons, consistent with prior results [42]. These results confirm that the neuropathology in

the hypothalamus after overexpression of TDP-43 is not simply a result of overexpression of any protein.

TDP-43 overexpression in the hypothalamus induces obesity and hyperglycaemia in mice

Hypothalamic overexpression of TDP-43 induced selective loss of neuropeptide-expressing neurons involved in the regulation of metabolism and emotion, including hypocretin, MCH and oxytocin. Therefore, we next wanted to investigate the effects of TDP-43 overexpression in the hypothalamus on metabolism and behaviour in mice. Mice injected with AAV-TDP43 gained more weight compared to control mice injected with vehicle starting from 2 weeks post-injection ($p = 0.001$ at 2 weeks post-injection and $p < 0.001$ at subsequent time-points, AAV-TDP43 $n = 19$, vehicle $n = 20$, Fig. 3a). At 20 weeks post-injection, mice injected with AAV-TDP43 had increased in body weight by 92% ($28\text{--}156\%$) compared to 14% ($9\text{--}47\%$) in mice injected with vehicle. At that time-point, mice overexpressing TDP-43 in the hypothalamus had more than doubled in body fat percentage (43% , $22\text{--}51\%$, $p < 0.001$, $n = 19$) compared to control (20% , $15\text{--}37\%$, $n = 20$, Fig. 3b-d).

We also detected a 20% increase in serum glucose levels in mice overexpressing TDP-43 (12.7 mmol/L , $9.4\text{--}20.1 \text{ mmol/L}$, $p = 0.003$, $n = 19$) compared to vehicle injected mice (10.7 mmol/L , $7.2\text{--}15.7 \text{ mmol/L}$, $n = 20$, Fig. 3e). Spearman's rank correlation test revealed a negative correlation between hypothalamic volume and body weight gain in mice overexpressing TDP-43 ($\rho: -0.475$, $p = 0.040$, $n = 19$, Fig. 3f). This indicates that mice with more pronounced hypothalamic atrophy exhibited greater body weight gain. There was no significant correlation for hypothalamic volume and serum glucose levels ($\rho: -0.204$, $p = 0.401$, $n = 19$, Fig. 3g).

A separate cohort of pair-housed mice was used to assess food intake per cage at 4 and 8 weeks following injection. We found no change in food intake in mice overexpressing TDP-43 at 4 weeks post-injection compared to control mice ($p = 0.161$, $n = 8$ cages/group at 4 weeks), while a trend of increased food intake was observed in mice overexpressing TDP-43 at 8 weeks post-injection compared to control, although not statistically significant ($p = 0.073$, AAV-TDP43 $n = 7$ cages, vehicle 8 cages, Fig. 3h). Despite unchanged food intake,

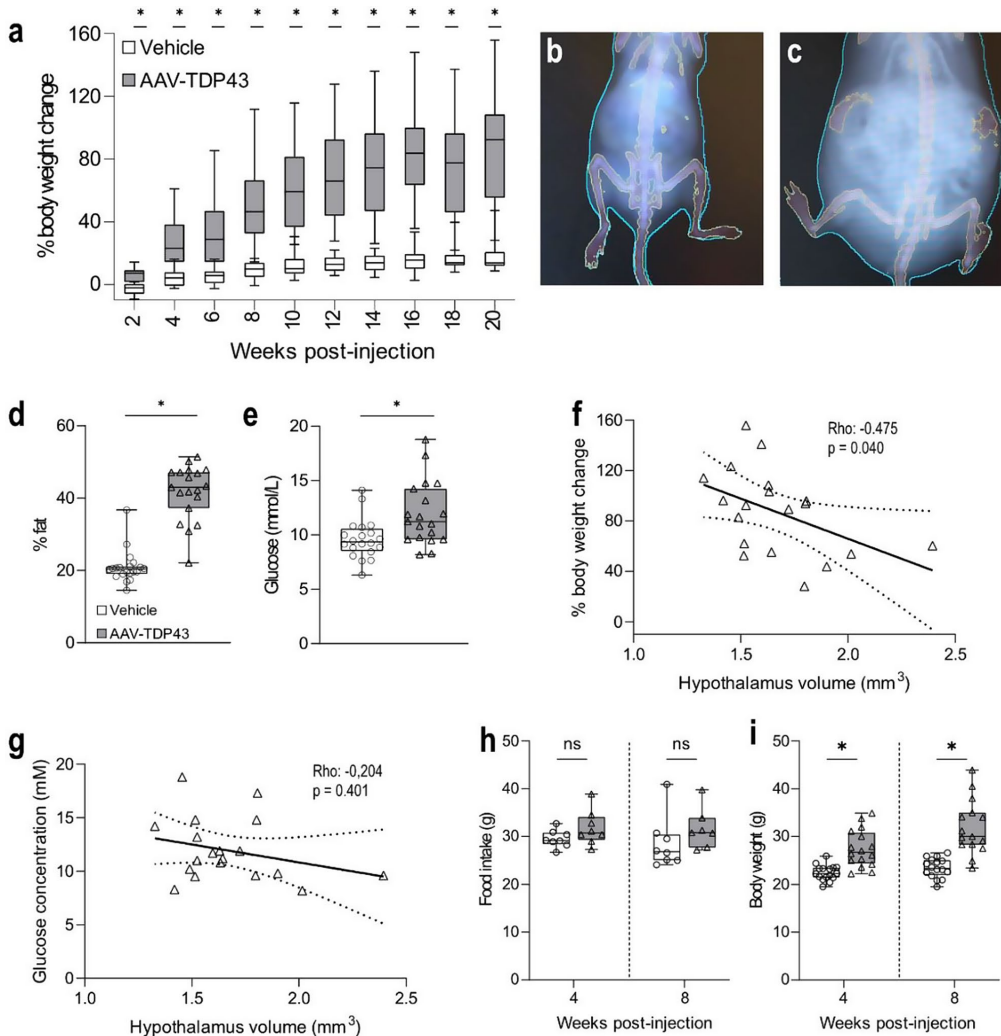


Fig. 3 TDP-43 overexpression in the hypothalamus induces obesity and hyperglycaemia in mice. **a**. Mice overexpressing TDP-43 in the hypothalamus gained more weight compared to control mice injected with vehicle starting from 2 weeks post-injection. Dual energy X-ray absorptiometry scans of vehicle-injected control (**b**) and AAV-TDP43-injected mice (**c**) revealed that mice overexpressing TDP-43 had higher body fat percentage than control mice ($p < 0.001$) (**d**, **e**). We observed a significant increase in serum glucose levels in mice overexpressing TDP-43 compared to controls ($p = 0.003$). Spearman's rank correlation test revealed a negative correlation for hypothalamic volume and percent body weight change (**f**), but no significant correlation for hypothalamic volume and serum glucose levels (**g**). In a separate cohort, mice were pair-housed and food intake was measured after 4 days at 4 and 8 weeks post-injection. Hypothalamic TDP-43 overexpression did not affect food intake at 4 and 8 weeks post-injection (**h**), despite higher body weight (**i**, $p < 0.001$ at both time-points). Data are presented as box-and-whisker plots with whiskers representing minimum and maximum values, * = $p < 0.01$ (2-tailed). Statistical analysis was performed using Mann-Whitney U test (**a**, **d**, **e**, **h**, **i**) and adjusted for multiple-comparison using Bonferroni (**a**)

mice overexpressing TDP-43 had a higher body weight compared to control mice at both 4 and 8 weeks post-injection also in this cohort. Here, TDP-43 overexpressing mice had a median body weight of 26.7 g at 4 weeks (22.2–34.9 g, $p < 0.001$, $n = 16$) and 30.0 g at 8 weeks (23.4–43.9, $p < 0.001$, $n = 15$) post-injection compared to control mice of 22.3 g (19.5–25.9 g, $n = 16$) and 23.4 g, respectively (19.5–26.6 g, $n = 16$, Fig. 3i). Taken together, these results demonstrate that hypothalamic TDP-43 overexpression disrupts metabolic homeostasis, leading to obesity and hyperglycaemia. Increased body weight gain in mice overexpressing TDP-43 correlates with hypothalamic atrophy and appears to occur independent on food intake.

Mice overexpressing TDP-43 in the hypothalamus display significantly reduced motor activity but maintain ability to swim

Motor impairment and psychiatric features, including anxiety, depression, and apathy, are seen in individuals with ALS/FTD and HD [25, 60]. To determine the effects of TDP-43 overexpression in the hypothalamus on motor activity and behavior, we first investigated motor activity using the open field test and rotarod test. In the open field

test, mice overexpressing TDP-43 in the hypothalamus travelled a shorter distance by 67% (73.1 m, 9.9–137.4 m, $p < 0.001$, $n = 19$) compared to control mice injected with vehicle (218.5 m, 155.1–966.0 m, $n = 20$, Fig. 4a). Similarly, on the rotarod test, mice overexpressing TDP-43 exhibited a pronounced 82% decrease in latency to fall (34 s, 4–199 s, $p < 0.001$, $n = 19$) compared to controls (187 s, 71–295 s, $n = 20$, Fig. 4b). These results demonstrate that TDP-43 overexpression in the hypothalamus leads to decreased motor activity and may impair motor coordination in mice.

Next, we used the elevated plus maze and the forced swim test to assess the emotional phenotype in mice overexpressing TDP-43. In the elevated plus maze, mice overexpressing TDP-43 in the hypothalamus displayed reduced distance travelled by 93% (97 cm, 42–965 cm, $p < 0.001$, $n = 19$) compared to control mice (1473 cm, 345–2467 cm, $n = 20$, Fig. 4c). Mice overexpressing TDP-43 spent more time in the center of the arena (39%, 0–100%, $p = 0.001$), where mice were placed before start of the trial, compared to control mice (21%, 1–34%, Fig. 4d). Median time spent for mice overexpressing TDP-43 was 0% on closed arms (0–100%, $p = 0.011$) compared to control mice at 55% (14–79%, Fig. 4e) and 0% on

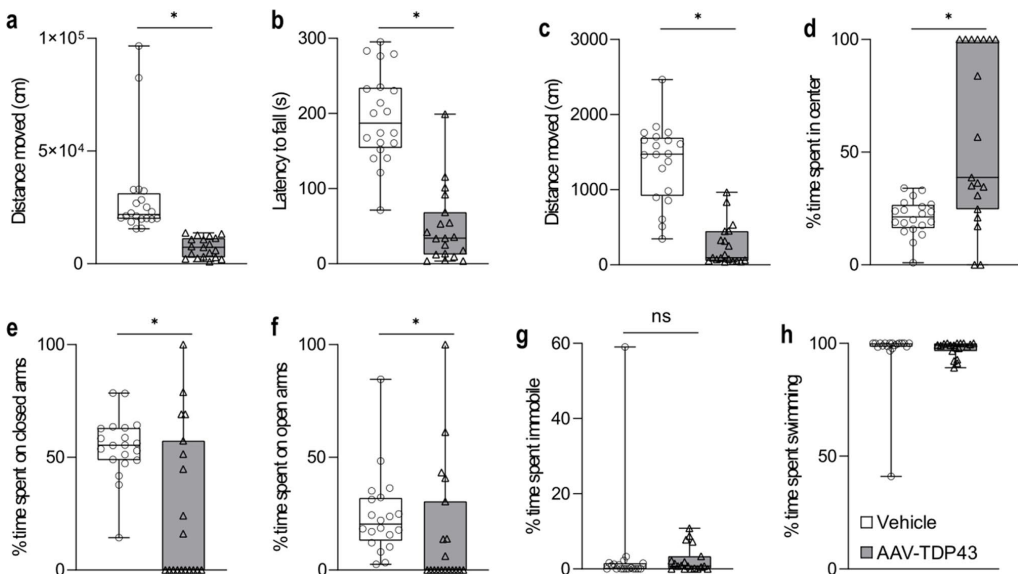


Fig. 4 Mice overexpressing TDP-43 in the hypothalamus show significantly reduced motor activity but maintain ability to swim. **a** Mice overexpressing TDP-43 travelled a shorter distance in the open field test than control mice injected with vehicle ($p < 0.001$). **b** The rotarod test revealed that mice overexpressing TDP-43 had a shorter latency to fall from the device than control mice ($p < 0.001$). Results from the elevated plus maze revealed that mice overexpressing TDP-43 in the hypothalamus also travelled shorter distances (**c**, $p < 0.001$), and that they spent more time in the center of the arena (**d**, $p = 0.001$) where the mice were placed at the start of the trial and less time on both closed (**e**, $p = 0.011$) and open arms (**f**, $p = 0.012$) compared to control mice. However, overexpressing TDP-43 does not limit swimming ability (**g-h**). Data are presented as box-and-whisker plots with whiskers representing minimum and maximum values, * = $p < 0.05$ (2-tailed). Statistical analyses were performed using Mann-Whitney U test

open arms (0–100%, $p = 0.012$) compared to control mice at 20% (3–85%, Fig. 4f), suggesting that mice overexpressing TDP-43 display reduced exploratory behavior.

In the forced swim test, commonly used to assess depressive-like behavior, there was no difference in immobility between mice overexpressing TDP-43 and vehicle-injected mice, indicating no depressive-like behavior induced by TDP-43 overexpression in the hypothalamus ($p = 0.181$) (Fig. 4g). In fact, mice with TDP-43 overexpression spent most of the time swimming similar to control mice and thus TDP-43 overexpression does not seem to limit the ability to swim (Fig. 4h). Taken together, TDP-43 overexpression in mice leads to reduced motor activity and reduced exploratory behavior but this is not caused by inability to move as indicated by maintained swimming ability.

Mice overexpressing TDP-43 in the hypothalamus develop apathy-like behavior

Mice overexpressing TDP-43 displayed reduced motor activity but preserved swimming ability, suggesting reduced motivation or drive, such as apathy, rather than severe motor impairment. Apathy is a common feature of patients with ALS/FTD [53, 70] as well as HD [19]. To investigate whether an apathy-like phenotype was present, another cohort of AAV-TDP43- and vehicle-injected mice was generated. Mice were housed in duplicate per treatment group for the analyses and nesting behavior (scored 0–3) was measured after 4 days at 4 and 8 weeks post-injection. No difference in nesting behavior was detected at 4 weeks post-injection ($p = 0.290$, $n = 8$ cages/group, Fig. 5a–c). However, mice overexpressing TDP-43 in the hypothalamus showed reduced nesting behaviour at 8 weeks post-injection compared to control mice injected with vehicle. The median nesting score of mice overexpressing TDP-43 was 1 (0–3, $p = 0.016$, $n = 7$ cages) compared to a median nesting score of 2 in vehicle-injected mice (2–3, $n = 8$ cages). These findings, combined with decreased motor activity, may suggest the development of apathy-like behavior in mice overexpressing TDP-43 in the hypothalamus.

In this cohort of mice, we confirmed overexpression of TDP-43 based on total *TARDBP/Tardbp* mRNA level using qRT-PCR (Fig. 5d). Median normalized relative mRNA levels were 34.6 (22.2–44.8, $n = 3$) in mice overexpressing TDP compared to 1.0 (0.8–1.2, $n = 3$) in vehicle-injected control mice. We also performed bulk RNA sequencing, which revealed alterations in genes associated with hypocretin/orexin, MCH, oxytocin, as well as neuropeptide Y and agouti-related peptide systems. Consistent with the neuropathological results in this study, mRNA levels of both the neuropeptide precursor and its corresponding receptor were reduced in the AAV-TDP43-injected mice (Table 1). Specifically, while the precursor for expression of MCH, oxytocin, and agouti-related peptide systems was significantly decreased, the hypocretin/

orexin and neuropeptide Y precursors exhibited a trend towards decreased expression without achieving statistical significance. In contrast, the expression of all receptors was significantly downregulated in the AAV-TDP43-injected mice compared with the vehicle-injected control mice. This differential impact on precursor and receptor mRNA levels in the context of observed neuronal loss suggests a specific vulnerability or loss of cell populations in this model of TDP-43 overexpression.

Nuclear and cytoplasmic localization of TDP-43 with inclusions following AAV-mediated TDP-43 expression in the hypothalamus

Pathogenic TDP-43 is known to undergo nuclear-to-cytoplasmic mislocalization and form both nuclear and cytoplasmic inclusions in patients with ALS/FTD [5, 37, 63]. To investigate the molecular effects following TDP-43 overexpression, we performed immunofluorescent analyses of brains from mice injected with AAV-TDP43 or vehicle in the hypothalamus 21 weeks post-injection. Brains were processed for immunofluorescence with an antibody against TDP-43 and counterstained with Hoechst for nuclear localization (Fig. 6a–b). TDP-43 was detected predominantly within the nucleus (Fig. 6c) but was also present in the cytoplasm (Fig. 6d). Furthermore, TDP-43-immunopositive inclusions were also present in both the nucleus (Fig. 6e) and cytoplasm (Fig. 6f), although they were more prevalent in the cytoplasm. Interestingly, we did not observe simultaneous overexpression of TDP-43 in both the nucleus and cytoplasm within the same cell. These results suggest that AAV-mediated TDP-43 overexpression in the hypothalamus recapitulates the molecular phenotype of patients with ALS/FTD, including TDP-43 mislocalization and the formation of both nuclear and cytoplasmic inclusions.

Mice overexpressing TDP-43 in the hypothalamus exhibited significant repression of constitutive exons

To further determine the effects of TDP-43 overexpression on gene expression and splicing, we performed bulk RNA sequencing of enriched polyA RNA from the hypothalamus (Fig. 7a–b). Mice overexpressing TDP-43 displayed increased exon skipping on constitutive exons, such as *Ddi2* exon 9 (2.5, 0–9%, $n = 6$ for vehicle; 10.5, 6–24%, $p < 0.05$, $n = 6$ for AAV-TDP43), *Psmd14* exon 5 (0, 0%, $n = 6$ for vehicle; 14.5, 7–24%, $p < 0.01$, $n = 6$ for AAV-TDP43), and *Mrlnd45* exon 7 (0, 0%, $n = 6$ for vehicle; 13, 10–25%, $p < 0.01$, $n = 6$ for AAV-TDP43), similar to what has been reported in the murine spinal cord overexpressing TDP-43 [15]. Analysis of the mapped RNA sequencing reads did not reveal expression of cryptic exons, including those previously described in *Ap3b2* (Fig. 7c), and *Camk1g* (Fig. 7d) in mice after deletion of TDP-43 in excitatory neurons in cortex and hippocampus [44].

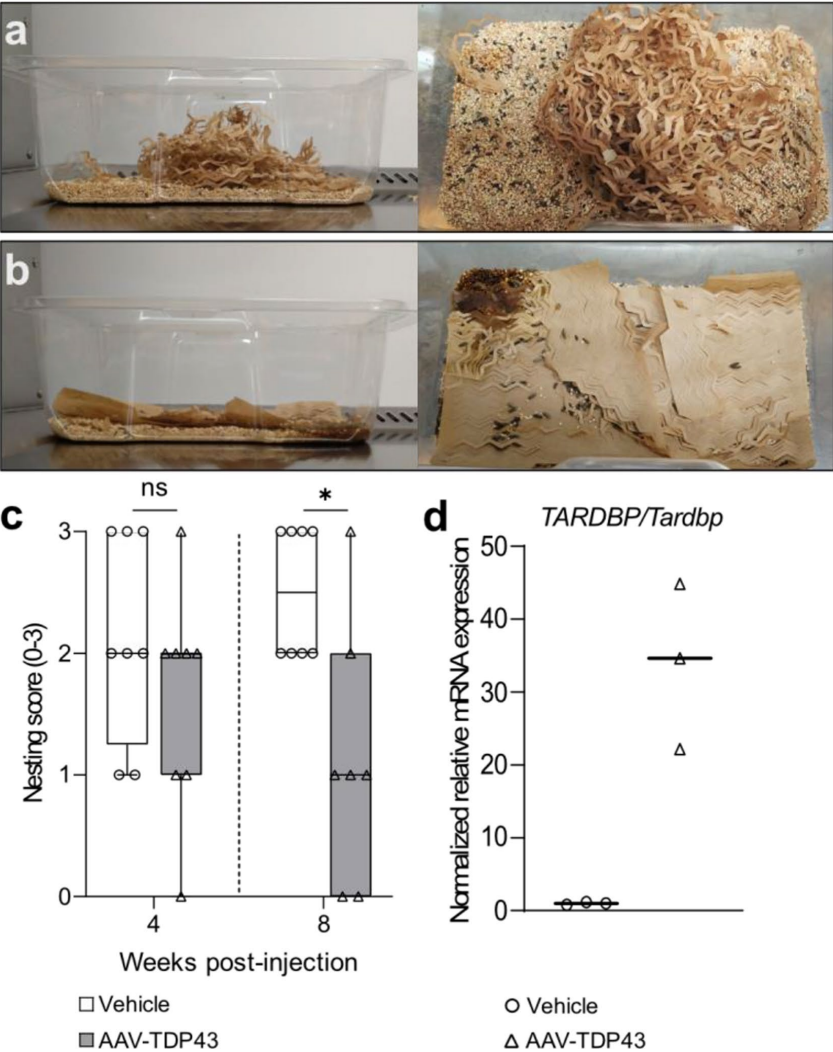


Fig. 5 Mice overexpressing TDP-43 in the hypothalamus displayed reduced nesting behavior. Representative images of cages containing mice with hypothalamic injections of either vehicle control (**a**) or AAV-TDP43 (**b**), with the corresponding nesting scores are 3 (highest score) and 0 (lowest score), respectively. **c**. Mice overexpressing TDP-43 in the hypothalamus displayed decreased nesting behavior compared to control mice at 8 weeks post-injection ($p = 0.016$), but no difference was observed at 4 weeks post-injection. **d**. Increased levels of *TARDBP/Tardbp* mRNA confirmed TDP-43 overexpression in the hypothalamus. Data are presented as box-and-whisker plots with whiskers representing minimum and maximum values (**c**) or as median (**d**), * = $p < 0.05$ (2-tailed). Statistical analysis was performed using Mann-Whitney U test (**c**)

Visually inspection did not indicate obvious increases in cryptic exons in *Stmn2*, *Sncb*, and *Unc13a* (data not shown). These results indicate that TDP-43 overexpression in the hypothalamus results in significant repression of constitutive exons, without clear evidence of increased cryptic exons.

Discussion

This study aimed to investigate whether there is a causative relationship between overexpression of wild-type human TDP-43 in the hypothalamus and development of neuropathology and metabolic and behavioral dysfunction. The wild-type form of TDP-43 is associated with the

Table 1 Overexpression of TDP-43 in the hypothalamus results in a specific decrease in neuropeptide-related transcripts

Neuropeptide pathway	Gene Symbol	Gene name	Log fold change	P-value
Hypocretin/Orexin	Hcrt	hypocretin	-0.59	0.0863
Hypocretin/Orexin	Hcrtr1	hypocretin receptor 1	-0.39	0.0206
Hypocretin/Orexin	Hcrtr2	hypocretin receptor 2	-0.74	< 0.0001
MCH	Pmch	pro-melanin-concentrating hormone	-0.49	0.0108
MCH	Mchr1	melanin-concentrating hormone receptor 1	-0.21	0.0947
Oxytocin	Oxt	oxytocin	-0.59	0.4389
Oxytocin	Oxtr	oxytocin receptor	-0.61	0.0093
Neuropeptide Y	Npy	neuropeptide Y	-0.15	0.6711
Neuropeptide Y	Npy1r	neuropeptide Y receptor Y1	-0.44	0.0079
Neuropeptide Y	Npy2r	neuropeptide Y receptor Y2	-0.46	0.1068
Neuropeptide Y	Npy5r	neuropeptide Y receptor Y5	-0.37	0.0414
Agouti-related peptide	AgRP	agouti related neuropeptide	-0.88	0.0990
Agouti-related peptide	Mc3r	melanocortin 3 receptor	-0.59	0.0022
Agouti-related peptide	Mc4r	melanocortin 4 receptor	-0.55	0.0444

majority of familial and sporadic cases of ALS/FTD [54, 87] as well as to HD [7, 64, 73, 78]. We generated an AAV vector for targeted expression of TDP-43 in hypothalamic neurons of mice. Our results demonstrate a dose-dependent effect, where higher titers of the AAV-TDP43 vector were associated with cellular loss and the formation of ubiquitinated inclusions within the hypothalamus. Additionally, TDP-43 overexpression caused hypothalamic atrophy and a selective reduction in the number of neurons expressing hypocretin, MCH, and oxytocin. We also observed TDP-43 nuclear-to-cytoplasmic mislocalization and the formation of both intranuclear and cytoplasmic inclusions, which are hallmarks of ALS/FTD pathology [5, 37, 63]. Dysfunction of key metabolism and emotion regulation neuronal populations may drive the development of the metabolic changes and apathy-like phenotype we observed in mice overexpressing TDP-43 in the hypothalamus. The results from our novel AAV-TDP43 mouse model strengthens the growing body of evidence implicating wild-type TDP-43 in neurodegeneration [31, 56, 89, 90]. Our study also provides new insight on the hypothalamus as a critical region directly susceptible to TDP-43-mediated pathological effects and

may contribute to early manifestation of clinical features observed in neurodegenerative disorders.

Selective loss of neuropeptide-expressing neuronal populations of the hypothalamus appears to be a common feature in several TDP-43 proteinopathies. We observed hypothalamic atrophy with loss of hypocretin-, MCH- and oxytocin-expressing neurons while sparing of vasopressin-expressing neurons in mice overexpressing TDP-43 consistent with previous studies in postmortem human ALS cases [12, 28]. Postmortem cases from persons with HD, Alzheimer’s disease and Parkinson’s disease also show loss of hypocretin-expressing neurons, but loss of oxytocin-expressing neurons has only been described in HD [6, 26, 29, 30, 52, 80, 88]. Loss of MCH neurons has also been reported in postmortem cases with Parkinson’s disease [80]. The degeneration of these neuropeptide-expressing neurons may play a role in the pathogenesis and development of the disease phenotype. For instance, near-complete loss of hypocretin neurons occurs in narcolepsy [81], and depletion of hypocretin in animal models leads to obesity and disruption of glucose homeostasis [27, 36, 43]. MCH deficiency has been linked to reduced food intake and weight loss in ALS patients [12, 49]. Furthermore, administration of MCH or a dual orexin receptor antagonist in ALS mice can ameliorate changes in sleep [35]. Oxytocin neuronal loss may at least partly explain social deficits seen in ALS/FTD and HD [9]. Further studies are however required to identify which specific neuronal populations in the hypothalamus are responsible for the link between TDP-43 overexpression and the development of metabolic and emotional dysregulation.

Metabolic dysregulation occurs in ALS, FTD and HD [22, 50]. In this study we show that overexpression of wild-type TDP-43 dysregulates metabolism, and leads to the development of obesity and hyperglycaemia without obvious effects on food intake. This result aligns with our previous studies where mutant huntingtin expression in the hypothalamus caused obesity and disrupted glucose homeostasis in mice [42, 75]. Metabolic assessment in metabolic cages using indirect calorimetry would be needed to further understand feeding behavior and energy expenditure after overexpression of TDP-43 in the hypothalamus. Importantly, the results on body weight gain are opposite to observations in persons with ALS and HD, who often exhibit reduced BMI even before the onset of motor symptoms [22, 50]. Potentially, it may be related to the increase in BMI reported in persons with FTD [3, 4], a disease with behavioral deficits more prominent than in ALS.

Our study shows that TDP-43 overexpression in the hypothalamus significantly reduces motor activity assessed by the open field, rotarod and elevated plus maze despite normal swimming ability. Importantly, the

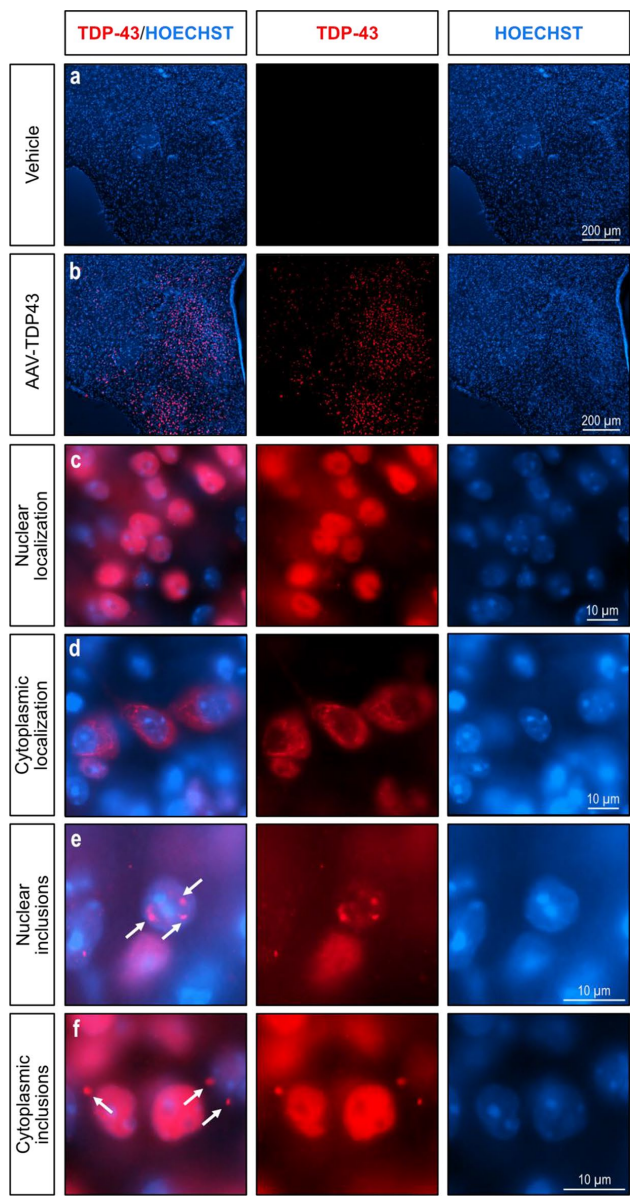


Fig. 6 Nuclear and cytoplasmic localization of TDP-43 with inclusions following AAV-TDP43 vector injection in the hypothalamus of mice. Immunohistochemistry for TDP-43 was performed on brains with hypothalamic injections of either AAV-TDP43 (**a**) or vehicle (**b**) and counterstained with Hoechst for nuclear localisation. TDP-43 immunoreactivity was present in both the nucleus (**c**) and the cytoplasm (**d**). Both nuclear (**e**) and cytoplasmic (**f**) inclusions of TDP-43 were present

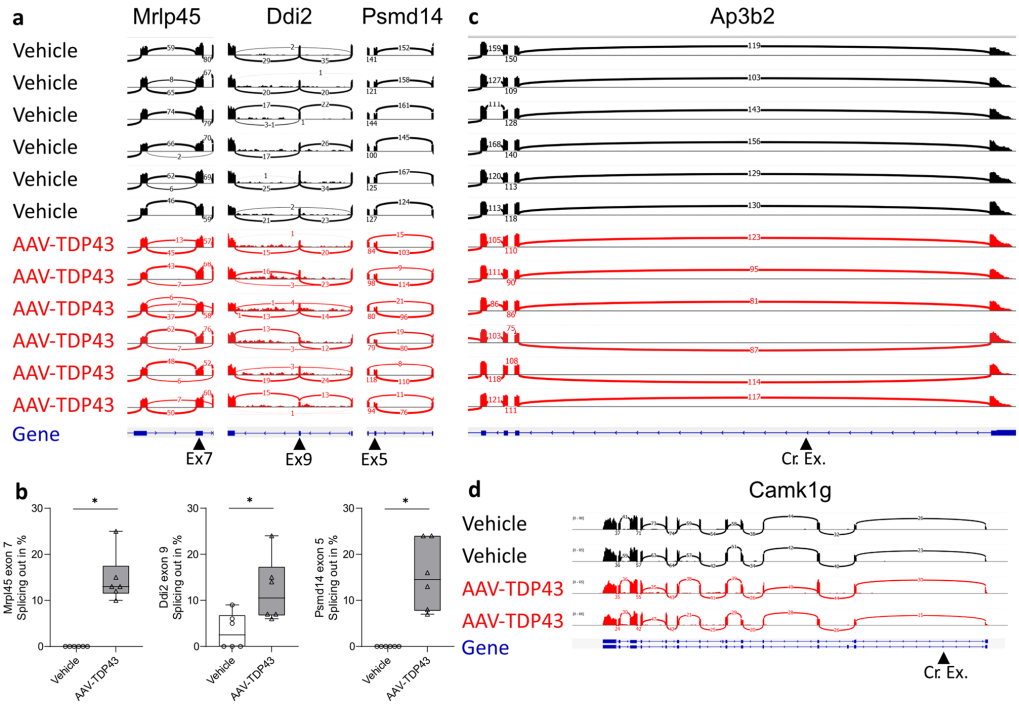


Fig. 7 Overexpression of TDP-43 leads to skipping of specific constitutive exons, but not to the inclusion of neuron-specific cryptic exons. RNA-sequencing analysis of frozen hypothalamic tissues revealed several examples of exon skipping in *Mrlp45*, *Ddi2*, and *Psmd14* (black arrowhead) in mice with TDP-43 overexpression **(a)**. Quantification of events was performed by calculating the frequency of exon skipping events in the AAV-TDP43 or vehicle injected group **(b)**. RNA-sequencing analysis does not reveal the expression of cryptic exons in *Ap3b2* **(c)** *Camk1g* **(d)** (black arrowheads). Data are presented as box-and-whisker plots with whiskers representing minimum and maximum values, * = *p* < 0.05 (2-tailed). Statistical analysis was performed using Mann-Whitney U test **(b)**

lateral hypothalamus is thought to provide an important drive to a number of vital behaviours including locomotion. Severe bilateral damage to the lateral hypothalamus is known to cause physical inactivity in mammals [51, 55]. Interestingly, recent studies have implicated several of the TDP-43 affected hypothalamic neuropeptide populations in regulating motor activity, including the hypocretin and the MCH systems [18, 46, 51, 79]. Our study also shows a deficit in nest-building behavior. This test is commonly used to assess motivation and daily activities in mice, indicative of goal-directed behavior and as a proxy to apathy [71]. Apathy, i.e. the lack of goal-directed behaviour and motivation is the most common behavioural change in the spectrum of ALS/FTD as well as other TDP-43 proteinopathies [1, 61, 77, 85]. The neurobiological substrate of apathy in neurodegenerative disorders has not been fully elucidated and there are no effective therapies to reduce apathy. The results from this study indicate that TDP-43 mediated pathology in the hypothalamus may be important in causing apathy-like

behavior and further understanding of the underlying mechanisms may reveal novel targets for therapeutic interventions for apathy.

An important question is whether non-motor features manifest before motor and cognitive symptoms in ALS/FTD, and more broadly in TDP-43-proteinopathies. Interestingly, a recent study shows that sleep disturbances are present already in pre-symptomatic ALS risk gene carriers [35], which was also linked to loss of MCH neurons in the hypothalamus. Changes in BMI have also been detected in ALS risk gene carriers at pre-symptomatic stages, which were linked to reduced hypothalamic volume [34]. These results open for the possibility of the hypothalamus to be affected very early in the disease process. As hypothalamic neuronal populations are part of neurocircuits to other brain regions, i.e. the cerebral cortex, the ventral striatum, the ventral tegmental area and the spinal cord, it is possible that pathological changes in the hypothalamus can exert direct effects also on these areas. Alternatively, pathology in these areas

may also contribute to hypothalamic dysfunction. Further experimental studies using viral vector designs and conditional gene expression technologies may shed light on how different parts of the neurocircuits may contribute to hypothalamic pathology and development of relevant phenotypes. This may also have relevance for the development of novel therapies as a recent study shows preservation of motor neurons in an animal model of ALS by treatment with the hypothalamic neuropeptide MCH [35].

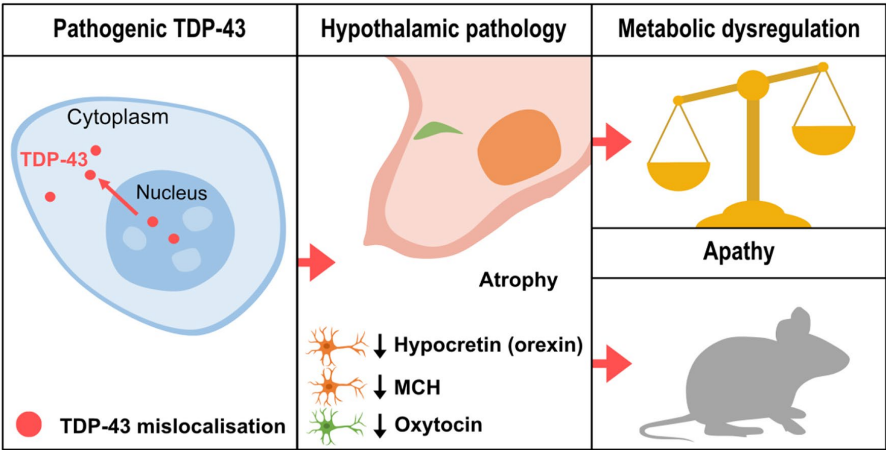
TDP-43, which is natively a nuclear protein, is mislocalized to the cytoplasm and forms both nuclear and cytoplasmic inclusions in ALS/FTD cases [5, 37, 63]. Here, AAV-mediated TDP-43 overexpression resulted in TDP-43 localization to both the nucleus and the cytoplasm, with the formation of both nuclear and cytoplasmic inclusions. Hence, our model recapitulates the molecular phenotype of TDP-43 pathology. In a recent study, mis-localization of TDP-43 in zebrafish induced using a modified nuclear localization signal of endogenous Tardbp caused metabolic dysfunction with hypothalamic pathology [40], which aligns with the results of our study. Elevated nuclear TDP-43 and cytoplasmic mislocalization of TDP-43 could lead to both gain- and loss-of-function as TDP-43 plays an important role in the regulation of splicing [39]. High levels of nuclear TDP-43 have been proposed to results in constitutive exon skipping whereas low nuclear TDP-43 protein levels may lead to cryptic exon incorporation [15]. Here we show that hypothalamic TDP-43 overexpression leads to exon skipping similar to what has been reported previously in mouse models overexpressing TDP-43 in spinal cord [15]. Previous studies have shown that deletion of TDP-43 in excitatory neurons in the cerebral cortex and the hippocampus leads to generation of specific cryptic exons but we were not able to detect these in our model overexpressing TDP-43 [44]. This study also suggested that cryptic exons may be highly variable between cell types and may play a role in selective vulnerability to TDP-43. Hence, further studies on the generation of cryptic exons in specific hypothalamic neurons would be interesting.

The present study was designed to test the hypothesis that overexpression of wild-type TDP-43 could cause direct toxicity to hypothalamic neurons resulting in the development of changes in behavior and metabolism. Choices made in experimental designs come with different limitations. In this study, we chose to test our hypothesis in mice as we have previously established a model of hypothalamic expression of transgenes in this species using stereotactic delivery of AAV-vectors [42]. However, effects of disease-causing transgenes may be species-specific including TDP-43 mediated exon skipping [15] and similar studies in other species may therefore

be interesting. Our AAV-vector based model uses the promoter synapsin to allow for neuronal expression and hence tests the effects of TDP-43 only in neurons. This choice was made as we have previously found inclusions of TDP-43 in several hypothalamic neuronal populations in postmortem cases with ALS [28]. It is not fully known how TDP-43 overexpression may affect glial cells in the hypothalamus. Delivery of AAV-vectors to specific brain regions could potentially lead to diffusion of the vector solution beyond the region of interest. For hypothalamic targeting, there is no promoter that can be used for pan-hypothalamic targeting. Further research could however employ promoters specific for certain neurocircuits in the hypothalamus and/or use a flex-switch cre-recombinase model, similar to the one used in Bergh et al. to target mHTT to oxytocin neurons [11]. In the present study, we observed limited TDP-43 overexpression outside the hypothalamus with single cells overexpressing TDP-43 in the striatum, hippocampus and cortex. This could be due to TDP-43 propagation, which has previously been reported [45, 83], and requires further investigation. The use of appropriate controls to an AAV-vector mediated overexpression of a transgene is also important to consider. Here we used both AAV-vector mediated expression of GFP and another disease-causing protein, mHTT, as well as a vehicle solution. Although GFP did not show any neurotoxicity in this paradigm, other studies have demonstrated toxicity after AAV-mediated overexpression of GFP already at very low titers [48]. An empty AAV-vector could have been tested, although it does not control for overexpression of a protein and cannot be visualized in the targeted brain region. As sex differences may play a role in the sensitivity to disease causing genes and this study was conducted only in female mice, further analyses in male mice would be necessary for a broader generalization of the effects of TDP-43 in the hypothalamus. Moreover, interpretation of results from behavioral testing requires consideration of several parameters. Effects on activity including reduced nesting behavior could suggest apathy-like behavior but would be interesting to explore further using other tests assessing motivation. The concurrent manifestation of both a metabolic phenotype with obesity and a behavioral phenotype with reduced motor activity also complicates the interpretation of the data. Obesity could result in reduced motor activity and reduced activity could contribute to weight gain. Further research is therefore needed to understand the temporal relationships between these different phenotypes.

In conclusion, this study shows that wild-type TDP-43 overexpression in the hypothalamus can lead to the development of neuropathological and molecular features similar to what have been reported for ALS/FTD including hypothalamic pathology with TDP-43

AAV-TDP43 Mouse Model



Clinical ALS/TDP-43 proteinopathies

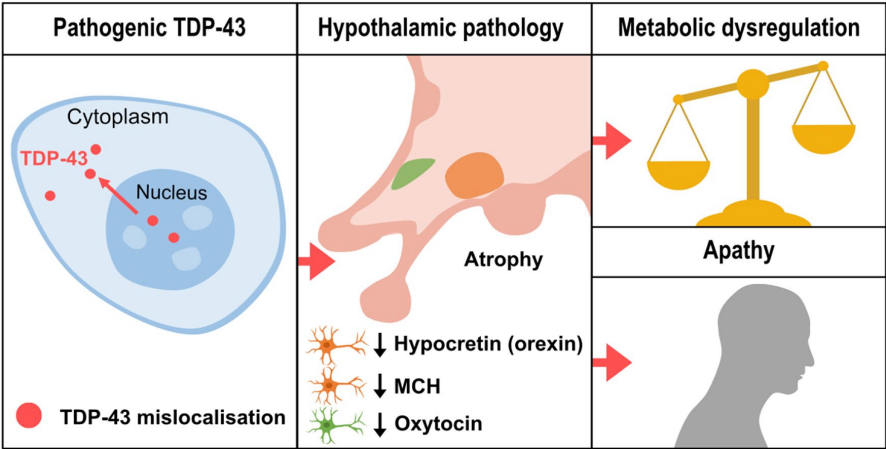


Fig. 8 Schematic representation of the molecular phenotype, hypothalamic pathology, and behavioral dysfunction induced by AAV-mediated over-expression of human wild-type TDP-43 in the hypothalamus, recapitulating key neuropathological features observed in clinical ALS and metabolic and psychiatric features seen in TDP-43 proteinopathies

mislocalization and formation of TDP-43 nuclear and cytoplasmic inclusions (Fig. 8). TDP-43 overexpression in the hypothalamus triggers a cascade of pathological events leading to metabolic and emotional alterations, likely involving dysfunction and/or loss of neuronal population expressing hypocretin, MCH and oxytocin. These results suggest that wild-type human TDP-43 can exert gain-of-toxic function within the hypothalamus, with the potential to contribute to the pathogenesis of neurodegenerative diseases. Therefore, elucidating the molecular

and cellular mechanisms of the effects of TDP-43 in the hypothalamus may provide novel therapeutic targets for TDP-43 proteinopathies.

Abbreviations	
AAV	Adeno-associated virus
AAV-GFP	AAV-vector expressing GFP
AAV-mHTT	AAV-vector expressing huntingtin with 78 polyglutamines
AAV-TDP43	AAV-vector expressing TDP-43
ALS	Amyotrophic lateral sclerosis
BMI	Body mass index
cpm	Counts per million

CV	Cresyl violet
DAB	3,3'-diaminobenzidine
DEXA	Dual energy X-ray absorptiometry
FTD	Frontotemporal dementia
gc	Genome copies
GFP	Green fluorescent protein
GFAP	Glial neurofibrillary protein
HD	Huntington disease
Iba1	Ionized calcium binding adaptor molecule 1
KPBS-T	Triton X-100 in potassium phosphate-buffered saline
MCH	Melanin-concentrating hormone
mHTT	Mutant huntingtin
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
RIN	RNA integrity number
rpm	Revolutions per minute
TDP-43	TAR DNA-binding protein 43

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40478-025-02018-8>.

Supplementary Material 1

Acknowledgements

We would like to thank Ulrika Sparrhult-Björk, Björn Anzelius, Anneli Josefsson and Anna Hansen for excellent technical assistance.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection or data analyses were performed by all authors. The first draft of the manuscript was written by SB and AP. All authors read and commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

Open access funding provided by Lund University. This study was funded by research grants to AP from the Swedish Research Council (grant number 2022/01092), the Swedish Brain Foundation, the Swedish governmental funding of clinical research (ALF) at Region Skåne as well as the Knut and Alice Wallenberg Foundation (# 2019.0467). NGS sequencing methods were performed with the support of the DFG-funded NGS Competence Center Tübingen (INST 37/1049-1). In addition, this research was supported by institutional funds of the Department of Human Genetics at Ruhr University Bochum.

Data availability

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The experimental procedures performed in animals were approved by the Regional Ethical Committee in Lund, Sweden, Permit number: 17113/2022.

Consent for publication

All authors have read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Received: 4 March 2025 / Accepted: 28 April 2025

Published online: 27 May 2025

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Paper II



The DNA/RNA autophagy protein SIDT2 as a novel neuropathological hallmark in Huntington disease

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Funding information

Vetenskapsrådet, Grant/Award Number: 2022/01092; Hjärnfonnen; Foundation of King Gustav V and Queen Victoria; Region Skåne; Knut och Alice Wallenbergs Stiftelse, Grant/Award Number: 2019.0467; Deutsche Forschungsgemeinschaft, Grant/Award

Abstract

The pathogenic mechanisms leading to neurodegeneration in Huntington disease (HD) are not fully understood but involve accumulation of toxic mRNA and protein products in the brain. Recent studies described an unconventional autophagic pathway involving DNA and RNA degradation through DNautophagy and RNautophagy that is regulated by the lysosomal protein SID1 transmembrane family member 2 (SIDT2). Interestingly, SIDT2 has been shown to bind to the expanded CAG repeat in the mutant huntingtin (mHTT) transcript and lower mHTT in vitro. The aim of the present study was to determine whether SIDT2 levels are altered in HD and whether manipulation

Abbreviations: AAV, adeno-associated virus; ACC, anterior cingulate cortex; ASO, anti-sense oligonucleotides; ARM, arginine-rich motif; BSA, bovine serum albumin; DAB, 3,3'-diaminobenzidine; DAPI, 4',6-diamidino-2-phenylindole; DPX, distyrene-plasticizer-xylene; DPBS, dulbecco's phosphate-buffered saline; DTT, dithiothreitol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EDTA, ethylenediaminetetraacetic acid; HD, Huntington disease; HTT, huntingtin; HTTex1, huntingtin exon 1; K48-pUb, lysine-48-linked polyubiquitin chains; KPBS-T, potassium phosphate-buffered saline containing Triton X-100; LC3B, MAP1LC3B (Microtubule-associated proteins 1A/1B light chain 3B); LDS, lithium dodecyl sulfate; mHTT, mutant huntingtin; p62, p62/SQSTM1 (sequestosome 1); PAGE, polyacrylamide gel electrophoresis; PFA, paraformaldehyde; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; qRT-PCR, quantitative real-time polymerase chain reaction; RT, room temperature; SID1, systemic RNA interference deficient-1; SD, standard deviation; SDS, sodium dodecyl sulfate; S.E.M., standard error of the mean; SIDT2, SID1 transmembrane family member 2; TBS-T, Tris-buffered saline containing Tween 20; UPS, ubiquitin-proteasome system; wks, weeks.

Sanaz Gabery and Sofia Bergh are considered as first authors.

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Numbers: NG-101/6-1, 6585/1-1; National Health and Medical Research Council of Australia, Grant/Award Number: 1176607; Deutsche Huntington Hilfe, Grant/Award Number: 4908100115

of SIDT2-mediated RNautophagy can alter HD pathology. We demonstrate a significant reduction of SIDT2 protein levels in the striatum and in the lateral hypothalamic area in postmortem HD brains compared to control cases without effects on SIDT2 mRNA levels. In frontal cortical postmortem HD tissue, we show a CAG-repeat-length-dependent increase in the frequency of SIDT2-immunoreactive intranuclear inclusions. In postmortem tissue of an HD case with Vonsattel grade 0, we demonstrate SIDT2- and mHTT-immunoreactive inclusions not only in the frontal cortex, but also in the striatum and the lateral hypothalamic area. In the R6/2 mouse model of HD, we show that SIDT2 inclusions form at later stages than mHTT inclusions. Overexpression of SIDT2 using adeno-associated viral vectors injected into the hypothalamus of R6/2 mice led to a reduction of mHTT inclusions in the lateral hypothalamic area. Similarly, in a neuronal cell model, overexpression of SIDT2 reduced soluble and insoluble mHTT exon 1 protein levels. Taken together, our results reveal novel pathology in clinical HD cases and in experimental models, characterized by the accumulation of SIDT2-immunoreactive inclusions, while demonstrating the efficacy of overexpressing SIDT2 for lowering detrimental mHTT species. Targeting SIDT2-mediated RNautophagy may offer a potential strategy to ameliorate the molecular pathology in HD.

KEYWORDS

aggregation, huntingtin, huntingtin lowering, inclusions, neuropathology, SIDT2

1 | INTRODUCTION

Huntington disease (HD) is a fatal neurodegenerative disorder without any disease modifying therapy [1]. It is caused by an expanded CAG repeat in the huntingtin gene and there is a negative correlation between the number of CAG repeats and age of onset [1]. The huntingtin (HTT) protein is expressed in multiple brain areas, yet pathology evoked by mutant HTT (mHTT) is most pronounced in the striatum, the cerebral cortex, and in the hypothalamus, including the lateral hypothalamic area [2–5]. The extent of striatal atrophy is classified according to the Vonsattel grading scale 0-IV [6]. Neuropathological hallmarks include formation of mHTT-containing neuronal inclusions in many HD affected brain regions [7] and in several animal models expressing different variants of mHTT [8]. The pathogenic mechanisms in HD are not fully understood but are likely to involve accumulation of toxic RNA and protein products due to alterations in protein degradation by the ubiquitin-proteasome system (UPS) and autophagy [9–13].

Recent study has described an unconventional autophagic pathway involving DNA and RNA degradation, DNautophagy and RNautophagy is regulated by the lysosomal protein SID1 transmembrane family member 2 (SIDT2) [14–17]. SIDT2 is the lysosomal vertebrate ortholog of the *Caenorhabditis elegans* RNA channel systemic RNA interference deficient-1 (SID1) protein and is expressed in many cell types [18, 19]. SIDT2 can bind directly to DNA and RNA via an arginine-rich motif (ARM) and mediates uptake into the lysosome for degradation. Interestingly, SIDT2 has been found to bind to exon 1 of the *HTT*

transcript through its ARM in a CAG-dependent manner [20]. Furthermore, overexpression of murine SIDT2 resulted in the degradation of *HTT* mRNA and reduced the levels of soluble and insoluble mHTT aggregates as assessed using filter trap and Western blot analyses in mHTT exon 1-transfected Neuro2a cells [20]. The degradation of mHTT exon 1 is suggested to occur via SIDT2-mediated RNautophagy. These results suggest that this novel pathway may play a role in HD and could be explored as a potential therapeutic target. Effects on SIDT2 or SIDT2-mediated RNautophagy have not been studied previously in vivo in HD. In this study, we investigated whether the SIDT2 system is affected in HD by examining human postmortem tissue, multiple HD mouse models, and neuroblastoma cell cultures expressing exon 1 of mHTT. We show for the first time that SIDT2-immunoreactive inclusions are formed in affected HD brain tissues, while SIDT2 protein levels are diminished in selected human brain areas with higher Vonsattel grades. Moreover, the occurrence of SIDT2-positive inclusions was reproduced in two HD mouse models. Our results from SIDT2 overexpression both in vivo and in vitro further support beneficial effects on mHTT protein levels and inclusion load, paving the way for further developments targeting this pathway in HD.

2 | MATERIALS AND METHODS

2.1 | Overview of study design

In the present study, we examined the involvement of the SIDT2 system in human postmortem HD brains, in three

TABLE 1 Demographic data for HD and control cases used for cortical and striatal Western blot and qRT-PCR analyses.

Case	Age/sex	CAG	DD	Cause of death	PMD	Brain	Grade	RIN: CTX	RIN: STR
<i>HD</i>									
HD1	68/m	44	13	Pneumonia	10	1184	3	7.5	6.8
HD2	69/f	42	20	Cardiorespiratory failure	2	1149	2	7.0	4.8
HD3	57/f	44	22	Pneumonia	22	800	4	4.8	5.9
HD4	61/m	43	17	HD endstage	17	1280	4	6.1	5.5
HD5	71/m	42	12	Myocardial infarct	41	1270	2	7.0	3.6
HD6	39/m	54	13	Cardiorespiratory failure	10	1047	4	7.4	5.7
HD7	39/f	46	11	HD endstage	36	680	3	8.0	7.9
HD8	58/m	46	11	HD endstage	32	1260	3	7.9	6.4
HD9	61/m	45	14	Sepsis	40	1500	4	7.6	3.6
HD10	62/m	43	12	HD endstage	22	1185	3	7.8	3.1
HD11	62/m	43	10	Pneumonia	24	1380	3	5.8	4.5
HD12	67/m	43	15	HD endstage	37	1200	2	6.9	4.6
HD13	67/m	45	15	Pneumonia	19	952	n.d.	6.6	3.0
HD14	71/m	40	10	Pneumonia	39	1150	3	4.2	3.2
HD15	72/m	43	33	Pneumonia	22	940	2	5.0	3.4
HD16	74/m	40	12	Cardiorespiratory failure	27	1475	2	6.5	6.0
HD17	77/m	41	20	Pneumonia	9	1018	4	7.6	7.0
Mean $\pm$ SD	63 $\pm$ 11 m: 14 f: 3		15 $\pm$ 6		24 $\pm$ 12	1114 $\pm$ 219		6.7 $\pm$ 1.2	5.0 $\pm$ 1.5
<i>Control cases</i>									
C1	69/m			Pulmonary embolism	24	1290		6.9	6.7
C2	67/f			Pulmonary embolism	26	1298		7.5	6.1
C3	57/m			Ischemic heart disease	48	1532		6.6	7.2
C4	73/m			Ischemic heart disease	43	n.d.		6.4	6.8
C5	64/m			Ischemic heart disease	32	1335		6.8	2.5
C6	64/m			Ischemic heart disease	24	1492		7.5	7.7
C7	66/f			Metastatic carcinoma	43	1233		6.8	6.5
C8	69/m			Ischemic heart disease	34	1240		6.6	6.5
C9	76/m			Aortic aneurysm	46	1459		7.8	7.6
C10	78/m			Ischemic heart disease	46	1471		6.9	6.5
Mean $\pm$ SD	68 $\pm$ 6 m: 8 f: 2				37 $\pm$ 10	1372 $\pm$ 116		7.0 $\pm$ 0.5	6.4 $\pm$ 1.5

Note: 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 [53].

Abbreviations: CTX, cerebral cortex; n.d., not determined; RIN, RNA integrity number; STR, striatum.

HD animal models and in one in vitro system for HD. Frozen human brain tissue from 17 HD and 10 control cases were analyzed by Western blot and quantitative real-time polymerase chain reactions (qRT-PCR) in order to measure SIDT2 protein and mRNA levels (Tables 1 and 2). We assessed SIDT2 and mHTT inclusion formation in fixed postmortem human brain tissue from four HD patients with Vonsattel grades I-IV and CAG repeats ranging from 42 to 66, one HD case with Vonsattel grade 0 with 44 CAG repeats and two control cases (Table 3). We investigated effects on SIDT2 inclusion formation in three animal models of HD: the R6/2

mouse, the BACHD mouse as well as in mice overexpressing fragments of mHTT or wild-type HTT after hypothalamic injections of adeno-associated viral (AAV) vectors. We also assessed effects of SIDT2 overexpression using AAV vectors injected into the hypothalamus of R6/2 mice. Finally, we analyzed effects of SIDT2 overexpression in an SH-SY5Y neuroblastoma cell model transfected with mHTT exon 1 constructs, focusing on soluble protein levels, mHTT aggregate load, dysregulation of proteolytic pathways (specifically UPS and autophagy), and microscopy analysis for assessing subcellular protein distribution.

TABLE 2 Demographic data for HD and control cases used for Western blot analyses of the lateral hypothalamic area.

Case	Age/sex	CAG	DD	Cause of death	PMD	Brain	Grade
HD							
HD1	68/m	44	13	Pneumonia	10	1184	3
HD2	69/f	42	20	Cardiorespiratory failure	2	1149	2
HD3	57/f	44	22	Pneumonia	22	800	4
HD4	61/m	43	17	HD endstage	17	1280	4
Mean ± SD	64 ± 6 m: 2 f: 2		18 ± 4		13 ± 9	1103 ± 210	
Control cases							
C1	69/m			Pulmonary embolism	24	1290	
C2	67/f			Pulmonary embolism	26	1298	
C3	57/m			Ischemic heart disease	48	1532	
Mean ± SD	64 ± 4 m: 2 f: 1				32 ± 13	1373 ± 138	

Note: 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 grams. Grade refers to Vonsattel grade for neuropathological classification of HD [53].
Abbreviation: HYP, hypothalamus.

TABLE 3 Demographic data for HD and control cases used for immunohistochemical SIRT2 and HTT analyses.

Case	Age/sex	CAG	DD	Cause of death	PMD	Brain	Grade
HD1	67/m	42/17	7	Cardiac failure	74	1343	1–2
HD2	55/f	44/17	12	Suicide	110	1284	2–3
HD3	40/f	50/18	15	Pneumonia	20	1070	3
HD4	32/f	66/20	14	Pneumonia	44	695	4
HD5	52/m	43/19	0	Cardiac arrest	96	1282	0
C1	69/f	21/15	n/a	Cardiac failure	12	1282	n/a
C2	79/m	25/20	n/a	Cardiac failure	27	1364	n/a

Note: Age is indicated in years. DD (disease duration) is indicated in years. PMD (postmortem delay) is indicated in h. Brain (total brain weight) is indicated in g. Grade refers to Vonsattel grade for neuropathological classification of HD [53].

2.2 | Methods related to postmortem human brain tissue

2.2.1 | Human postmortem brain tissue

Tissue from the caudate nucleus of the striatum and frontal cortex from 17 HD and 10 control cases were collected in blocks and frozen on dry ice upon autopsy and stored at –80°C for Western blot analyses and qRT-PCR analyses as reported previously [21]. Tissues from the entire hypothalamus from four HD and three control cases were frozen on dry ice upon autopsy and stored at –80°C. The lateral hypothalamic area was dissected as previously described [21] and used for Western blot analysis in the present study. Demographic data are shown in Tables 1 and 2. This set of human postmortem tissue was obtained from Victorian and Sydney Brain Banks in 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.

Paraformaldehyde (PFA)-fixed anterior cingulate cortex (ACC) from four HD and two control cases were used for immunohistochemistry. The two control patients had no clinical history of neurological or psychiatric diseases and the HD mutation was genetically excluded. Demographic data is shown in Table 3. This set of human postmortem tissue was obtained from the Huntington brain collection of Bochum (EPP and CS). All individuals had given written consent to examine the brains for research purposes. The study was approved by the Ethic Committee of the Medical Faculty of the Ruhr-University Bochum, Germany (Reg. No. 17-5939).

Formalin-fixed tissue from the frontal cortex, the striatum, and the hypothalamus of an HD case with Vonsattel grade 0 was obtained from the Department of Neuropathology in Lund, Sweden [22]. Demographic data is shown in Table 3. The analysis was approved by the regional ethical review board at Lund University

(reference number 2014/466), and written informed consent was obtained from the patient. All analyses performed on human postmortem tissue in Sweden were approved by the Swedish Ethical Review Authority (reference number 2022–05348-01).

2.2.2 | Western blots

Human postmortem tissue from frontal cortex, the caudate nucleus of the striatum, and the lateral hypothalamic area were lysed in 1:10 weight/volume in lysis buffer (50 mM NaCl, 100 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% SDS) supplemented with protease and phosphatase inhibitors (Roche). The homogenization of the tissue was performed by sonication (15× for 1 s at 40 Hz). The samples were then incubated for 15 min on ice and consecutively spun at 13,000 rpm for 10 min at 4°C. Total protein concentration was determined using the DC Protein Assay Kit (Bio-Rad) according to manufacturer's instructions. Before loading on the SDS-PAGE, the samples were boiled at 95°C for 5 min in the presence of Laemmli Loading Buffer (Bio-Rad). 40 µg of total protein was loaded on each well on a 4–15% gradient polyacrylamide gel (Bio-Rad Mini-PROTEAN TGX Precast Gels). The samples were run for 30 min at 90 V, followed by 1 h at 120 V. Proteins were then transferred onto the PVDF membrane (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad) for 7 min at 25 V. Blocking was performed for 1 h at room temperature (RT) using 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 (Sigma) (TBS-T), followed by incubation overnight at 4°C with a primary antibody against SIDT2 (at 1:1000, made in rabbit, Abcam ab85847 for cortical and striatal samples; at 1:1000, made in rabbit, Abnova PAB27211 for hypothalamic and striatal samples, due to discontinuation of the Abcam antibody) or β-actin (at 1:10000, made in mouse) diluted in 2% milk in TBS-T. After three 10 min washes in TBS-T, the membranes were incubated for 1 h at RT with a secondary antibody (mouse anti-rabbit, Santa Cruz Biotechnology, sc-2357 at 1:100; or donkey anti-mouse, ab6789 at 1:10000). After three 10 min washes in TBS-T, the membranes were developed using enhanced chemiluminescence (BioRad). The images were acquired with the VersaDoc instrument (Bio-Rad). Band intensities were quantified using the ImageLab software (Bio-Rad) and expressed as a ratio relative to β-actin.

2.2.3 | qRT-PCR

Total RNA was isolated from the 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, an indicator for appropriate preservation of RNA integrity, was used to assess the RNA quality of the human postmortem tissue [23]. RNA samples were analyzed by SCIBLU Genomics, Affymetrix unit at Lund University using Agilent 2100 Bioanalyzer and RNA integrity was determined for all samples before proceeding with the analyses as reported previously [21] (Table 1). 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 $\Delta\Delta C_T$ -method and were normalized to the expression of the two housekeeping genes β-actin (*ACTB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). All primers were designed with Beacon Designer™ (Premier Biosite). All values are presented as ratios to the mean of the control group. The primer sequences used for the gene expressions analyses were (5'–3'): *ACTB* (Fwd: ATATGAGATGCGTTTGTTA, Rev.: AAGTATTAAGGCGAAGAT), *GAPDH* (Fwd: CTCTGGTAAAGTGGATATTGT, Rev.: GGTGGAATCATATTGGAACA), and *SIDT2* (Fwd: TGAACGTCCTGAACAAGCAG, Rev.: CACAGGGTTCGTTCCACTTT).

2.2.4 | Immunohistochemistry of human postmortem brain tissue

We examined three frontal 80 µm vibratome sections of the ACC per HD and control brains (Table 3, HD1–4; C1 and 2), all of which were located at the same supracallosal level. All human brains were fixed by immersion in 4% PFA. For immunohistochemistry all sections were rinsed free floating in phosphate buffered saline (PBS) and subjected to SIDT2 and EM48 antibodies according to a protocol described previously [24]. Briefly, the sections were treated with 1% NaBH₄ and incubated for 30 min in PBS with 10% normal goat or horse serum and 0.3% Triton X-100 (Serva 37,240), followed by an incubation with antibodies against SIDT2 (1:1000, made in rabbit, Abnova PAB272) or HTT (1:1000, anti-mouse, EM48, MAB5374, Merck Millipore) for 72 h at 4°C in the same solution. After rinsing in PBS and a preincubation with 0.1% bovine serum albumin in PBS for 1 h the sections were incubated overnight at 4°C with a biotinylated goat anti-rabbit secondary antibody (CA 94010, Vector Laboratories) or horse anti-mouse antibody (Thermo Fisher Scientific). After blocking in PBS-albumin at RT, sections were incubated with the avidin-biotinylated peroxidase complex (Vector Laboratories) for another 3 h at RT. Peroxidase activity was visualized with 3,3'-diaminobenzidine (DAB). The reaction was controlled microscopically, with a maximum of 15 min. Finally, sections were mounted on Superfrost Plus slides

(Thermo Fisher Scientific), air-dried for 1.5 h, dehydrated and coverslipped. As negative controls for HD-specific immunolabeling, we used cases without known history of neurological or psychiatric diseases. HD was excluded by genetic testing of postmortem blood in these controls. In order to exclude the possibility of nonspecific staining, we performed omission of primary antibodies in normal goat or horse serum, which both lead to absence of immunoreactivity in the sections.

For immune electron microscopic analyses, sections were treated as described above with the omission of Triton X-100. These sections were photodocumented after the immunohistochemical procedure for later orientation, postfixed with 1% osmium tetroxide in PBS for 1 h, dehydrated and flat-embedded in Araldite (Serva). Alternating semi- and ultrathin sections were cut with a Leica Ultracut R microtome. Semithin sections (0.75 μ m) were lightly counterstained with 1% toluidine blue 0 (T3260, Sigma-Aldrich), ultrathin sections (100 nm) contrasted with 5% aqueous uranyl acetate (E22400, Science Services) for 10 min and 2% aqueous lead citrate (228,621, Sigma-Aldrich) for 5 min. Ultrathin sections were viewed by the LVEM25 (Delong Instruments, Brno, Czech Republic).

For the HD case with Vonsattel grade 0, formalin-fixed coronal tissue blocks of the striatum, the cerebral cortex and the hypothalamus were cryoprotected in 30% sucrose and serially cut at a thickness of 50 μ m on a freezing microtome in 15 series. Briefly, antigen retrieval was performed on free-floating sections with Tris-EDTA buffer with pH 9.0 at 80°C for 1 h, quenched in 3% hydrogen peroxide and 10% methanol for 30 min and blocked in TBS-T containing 5% serum (from the same species as the corresponding secondary antibody was raised in) for 1 h at RT. Subsequently, the sections were incubated overnight using primary antibodies against SIRT2 (1:2500, anti-rabbit, PAB27211, Abnova) and HTT (1:200, anti-mouse, EM48) in antibody solution containing 1% BSA and TBS-T. The following day, the sections were incubated with secondary antibody for 1 h at RT (1:200 in 1% BSA TBS-T). The sections were visualized using hydrogen peroxide and DAB development. Finally, the sections were mounted on chromatin-gelatin coated glass slides and coverslipped with Distyrene-Plasticizer-Xylene (DPX) (Sigma Aldrich) after a series of dehydration steps in ascending concentrations of ethanol and xylene.

For immunofluorescence microscopy, dissected ACC were rinsed in PBS, immersed overnight in 30% saccharose in PBS, then shock-frozen and immunostained as previously described [25]. Briefly, immunohistochemistry was performed on cryosections (14 μ m) mounted on Superfrost Plus slides and dried for 1.5 h at 40°C. Sections were simultaneously incubated with antibodies against SIRT2 (1:200 anti-rabbit, Abnova PAB27211) and HTT (1:300 anti-mouse, EM48) overnight at 4°C and then treated with secondary antibodies conjugated with Alexa Fluor 594 or Alexa Fluor 488 (1:500) for 2 h

at RT. To reduce unspecific background staining, we applied True Black autofluorescence quencher (1:20 in 70% ethanol) (BIOZOL Diagnostica Vertrieb, Germany) for 1 min. Finally, all sections were counterstained with the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI; 1:1000; Roche Diagnostics). All sections were documented in a confocal fluorescence microscope (Nikon Spinning Disk) using a 60 $\times$ water magnification immersion objective. For immunofluorescent staining of the HD case with Vonsattel grade 0, antigen retrieval was performed in Tris-EDTA buffer with pH 9.0 at 80°C for 1 h; samples were blocked in potassium phosphate-buffered saline in 0.25% Triton X-100 (KPBS-T) containing 5% donkey serum for 1 h. Subsequently, the sections were incubated overnight using primary antibodies against SIRT2 (1:300, anti-rabbit, PAB27211, Abnova) and HTT (1:100 anti-mouse, EM48) in KPBS-T containing 5% donkey serum. Sections were labelled with biotin-conjugated secondary antibody (1:200, donkey anti-rabbit) followed by Alexa Fluor 647-conjugated streptavidin and Alexa Fluor 488-conjugated secondary antibody (1:200, donkey anti-mouse, Jackson Laboratories). Sections were mounted on chromatin-gelatin-coated glass slides and coverslipped using VECTASHIELD Mounting Medium (Vector Laboratories).

2.2.5 | Image acquisition

Photodocumentation of vibratome and semithin sections was performed using an Olympus Microscope BH-2 equipped with an Olympus DP28-CU Camera (Olympus Optical) and the computer-assisted cellSense Entry program version 3 (Olympus). Images of 80 μ m vibratome sections were documented on Z-stacks created with Zene Stack (Zerene Systems, Cherrystone, Loop Richland, WA, USA) in ImageJ to show the representative distribution of SIRT2-immunoreactive inclusions and HTT (mEM48)-immunoreactive inclusions. Images used in the same figure were adapted for brightness and contrast. Brightfield and fluorescence image acquisitions of the HD case with Vonsattel grade 0 were performed using a Zeiss Axio Imager M2 microscope (Zeiss, Göttingen, Germany) or a Zeiss AxioCam 305 color camera. For each figure, images were acquired with consistent settings, and contrast was optimized by adjusting the histogram using the Zeiss Zen 3.7 software. Image panels were created and edited using Affinity Designer 1.10 (Affinity).

2.3 | Methods related to animal models

2.3.1 | Animal models

All mice were housed in cages of 2–5 animals and maintained in a 12-h light/dark cycle with ad libitum access to food and water. All described experimental procedures performed in animals were approved by the Regional

Ethical Committee in Malmö/Lund, Sweden under permit number 17113/2022. Brain tissue from the R6/2 mouse, a well-established transgenic model of HD expressing exon 1 of the mutant *HTT* gene [26] was used for neuropathological analyses at 6 ($n = 4$ /genotype) and 16 weeks of age ($n = 6$ R6/2 and $n = 2$ wild-type littermates). The R6/2 mice used in this study had a CAG repeat size range between 276 and 296 resulting in a slower disease progression compared to R6/2 mice with 150 CAG repeats [27, 28]. Brain tissue from the BACHD mouse, another well-established transgenic HD mouse expressing the full-length mHTT with around 97 glutamines [29] in the colony at Lund University [30], was used for neuropathological analyses at 6 months of age ($n = 6$ for BACHD and $n = 3$ for wild-type littermates). Brain tissue from mice overexpressing fragments of 853 amino acids of HTT with either 79Q (mHTT) or 18Q (wild-type HTT) after hypothalamic injections of AAV vectors at 6 weeks of age in the experiments described in Hult et al., 2011 [31] was used for neuropathological analyses at 6 and 18 weeks post-injection ($n = 1$ /group).

In order to test the effects of overexpression of SIDT2 in the R6/2 mice, an AAV vector was produced to overexpress human SIDT2 (AAV-SIDT2). The transgene sequence was regulated under the synapsin 1 promoter and flanked with simian virus 40 polyadenylation signal sequence between the inverted terminal repeat from AAV2 genome and packaged in a AAV5 capsid, as previously described [31, 32]. Stereotactic injections of 0.5 μ L of the AAV vectors at 1.0 E14 genome copies/ml was performed bilaterally in the hypothalamus as previously described [33] at the following coordinates: anterior/posterior = -0.7 mm, medial/lateral: ± 0.55 mm, ventral/dorsal = -5.2 mm in four R6/2 mice at 6 weeks of age. The stereotactic coordinates were calculated relative to the bregma and dura. Neuropathological analyses were performed at 10 weeks post-injection, that is, at 16 weeks of age and AAV-SIDT2 injected R6/2 mice were compared to uninjected R6/2 mice of the same age ($n = 4$ –5/group).

2.3.2 | Immunohistochemistry of mouse brain tissue

Mice were sacrificed using sodium pentobarbital (~ 150 mg/kg i.p.) and transcardially perfused first with saline and then pre-cooled 4% PFA. Brains were removed, and then post-fixed for 24 h in 4% PFA and placed in 25% sucrose for cryoprotection. Brains were sectioned frozen in the coronal plane at a thickness of 30 μ m in six series. The sections were processed as previously described [33]. Primary antibodies used in three separate series of sections were against SIDT2 (1:2000, anti-rabbit, PAB27211, Abnova), the N-terminus of HTT (1:500, anti-goat, sc-8767, Santa Cruz Biotechnology; or 1:100 mEM48) or hypocretin (1:4000, anti-rabbit,

H-003-30, Phoenix Pharmaceuticals). Sections were labelled with suitable secondary antibody (1:200, anti-rabbit, anti-goat or anti-mouse) and visualised with DAB using hydrogen peroxidase. Lastly, sections were mounted on chromatin-gelatin coated glass slides, dehydrated in an ethanol and xylene series and coverslipped with DPX (Sigma Aldrich).

2.3.3 | Estimation of the number of hypocretin-immunopositive neurons and mHTT inclusions

Images were captured at consistent magnification and brightness settings on a Zeiss PrimoStar microscope (Zeiss) and quantified in ImageJ 1.53t. Images of EM48-immunoreactive mHTT inclusions were acquired bilaterally in the superior lateral part of the fornix at bregma -1.70 mm using a $40\times/0.6$ NA objective. Images were made binary and thresholded (0–130). Watershed segmentation was applied to separate overlapping inclusions. Inclusions with a pixel size range of 6–250 and a circularity value between 0.20 and 1.00 were automatically counted. The total number of mHTT inclusions was then calculated per square mm. Images of hypocretin-immunoreactive neurons were acquired bilaterally in the lateral hypothalamic area at a bregma range of -1.06 to -2.30 mm using a $4\times/0.1$ NA objective. Images were made binary and thresholded (0–48). Watershed segmentation was applied, and neurons with a pixel size range of 15–infinity and a circularity value 0.10–1.00 were counted. The total number of neurons was estimated by multiplying the total cell count by the number of series. Image acquisition was performed as previously described for the HD case with grade 0.

2.4 | Methods related to cell culture experiments

2.4.1 | Cell culture expression constructs

To overexpress human SIDT2, pcDNA3.1 vectors carrying C-terminally V5-tagged SIDT2 (UniProt ID: Q8NB9J-1) were synthesized (BioCat, Heidelberg, Germany). A pcDNA3.1 plasmid without an insert served as an empty vector control. The HTT exon 1-coding pCMV HTT_{Ex1} 16Q and pCMV HTT_{Ex1} 72Q constructs were a gift from Hilal A. Lashuel (EPFL, Lausanne, Switzerland).

2.4.2 | Cell cultures

All cell culture experiments were conducted in SH-SY5Y (ATCC: CRL-2266) cells, maintained in Dulbecco's modified Eagle medium (DMEM) GlutaMAX supplemented with 10% foetal bovine serum, 1% non-essential

amino acids and 1% Antibiotic-Antimycotic (A/A) (all Gibco, Thermo Fisher Scientific) in 5% CO₂ at 37°C. Cells were transiently transfected for 72 h with Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol.

2.4.3 | Protein extraction from cells

For generating protein extracts, cells were dissociated by trypsinization and centrifuged for 5 min at 500×g in a pre-cooled (4°C) centrifuge. Supernatant was discarded and cell pellets were washed with cold 1× Dulbecco's phosphate-buffered saline (DPBS) for 2.5 min at 500×g and 4°C. Cell pellets were resuspended in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (v/v) sodium deoxycholate, 1% (v/v) Triton X-100, and 10% (v/v) glycerol) supplemented with cComplete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor (Roche) and ultrasonicated using a Sonopuls ultrasonic homogenizer (Bandelin electronic) at 10% power and 10% pulse duration for 3 s. Protein concentrations were determined spectrophotometrically in a microtiter plate using Bio-Rad Protein Assay Bradford reagent (Bio-Rad Laboratories).

2.4.4 | Western blots of cell cultures samples

For protein extracts obtained from cells, Western blotting was performed according to standard procedures. In brief, 25 µg of total protein was mixed in a ratio 3:1 with 4× LDS sample buffer (1 M Tris pH 8.5, 43% (v/v) glycerol, 8% (w/v) LDS, 2 mM EDTA, 0.1% (v/v) Orange G) with 100 mM of dithiothreitol (DTT) and heat-denatured at 70°C for 10 min. Protein samples were then electrophoretically separated on 10% Bis-Tris gels using MOPS electrophoresis buffer (50 mM MOPS, 50 mM Tris pH 7.7, 0.1% (w/v) SDS, and 1 mM EDTA). Separated proteins were transferred onto Amersham Protran Premium 0.2 µm nitrocellulose membrane (Cytiva) using a Bicine/Bis-Tris transfer buffer (25 mM Bicine, 25 mM Bis-Tris pH 7.2, 1 mM EDTA, and 15% (v/v) methanol) at 80 V and a maximum of 250 mA for 2 h.

2.4.5 | Filter retardation assay

Detection of SDS-insoluble proteins was performed as previously described [34]. Briefly, 3 µg of total protein was diluted in 1× DPBS containing 2% (w/v) SDS and 50 mM DTT and heat-denatured for 5 min at 95°C. Using a Minifold II Slot Blot system (Schleicher & Schuell), an Amersham Protran 0.45 µm nitrocellulose membrane (Cytiva) was equilibrated with 1× DPBS containing 0.1% (w/v) SDS, and samples were subsequently

filtered through applying a vacuum. Afterwards, slots were washed once with 1× DPBS containing 0.1% (w/v) SDS and twice with 1× DPBS before the standard immunodetection protocol was applied.

2.4.6 | Visualization of Western blots and filter retardation assays of cell culture samples

Membranes obtained from Western blotting or filter retardation assays of cell culture samples were blocked with 5% (w/v) skim milk powder (Merck) in 1× TBS (10 mM Tris pH 7.5, 150 mM NaCl) at RT and subsequently probed with primary antibodies diluted in 1× TBS-T (TBS with 0.1% Tween 20) at 4°C overnight. A detailed listing of used primary antibodies can be found in Table S1. Afterwards, membranes were washed with 1× TBS-T and probed for 1 h at RT with the respective fluorescently labeled secondary IRDye antibodies (goat anti-mouse 680LT, goat anti-mouse 800CW, and goat anti-rabbit 800CW; 926-68020, 926-32210, 926-32211, all 1:5000; LI-COR Biosciences). After incubation, membranes were washed a final time with 1× TBS-T. Fluorescence signals were detected and quantified using the LI-COR ODYSSEY FC and the Image Studio 5.2 software (both LI-COR Biosciences).

2.4.7 | Immunocytochemistry and fluorescence microscopy

For immunocytochemistry, SH-SY5Y cells were seeded on poly-L-lysine (P4707, Sigma-Aldrich) pre-coated 8-well chamber slides (80,841, Ibidi), and transfected after 24 h. After 96 h, cells were fixed with 4% (w/v) PFA in 1× DPBS. For blocking and permeabilization, cells were incubated for 1 h at RT in 10% (w/v) bovine serum albumin, 0.5% (v/v) Triton X-100, and 0.02% (w/v) NaN₃ in 1× DPBS. Subsequently, cells were probed with primary antibodies anti-V5 (made in mouse, 1:500; clone SV5-Pk1, R960-25, Thermo Fisher Scientific) and anti-HTT (made in rabbit, 1:500; clone EPR5526, ab109115, Abcam) diluted in antibody diluent (1% (w/v) bovine serum albumin, 0.5% (v/v) Triton X-100, 0.02% (w/v) NaN₃ in 1× DPBS) at 4°C overnight. The following day, cells were washed and incubated for 1 h at RT with goat anti-mouse IgG AlexaFluor 594 or goat anti-rabbit AlexaFluor 488 (both 1:500; A32742, A27034, Thermo Fisher Scientific). After washing, cells were mounted and counterstained with VECTASHIELD Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories). Epifluorescence images were taken at a 630× (Plan-Neofluar 63×/1.4 oil objective) magnification on an Axioplan 2 imaging microscope equipped with an ApoTome and an AxioCam MRm camera, using the AxioVision 4.3 imaging software (all Zeiss, Oberkochen, Germany).

2.5 | Statistical analyses

Data on human and mouse tissues was analyzed using independent samples Mann–Whitney *U* test or Students *t* test in SPSS Inc. 18.0. Statistical significance was set at *p*-value <0.05. Graphs were generated using GraphPad Prism 10.2 (GraphPad Software). Data are presented as mean ± standard deviation (SD) or median with range. Statistical analyses of cell culture-based experiments were performed using GraphPad Prism 10.1.1 (GraphPad Software). Results are presented as bar charts representing mean ± standard error of mean (SEM). Two-way ANOVA with the respective post-hoc analyses were applied. Statistical significance was set at a *p*-value 0.05. Further information is provided in the respective figure legends.

3 | RESULTS

3.1 | SIDT2 levels are reduced in striatum and lateral hypothalamus in human HD cases

First, we investigated SIDT2 protein levels in human postmortem tissue from HD and control cases. Western blot analysis revealed a significant reduction of SIDT2 protein levels in the caudate nucleus of the striatum (93% with antibody 1 and 68% reduction with antibody 2) and a significant 67% reduction of SIDT2 in the lateral hypothalamic area of HD cases compared to control cases (Figure 1A,B,D and S1–S4). No differences in SIDT2 protein levels were detected between HD and control cases in the frontal cortex (Figure 1C). mRNA levels of SIDT2 in the striatum or the frontal cortex did not differ between HD and control cases (Figure 1E,F). This data indicate a depletion of soluble SIDT2 protein in the striatum and, to a lesser extent, in the lateral hypothalamic area during later stages of HD, independent of its transcription.

3.2 | CAG-dependent frequency of SIDT2-immunoreactive inclusions in the HD ACC

Given the preserved SIDT2 protein levels in the HD cortex, and since the ACC is characterized by frequent mHTT inclusions unlike striatal areas that display greater neuronal loss in later stages [35], we examined SIDT2 immunoreactivity in the ACC of postmortem human HD cases. SIDT2 immunoreactivity was observed in numerous neurons in both HD and control cases with a similar distribution pattern (Figure 2A,C,E,G,I). The reactivity was detected in the nucleus, cytoplasm, and dendritic processes, varying in expression and intensity. Interestingly, HD cases showed SIDT2-immunoreactive intranuclear inclusions of uniform size when compared to

control cases. In HD patients with CAG repeat lengths of 42 and 45, SIDT2 inclusions were rare (Figure 2C, data for CAG 45 not shown), whereas in HD individuals with CAG repeats of 50 and 66, intranuclear inclusions were frequent, suggesting that the frequency of SIDT2 intranuclear inclusions is dependent on the CAG repeat length (Figure 2E,G,I). Most inclusions were localized in layers V and VI of the ACC.

We then compared SIDT2 immunoreactivity with the distribution of mHTT inclusions in the ACC. The density of EM48-immunopositive inclusions also increased with the CAG repeat length (Figure 2, D, F, H, J), confirming previous observations in cortical areas [36]. However, in contrast to SIDT2-immunoreactive intranuclear inclusions, EM48-immunoreactive inclusions were heterogeneous in size and form, mainly localized in the cytoplasm and neuropil, while intranuclear inclusion bodies were rare. To elucidate the subcellular composition of SIDT2 intranuclear inclusions, transmission immunoelectron microscopy was performed, showing dense and membrane-less SIDT2-immunoreactive intranuclear inclusions that were clearly distinguishable from the nucleolus (Figure 3A–C). Co-localization of mHTT and SIDT2 was rarely observed, as illustrated by immunofluorescence triple staining (Figure 3D).

3.3 | SIDT2-immunoreactive inclusions are present in an HD case with Vonsattel grade 0

To investigate the early presence of SIDT2 immunoreactivity as well as mHTT inclusions, we analyzed a rare HD case with 43 CAG repeats and a Vonsattel grade 0 [22]. We observed frequent EM48-immunoreactive mHTT inclusions in the frontal cortex (Figure 4A,B), the caudate nucleus of the striatum (Figure 4F,G), and in the lateral hypothalamic area (Figure 4K,L). SIDT2 immunoreactive inclusions were detected in the same brain regions (Figure 4C–E, H–J, M, N). In the frontal cortex, SIDT2-immunoreactive inclusions were mainly formed in layer II and V–VI (Figure 4 C–E). Immunofluorescent analysis shows that only very few cells display co-localization of SIDT2 and mHTT inclusions (Figure 4O) and that most SIDT2 inclusions do not co-localize with mHTT inclusions (Figure 4P). This is consistent with observations in HD cases with higher Vonsattel grades (Figure 3D). Hence, most SIDT2-immunoreactive inclusions do not co-localize with EM48-immunoreactive mHTT inclusions in postmortem human brain tissue from clinical HD cases.

3.4 | SIDT2-immunoreactive inclusions appear later than the formation of mHTT inclusions in the R6/2 HD mouse model

We then examined whether SIDT2-immunoreactive inclusions were present in the brains of HD mouse

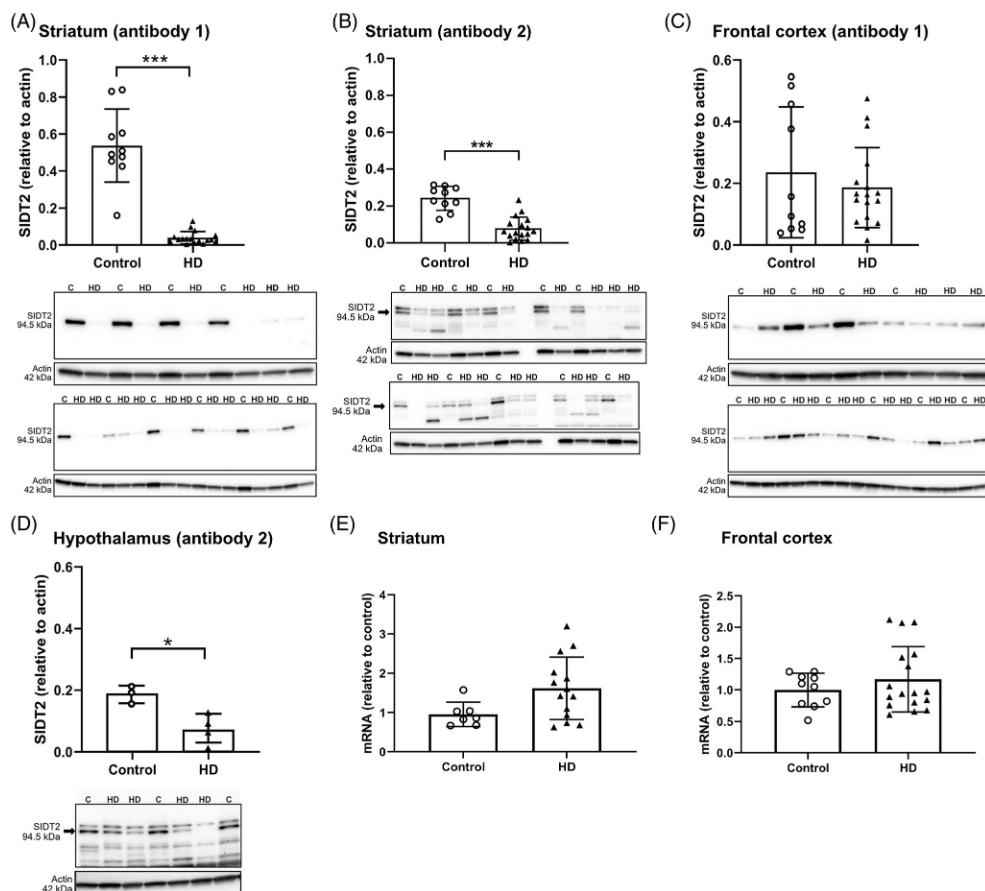
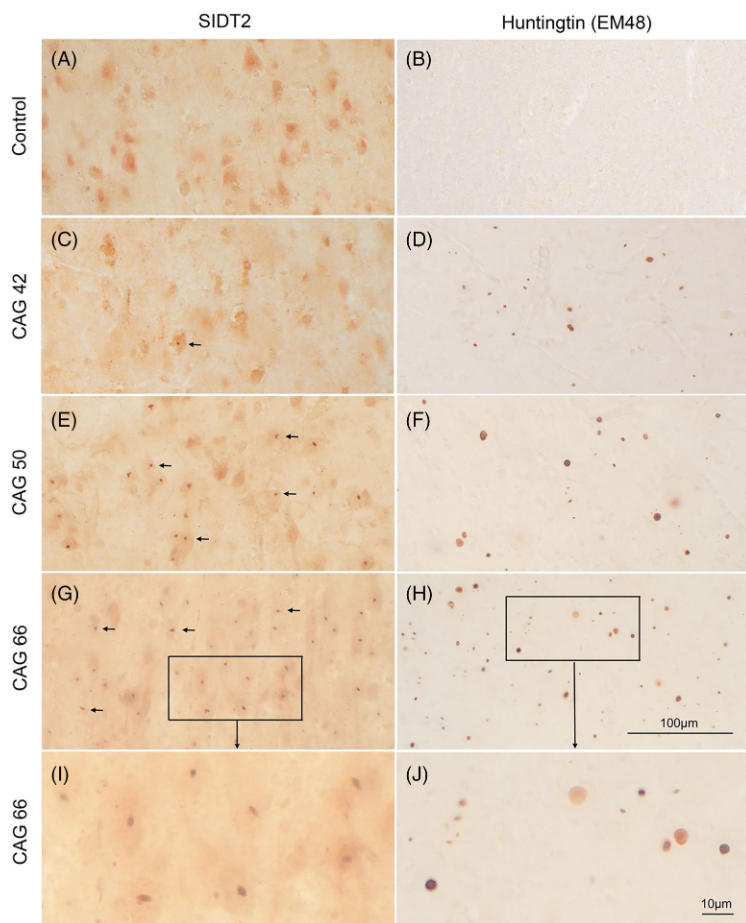


FIGURE 1 Reduced SIRT2 protein levels in the striatum and the lateral hypothalamic area in HD cases. Western blots of SIRT2 in the striatum (A, B), the cerebral cortex (C), and the lateral hypothalamic area (D) from HD cases compared to age- and sex-matched control cases. Antibody 1 is Abcam ab85847, and antibody 2 is Abnova PAB27211 that replaced the first antibody in this study due to the discontinuation of antibody 1 at the company level. qRT-PCR shows no differences in mRNA levels of SIRT2 in the striatum (E) and cerebral cortex (F) between HD and control cases. Data are expressed as mean $\pm$ SD. Student's *t*-test, * = $p < 0.05$.

models. In the R6/2 mouse model, we did not detect SIRT2-immunoreactive inclusions at 6 weeks of age in the striatum, hypothalamus, and cerebral cortex (Figure 5B,H,N). However, at 16 weeks of age, SIRT2-immunoreactive inclusions were present in these three regions in R6/2 mice (Figure 5E,K,Q). We did not detect any SIRT2-immunoreactive inclusions in the striatum, the hypothalamus, or the cerebral cortex in any wild-type littermates at these two time points (Figure 5A,D,G,J,M,P). As expected, EM48-immunoreactive mHTT inclusions were present in these three brain regions at 6 weeks of age and showed a higher density at 16 weeks of age (Figure 5C,F,I,L,O,R). Based on the analyses of R6/2 brain tissues, SIRT2-immunoreactive

inclusions appear at a later stage than EM48-immunoreactive mHTT inclusions. A similar observation was made in hypothalamic tissue of mice with AAV vector mediated overexpression of a large fragment of mHTT with 79 glutamines, where many cells formed mHTT inclusions at 6 weeks post-injection, as reported previously [31], but only a few cells displayed SIRT2-immunopositive inclusions (Figure 6A,B). At 18 weeks post-injection, the density of mHTT inclusions was increased, as well as the density of SIRT2-immunoreactive inclusions (Figure 6C,D). SIRT2-immunoreactive inclusions or mHTT inclusions were not present after overexpression of a similar HTT fragment with a normal length of 18 glutamines at 18 weeks post-

FIGURE 2 Formation of SIDT2-immunoreactive CAG-repeat dependent inclusions in human HD cortex. SIDT2 immunoreactivity is localized in multiple neuronal nuclei, cytoplasm, and dendrites of HD and in control anterior cingulate cortex (A, C, E, G, I). In the HD case with 42 CAG repeats, there are single SIDT2 intranuclear inclusions (C; black arrow) not observed in the control (A). HD cases with 50 and 66 CAG repeats present abundant inclusions (E, G, I). EM48-immunoreactive mHTT inclusions, lacking in the control (B), also become denser in distribution with increasing CAG length (D, F, H), but in contrast to the SIDT2 inclusions, the EM48-immunopositive mHTT inclusions are heterogeneous in size and form (J) and mainly localized in the cytoplasm and neuropil. Intranuclear EM48-immunoreactive inclusions are rare.



injection (Figure 6E,F). We then examined SIDT2-immunoreactivity in these brain regions in the BACHD mice expressing full-length mHTT with 97 glutamines [29]. We could not detect any SIDT2- or EM48-immunoreactive mHTT inclusions in the striatum, cerebral cortex, and hypothalamus of BACHD mice at 6 months of age, a stage when these mice display an overt phenotype (Figure S5). Taken together, SIDT2-immunoreactive inclusions appear to develop after the formation of mHTT inclusions in HD mice.

3.5 | Overexpression of SIDT2 using AAV vectors reduces the number of mHTT inclusions in the lateral hypothalamic area in R6/2 mice

Given that a previous study [20] demonstrated a reduction in mHTT inclusions following SIDT2 overexpression

in vitro, we conducted an in vivo proof-of-principle experiment. We designed an AAV vector expressing human SIDT2 that was injected into the hypothalamus of R6/2 mice at 6 weeks of age, and the effects were assessed 10 weeks post-injection. We confirmed the presence of AAV vector delivered SIDT2 by immunohistochemistry using an antibody against SIDT2 (Figure 7A,B). The R6/2 mice, similarly to persons with HD, show loss of hypocretin (orexin) neurons in the lateral hypothalamic area [5, 37]. Therefore, we examined the lateral hypothalamic area and found that AAV-mediated overexpression of SIDT2 resulted in a 45% reduction in the density of mHTT inclusions ($p = 0.01$) and a trend towards a 16% increase in hypocretin-immunoreactive neurons compared to uninjected R6/2 mice at the same age ($p = 0.17$). There were no significant differences in body weight between SIDT2-overexpressing R6/2 mice compared to uninjected R6/2 mice (data not shown).

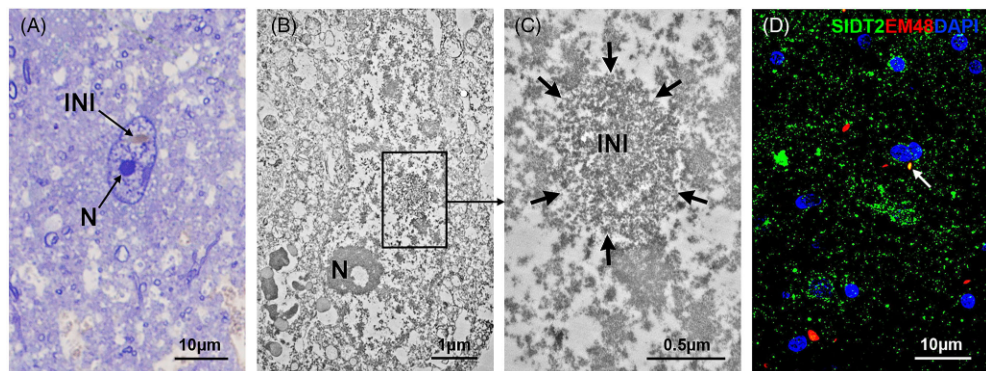


FIGURE 3 SIDT2 transmission electron and fluorescence microscopy in human HD cortex. Peroxidase immunohistochemistry (A–C) shows a large pyramidal cell in layer V of the anterior cingulate cortex with a SIDT2-immunoreactive intranuclear inclusion (INI) clearly distinguished from the nucleolus (N) as illustrated in a semithin section counterstained with Toluidine-blue (A) and a transmission electron microscopic picture (B). The enlargement (C) confirms membrane-less INI indicated by black arrows. (D) Triple fluorescence staining with SIDT2 (green), EM48 (red), and DAPI (blue) exhibits only rare co-localization of mHTT and SIDT2 (white arrow).

3.6 | Overexpression of SIDT2 reduces soluble and insoluble mHTT protein in SH-SY5Y cells without affecting major proteolytic degradation pathways

A previous study demonstrated that SIDT2 overexpression leads to a reduction in *HTT* mRNA, as well as reduced soluble and insoluble mHTT protein levels in murine Neuro2a cells co-transfected with vectors encoding EGFP-tagged *HTT* exon 1 and mouse *Sidt2* [20]. To validate both our observations in R6/2 mice and these earlier findings in mouse cells in a human cell model system, we replicated the corresponding condition in SH-SY5Y neuroblastoma cells overexpressing untagged *HTT* exon 1 16Q and 72Q along with human V5-tagged *SIDT2*. Western blot analysis confirmed an approximately 4-fold overexpression of SIDT2 resulted in a significant reduction of soluble HTT exon 1 16Q and, even more pronounced, polyQ-expanded HTT exon 1 72Q (Figure 8A,B). To assess potential effects on HTT exon 1 72Q aggregation, we performed filter retardation assays to detect SDS-insoluble HTT. Consistent with our findings on soluble HTT exon 1, insoluble HTT exon 1 72Q levels was significantly reduced upon SIDT2 overexpression (Figure 8C).

Since SIDT2 has been reported to not only modulate RNautophagy but also macroautophagy in general [38], we sought to determine whether the observed effects on soluble and insoluble HTT exon 1 levels were due to changes in baseline autophagy. To test this and to investigate potential effects on the UPS, we analyzed protein markers of both pathways. Western blot analysis revealed no significant changes in K48-linked polyubiquitinated proteins (a marker for the UPS), SQSTM1/p62 levels, or

the LC3B-II to LC3B-I ratio, indicating no alterations in baseline autophagy (Figure S6A,B). These findings rule out the activation of either proteolytic degradation pathway as the underlying cause of SIDT2-driven HTT exon 1 reduction in our cell model.

Given our previous observations of SIDT2-immunoreactive inclusions in both HD patient brain tissue and HD mice, we extended our microscopy analysis to the SH-SY5Y overexpression model. Epi-fluorescence microscopy confirmed the presence of inclusion-like structures in cells overexpressing SIDT2, which did not directly co-localize with HTT exon 1 72Q inclusion bodies (Figure 8D). In summary, our human neuroblastoma cell-based analysis confirms previous reports and our *in vivo* findings, demonstrating that SIDT2 overexpression lowers both soluble and insoluble mHTT levels. Additionally, our results support the potential of SIDT2 to form inclusion-like structures.

4 | DISCUSSION

SIDT2 is a lysosomal protein involved in RNA and DNA autophagy. Here we provide the first evidence of significant alterations of the SIDT2 system in postmortem brain tissue from persons with HD and in multiple HD models. We show that SIDT2-immunoreactive inclusions are present in brain tissue displaying mHTT inclusions, although they seldom co-localize. Inclusions with SIDT2 are readily detected in the caudate nucleus of the striatum, the frontal cortex, and the lateral hypothalamic area in human postmortem HD tissue as well as in animal models of HD. Moreover, SIDT2-immunoreactive inclusions appear to form in a CAG-repeat dependent fashion subsequent to the formation

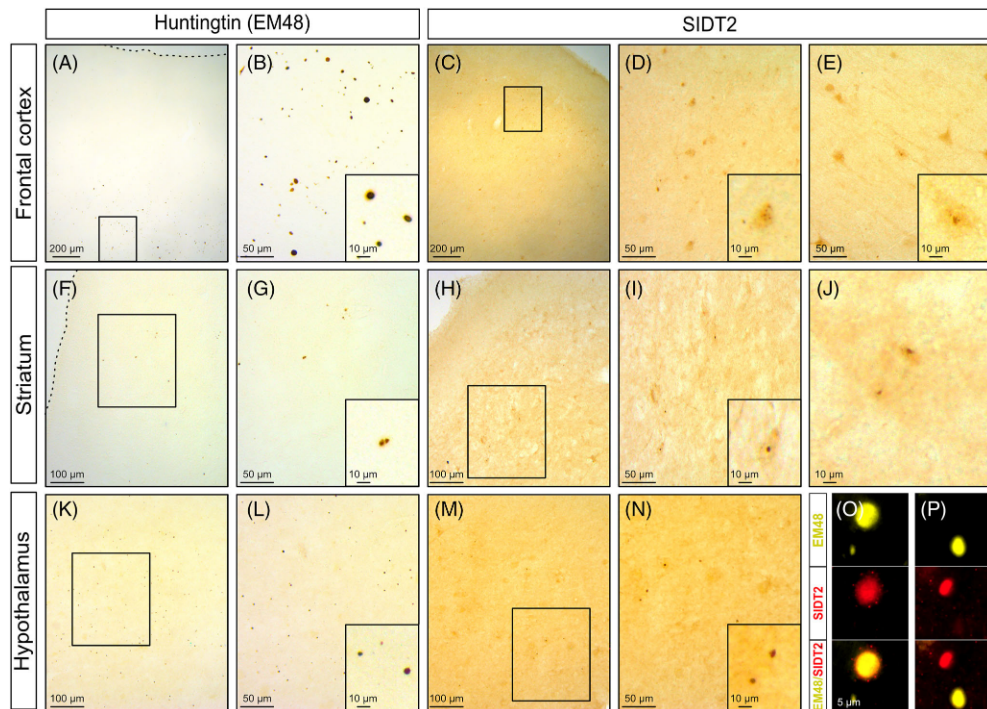


FIGURE 4 EM48- and SIDT2-immunopositive inclusions in human postmortem tissue from an HD case with Vonsattel grade 0. EM48-immunoreactive HTT inclusions are observed in the frontal cortex (A, B), the caudate nucleus of the striatum (F, G), and in the lateral hypothalamic area (K, L) of an HD brain with Vonsattel grade 0 (A). SIDT2-immunoreactive inclusions are detected in the same brain regions (C–E, H–J, M, N). Immunofluorescent analysis shows that only a very few cells have co-localization of SIDT2 and mHTT inclusions (O), whereas most SIDT2 inclusions do not co-localize with mHTT inclusions (P), suggesting independent aggregation pathways (D).

of mHTT inclusions. At later HD stages, SIDT2 protein levels are significantly reduced, particularly in the striatum but also in the lateral hypothalamic area, without changes in *SIDT2* mRNA levels. This suggests a potential depletion of soluble SIDT2 during attempted mHTT degradation. Interestingly, consistent with a prior in vitro study [20], we show a significant reduction of mHTT load after SIDT2 overexpression in both R6/2 mice and a neuroblastoma cell line overexpressing mHTT exon 1. Taken together, our data demonstrate novel neuropathological features involving SIDT2 in HD and further support the potential therapeutic effects of modulating this pathway.

Neuropathological hallmarks in HD include the presence of mHTT containing inclusions. The present study reveals that SIDT2-immunoreactive inclusions constitute an additional neuropathological hallmark. Since SIDT2-immunoreactive inclusions rarely co-localized with mHTT inclusions, as assessed with the EM48-antibody, these inclusions may occur independently. However, our analyses indicate that SIDT2 inclusions only form in brain tissue that also

contains mHTT inclusions, although at a later stage. Therefore, the formation of SIDT2 inclusions and mHTT inclusions may reflect similar underlying pathogenic processes occurring in parallel.

To our knowledge, only one prior published study has investigated SIDT2 in HD [20]. That study showed that SIDT2 binds to *HTT* mRNA in a CAG dependent fashion. Here we detected the formation of SIDT2-immunoreactive inclusions in affected HD brain tissues and observed an increased density of SIDT2 inclusions in HD cases with higher CAG repeats in the ACC. Both previous and recent studies have shown somatic expansion of the *mHTT* gene in the striatum and cerebral cortex in postmortem cases with HD [39–42]. It is possible that the SIDT2-containing inclusions also reflect the presence of somatic expansion in *mHTT* RNA. As SIDT2 binds to CAG repeats, the formation of SIDT2 inclusions may also be present in brains from persons with other CAG triplet repeat disorders such as the spinocerebellar ataxias. It would therefore be interesting to examine

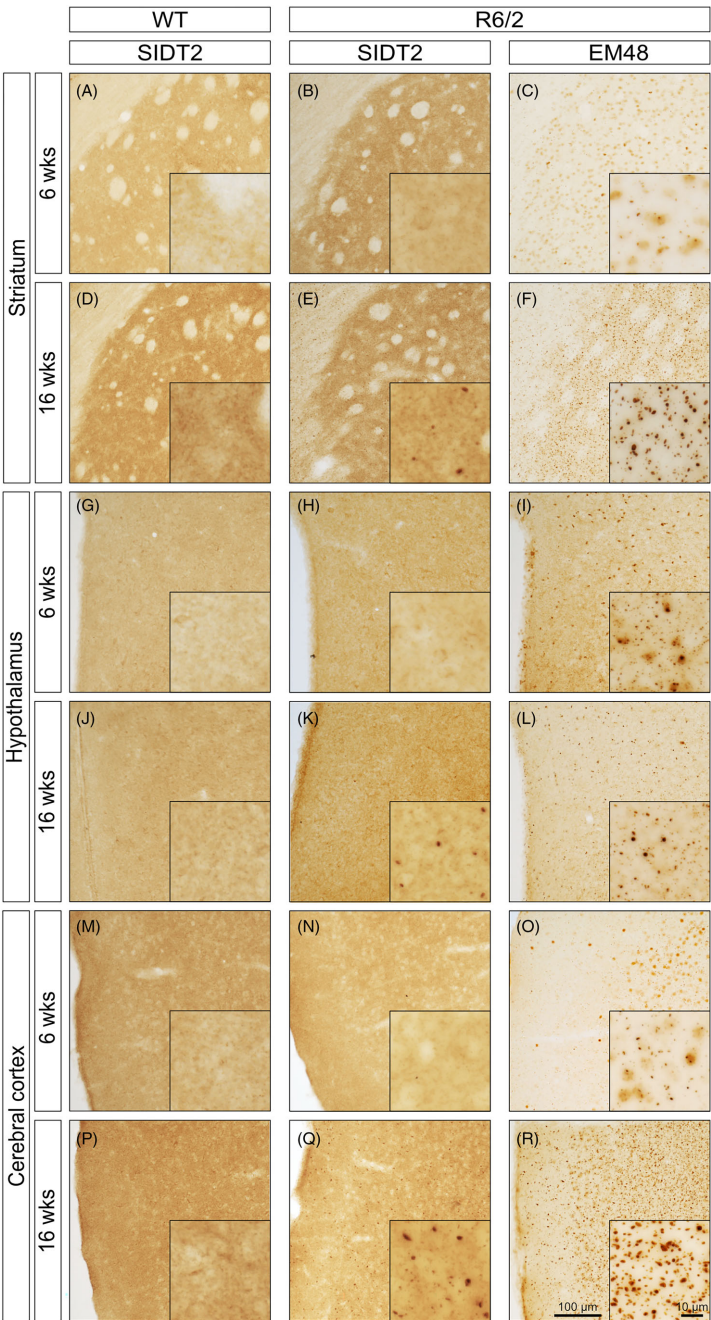
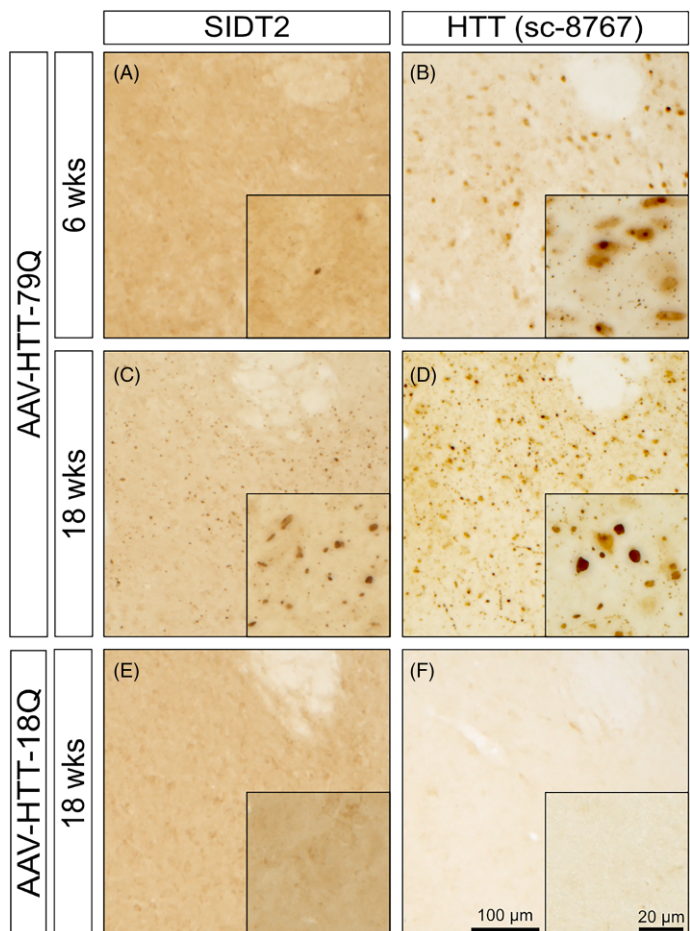


FIGURE 5 R6/2 mice form SIDT2-immunoreactive inclusions following the formation of mHTT inclusions. In R6/2 mice at 6 weeks (wks) of age, single SIDT2-immunoreactive inclusions are present in the cerebral cortex with no SIDT2 immunoreactivity in the striatum and hypothalamus (B, H, N). At this age, R6/2 mice display EM48-immunoreactive mHTT inclusions in these brain regions (C, I, O). At 16 weeks of age, R6/2 mice have formed SIDT2-immunoreactive inclusions in the striatum, hypothalamus, and cerebral cortex (E, K, Q) with an increased frequency of mHTT inclusions (F, J, R). Brain regions from wild-type (WT) littermates of the same age show no SIDT2 or mHTT immunoreactivity (A, D, G, J, M, P).

FIGURE 6 Time-dependent formation of SIDT2 inclusions in the hypothalamus following AAV vector mediated mHTT overexpression. Presence of rare SIDT2-immunoreactive inclusions and widespread mHTT inclusions in mice overexpressing mHTT in the hypothalamus following AAV-HTT-79Q injection at 6 weeks post-injection (A, B). An increase in the frequency of SIDT2 inclusions and mHTT inclusions 18 weeks post-injection of AAV-HTT-79Q (C, D). Control mice injected with AAV-HTT-18Q, expressing an 853 amino acid fragment of wild-type huntingtin with 18Q, showed no detectable SIDT2 or mHTT immunoreactivity (E, F).



whether SIDT2-immunoreactive inclusions are present in brains from persons with such disorders.

This study demonstrates for the first time that overexpression of SIDT2 can reduce the load of mHTT inclusions *in vivo*. Our proof-of-principle experiment using AAV vectors to overexpress SIDT2 led to a significant reduction of mHTT inclusions in the lateral hypothalamic area of R6/2 mice and trends towards beneficial effects on hypocretin neuronal loss. Further studies examining effects in a larger cohort of mice including in other HD animal models and in other affected brain regions with different concentrations of the AAV vector will be important to evaluate the full potential of SIDT2 overexpression on HD pathology. Moreover, in the present study we also demonstrate that overexpression of human SIDT2 in human SH-SY5Y neuroblastoma cells

leads to a reduction in both soluble and insoluble levels of untagged HTT exon 1, confirming findings from a murine cell model employing human GFP-tagged HTT exon 1 constructs [20]. Interestingly, SIDT2 overexpression alone resulted in the formation of inclusion-like structures that did not directly co-localize with inclusion bodies formed by polyQ-expanded HTT exon 1 72Q, mirroring, to a certain degree, our observations in HD patient and R6/2 mouse brains.

Since SIDT2 has been implicated in macroautophagy, including mitophagy [38, 43–45], we analyzed baseline levels of the autophagy markers SQSTM1/p62 and LC3B but did not detect any modulatory effects upon SIDT2 overexpression. This suggests that the observed HTT reduction does not occur outside the context of RNAutophagy. Additionally, our data showing unchanged

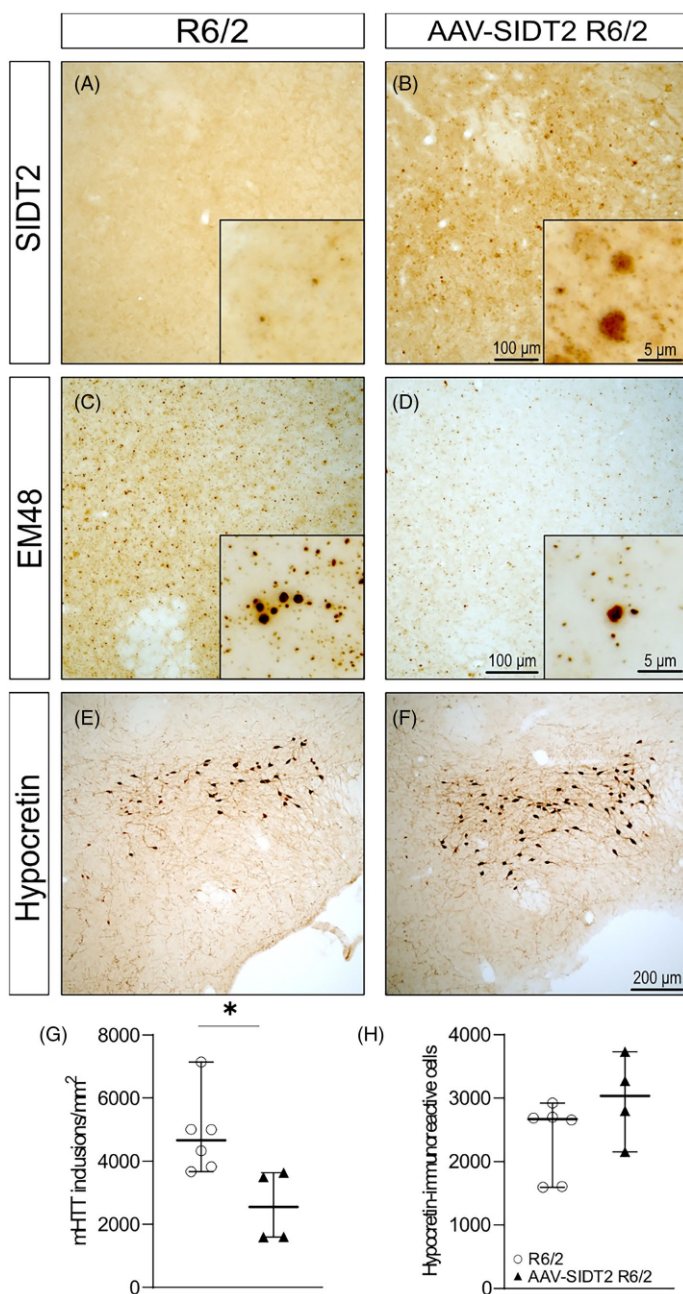


FIGURE 7 Overexpression of SIDT2 using AAV vectors reduces the number of mHTT inclusions in the hypothalamus in R6/2 mice. Immunohistochemistry for SIDT2 shows increased immunoreactivity in AAV-SIDT2-injected R6/2 mice at 10 weeks post-injection compared to uninjected R6/2 controls of the same age (16 weeks of age; A, B). mHTT immunoreactivity is reduced in AAV-SIDT2-injected R6/2 mice compared to R6/2 controls (C, D), with a significant decrease in the density of mHTT inclusions in the lateral hypothalamic area in mice overexpressing SIDT2 (G; $p = 0.010$). While hypocretin-immunoreactive neurons are decreased in R6/2 mice as reported in previous studies [37], mice overexpressing SIDT2 in the hypothalamus show a non-significant trend towards increased hypocretin-immunoreactive neurons (E, F, H; $p = 0.171$). The data are presented as median with range. Two-tailed, Mann-Whitney U test, * $p < 0.05$.

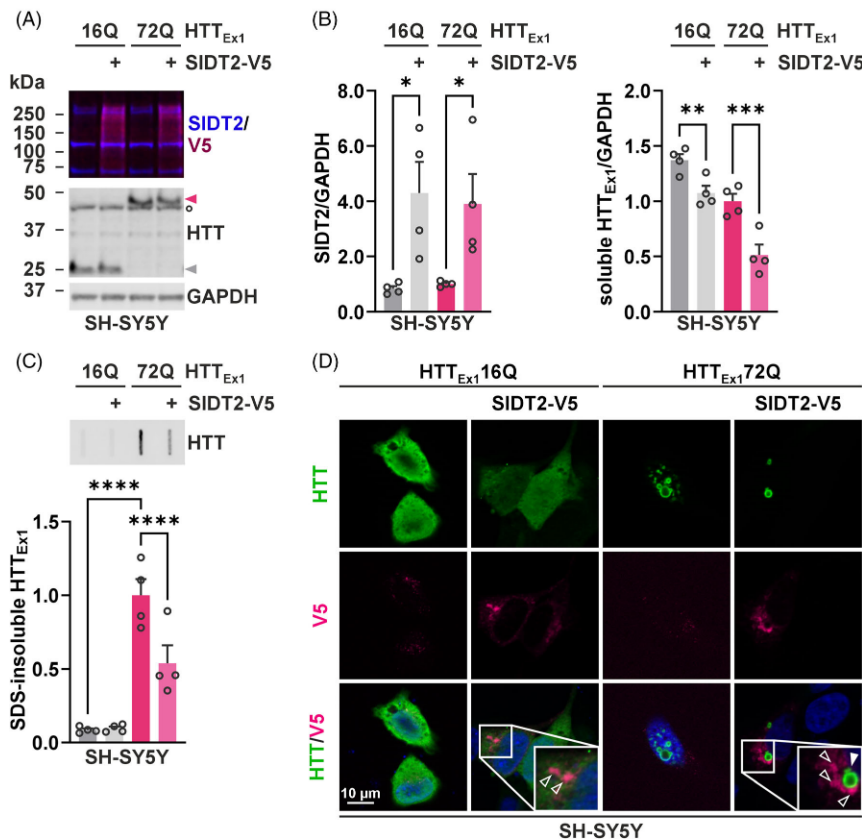


FIGURE 8 Overexpression of SIDT2 lowers soluble and SDS-insoluble forms of HTT exon 1 in SH-SY5Y cells. Protein extracts from SH-SY5Y cells co-transfected with HTT exon 1 (HTT_{Ex1}) with 16Q or 72Q, along with V5-tagged SIDT2 or an empty control vector, were analyzed by Western blotting and filter retardation assay. (A) Membranes were incubated with antibodies specific for SIDT2, the V5 tag, and HTT. GAPDH served as the loading control. The gray arrowhead marks HTT_{Ex1} 16Q, while the magenta arrowhead indicates HTT_{Ex1} 72Q. The black circle denotes an HTT_{Ex1}-unspecific band. (B) Quantitative analysis confirmed the overexpression of SIDT2 and demonstrated a significant reduction in soluble HTT_{Ex1} 16Q and 72Q levels upon SIDT2 overexpression. Signals were normalized to the loading control and then to the reference condition (HTT_{Ex1} 72Q/empty). Bars represent mean ± S.E.M. *n* = 4. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (Two-way ANOVA with Fisher's LSD test). (C) Filter retardation assay of SH-SY5Y cells co-transfected with HTT_{Ex1} with 16Q or 72Q with V5-tagged SIDT2 or an empty control vector. HTT was detected using an HTT-specific antibody, and densitometric analysis demonstrated a significant SIDT2-dependent reduction of SDS-insoluble HTT_{Ex1} 72Q. Signals were normalized to the respective control condition (HTT_{Ex1} 72Q/empty). Bars represent mean ± S.E.M. *n* = 4. *****p* < 0.0001 (Two-way ANOVA with Fisher's LSD test). (D) Immunocytochemistry staining and fluorescence microscopy of SH-SY5Y cells co-transfected with HTT_{Ex1} with 16Q or 72Q, along with V5-tagged SIDT2 or an empty control vector. HTT was detected using an HTT-specific antibody (green), while overexpressed SIDT2 was stained with V5 tag-specific antibody (red). Overexpressed SIDT2 formed inclusion-like structures (white-rimmed arrowheads), which did not co-localize with HTT_{Ex1} 72Q inclusion bodies (solid white arrowheads). DAPI was used as a nuclear counterstain (blue). Insets show 2-fold magnifications of selected areas (white boxes). Bars represent mean ± S.E.M. *n* = 4. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 (Two-way ANOVA with Fisher's LSD test).

K48-polyubiquitin levels suggest that SIDT2 does not impact the UPS. However, further investigations into a potential link between SIDT2 and macroautophagy, which is known to be dysregulated in HD and contributes to HTT clearance [46], are necessary to fully rule out additional regulatory effects.

Moreover, while incomplete splicing-derived HTT exon 1 is considered a primary contributor to HD pathology [47–49], and targeting this protein species or its coding RNA via SIDT2 appears to be an effective approach, further research into the effects on full-length HTT mRNA and protein may provide insights into molecular

interactions relevant to HD pathology. Additionally, exploring potential effects of other RNautophagy/uptake-linked proteins such as LAMP2C and SIDT1 [14, 50, 51] on *HTT* mRNA may be helpful to further elucidate and optimize this strategy for HTT lowering.

Lysosomal pathways have been implicated in other neurodegenerative disorders including synucleinopathies. In fact, SIDT2-immunoreactive inclusions have been described in the ACC of persons with these disorders [52]. Furthermore, SIDT2 may exert other important roles in the cell [53]. Recent studies have shown that SIDT2 can be localized to the plasma membrane and mediate the uptake of single and double-stranded oligonucleotides in cells [54–56]. This has relevance for the development of nucleic acid-based therapies, including those attempted for HD using anti-sense oligonucleotides (ASO) for reducing mHTT levels [1]. The mechanisms underlying the cellular uptake of such oligonucleotide therapeutics are unclear. Interestingly, a recent study showed that inducing SIDT2 expression in human cell lines increased knock-down activity of gapmer ASOs [56]. It is therefore possible that reductions of SIDT2 levels, as demonstrated in the present study in the striatum in HD and/or SIDT2 inclusion formation as shown in several affected HD brain tissue, may have a negative impact on the uptake of ASOs into cells. Further studies on the impact of SIDT2 on uptake of oligonucleotide therapeutics for HD are therefore warranted.

In conclusion, we identified a novel neuropathological hallmark with the formation of SIDT2-immunopositive inclusions in affected brain tissues in HD. Our results suggest that targeting SIDT2-mediated RNautophagy may have beneficial effects on reducing the load of mHTT inclusions independent of traditional protein degradation pathways. Recent studies suggest that SIDT2 may play a role in the uptake of extracellular nucleotides. Combined with our data showing significantly reduced SIDT2 protein levels in the striatum of advanced HD cases, this indicates that alterations in the SIDT2 system could impact the development of nucleotide-based therapeutics for HD.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection, or data analyses were performed by all authors. The first draft of the manuscript was written by Sanaz Gabery, Sofia Bergh, and Åsa Petersén. All authors read and commented on previous versions of the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We are very grateful to the donors of brain tissue that was obtained from the Sydney Brain Bank, which is supported by Neuroscience Research Australia and the University of New South Wales. Tissues were also received from the Victorian Brain Bank Network, supported by the Mental Health Research Institute, Alfred Hospital,

Victorian Forensic Institute of Medicine, The University of Melbourne, Helen Macpherson Smith Trust, Parkinson's Victoria, and Perpetual Philanthropic Service. We are also grateful to the donors of tissue to the Huntington brain collection of Bochum, Germany, and the Department of Neuropathology, Lund, Sweden. We would like to thank Björn Anzelius, Anna Hansen, Anneli Josefsson, Ulrika Sparrhult-Björk, Luzie Augustinowski, Robert Nadgrabski, Katja Rumpf, Corinna Wojczak and Claudia Schneider for excellent technical assistance.

FUNDING INFORMATION

This study was funded by research grants to AP from the Swedish Research Council (grant number 2022/01092), the Swedish Brain Foundation, the Foundation of King Gustav V and Queen Victoria, the Swedish governmental funding of clinical research (ALF) at Region Skåne as well as the Knut and Alice Wallenberg Foundation (2019.0467). HPN and JJW were funded by the German Research Foundation (DFG; research grant numbers NG 101/6-1 and WE 6585/1-1, respectively). GMH was funded by the National Health and Medical Research Council of Australia [Investigator Grant 1176607]. EPP was funded by the Deutsche Huntington Hilfe (RUB No. 4908100115).

CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Human postmortem tissues were obtained from the Victorian Brain Bank and the Sydney Brain Bank at Neuroscience Research, Australia after approvals from their Scientific Committees (PID073, PID0111, PID167). All persons had given their informed consent prior to the donation of their brains and the regional brain donor programs were approved by the Institutional Human Research Ethics Committees. The analyses of human postmortem tissue in Bochum were approved by the Ethic Committee of the Medical Faculty of the Ruhr-University Bochum, Germany (Reg. No. 17-5939). Analyses performed on human tissue in Sweden were approved by the Swedish Ethical Review Authority (reference number 2022-05348-01) and the regional ethical review board at Lund University (reference number 2014/466). The experimental procedures performed in animals were approved by the Regional Ethical Committee in Lund, Sweden under permit number 17113/2022.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Gabery S, Bergh S, Huridou C, Cheong RY, Baldo B, Scheunemann PG, et al. The DNA/RNA autophagy protein SIRT2 as a novel neuropathological hallmark in Huntington disease. *Brain Pathology*. 2026. e70088. <https://doi.org/10.1111/bpa.70088>

Paper III



Effects of mutant huntingtin in oxytocin neurons on non-motor features of Huntington's disease

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Funding information

This work was supported by research grants to RYC and ÅP from the Swedish Research Council (grant numbers 2013/03537 and 2018/02559), ALF grant from Region Skåne, the Royal Physiographic Society of Lund (grant numbers 2016-37869, 2018-39651 and 2019-40523), the Swedish Brain Foundation (grant numbers FO2016-0121 and FO2018-0080), the Åhlén Foundation (grant number mA28/h17), the Fredrik and Ingrid Thuring's Foundation (grant number 2017-00316) and NEURO Sweden (2017 and 2019). RYC was supported by a Swedish Society for Medical Research (SSMF) Postdoctoral Fellowship (grant number P15-0017).

Abstract

Background: Early non-motor features including anxiety, depression and altered social cognition are present in Huntington's disease (HD). The underlying neurobiological mechanisms are not known. Oxytocin (OXT) is involved in the regulation of emotion, social cognition and metabolism, and our previous work showed that the OXT system is affected early in HD. The aim of the study was to investigate the potential causal relationship between the selective expression of mutant huntingtin (mHTT) in OXT neurons and the development of non-motor features and neuropathology.

Methods: To express mHTT only in OXT neurons, we used a novel flex-switch adeno-associated viral vector design to selectively express either mHTT or wild-type HTT in the paraventricular nucleus of the hypothalamus using OXT-Cre-recombinase mice. We also performed a mirror experiment to selectively delete mHTT in OXT neurons using the BACHD mouse model. Mice underwent a battery of behavioural tests to assess psychiatric and social behaviours 3 months post-injection or at 2 months of age, respectively. Post-mortem analyses were performed to assess the effects on the OXT system.

Results: Our results show that selective expression of mHTT in OXT neurons was associated with the formation of mHTT inclusions and a 26% reduction of OXT-immunopositive neurons as well as increased anxiety-like behaviours compared with uninjected mice. However, selective deletion of mHTT from OXT neurons alone was not sufficient to alter the metabolic and psychiatric phenotype of the BACHD mice at this early time point.

Conclusions: Our results indicate that mHTT expression can exert cell-autonomous toxic effects on OXT neurons without affecting the non-motor phenotype at early time points in mice.

KEYWORDS

behavioural symptoms, huntingtin protein, Huntington's disease, hypothalamus, oxytocin, paraventricular hypothalamic nucleus

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INTRODUCTION

Huntington's disease (HD) is a monogenic disorder inherited in an autosomal dominant fashion with full penetrance. The disease-causing mutation is an expansion of a CAG trinucleotide repeat in the huntingtin (*HTT*) gene, which encodes an extended polyglutamine (Q) stretch within the N-terminus of the HTT protein.^{1,2} HD patients experience a spectrum of non-motor symptoms, including psychiatric disturbances (such as anxiety, depression, irritability and apathy as well as impaired social cognition) in addition to cognitive decline with executive dysfunction.^{3,4} Other non-motor aspects include metabolic and sleep disturbances. So far, HD research has mainly focused on the motor aspect of the disease with limited understanding of the non-motor aspects. Increasing our knowledge of the non-motor features of HD may lead to the identification of novel targets for early therapeutic interventions in HD.

The exact role of mutant huntingtin (mHTT) in HD pathology is still not fully understood, although the prevailing idea is that mHTT exerts its toxic function through both mHTT gain-of-function and wild-type (WT) HTT loss-of-function mechanisms. A major hallmark of HD pathology is the formation of intracellular mHTT inclusions, but whether these inclusions are cytotoxic per se is not clear.^{5–7} However, the presence of mHTT will ultimately result in neurodegeneration, with increased vulnerability in certain brain regions including the striatum and the cerebral cortex. The effects in the striatum are used to grade the severity of pathology in post-mortem HD cases according to a scale set by Vonsattel from 0 to 4.⁸ Neuronal loss has also been observed in the hypothalamus and may explain some of the early non-motor signs and symptoms, including psychiatric symptoms, metabolic disruption and sleep disorders.^{6,9–12} Inactivation of mHTT in the hypothalamus of the well-established BACHD mouse model restored the depressive phenotype, which supports a link between neuropsychiatric symptoms and hypothalamic pathology.^{13,14} Additionally, post-mortem neuropathological analyses of hypothalamic tissue have demonstrated a significant loss of several neuronal populations in HD cases with Vonsattel Grades 2–4 when compared with control cases. The most pronounced reduction occurs in oxytocin (OXT)-expressing neurons.¹¹ Furthermore, similar OXT neuronal loss was observed in post-mortem hypothalamic tissue from an HD Vonsattel Grade 0 case that did not exhibit any motor symptoms yet displayed psychiatric symptoms with anxiety and sleep disturbances.¹⁵ These observations suggest that OXT could play a central role in the manifestation of early non-motor symptoms in HD.

The neuropeptide OXT is synthesised in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus.¹⁶ Apart from its classical function in lactation and mother–child bond attachment, OXT also plays a pivotal role in complex social behaviour, including prosocial behaviour and pair bonding^{17–19} as well as metabolism and food intake.²⁰ Furthermore, OXT is known for its role in anxiety, depression and stereotypical stress responses.^{21–23} However, the role of OXT in anxiety and stress remains controversial and is associated with both anxiolytic and anxiogenic as well as stress-

Key points

- Mutant huntingtin inclusion formation is associated with a reduction in oxytocin-immunopositive cells.
- Overexpression of mutant huntingtin in oxytocin neurons using an AAV vector does not have a major impact on the early development of the psychiatric and metabolic phenotype in mice.
- Selective deletion of mutant huntingtin in oxytocin neurons in BACHD mice does not have a major impact on the early development of the psychiatric and metabolic phenotype of HD.

relieving and stress-inducing properties.^{24,25} Although the precise role of OXT in HD is not clear, interesting findings with the administration of OXT have been reported in both HD animal models and clinical HD. Our previous study reported that an acute supraphysiological dose of intranasal OXT prior to behavioural analyses improved the depressive phenotype in BACHD mice.²⁶ Moreover, in clinical HD, administering intranasal OXT restored the ability for facial emotional recognition in HD.²⁷ Together, both studies support the possibility that OXT can be used as a potential treatment for emotional and social cognitive deficits in HD. However, the potential causal link between the expression of mHTT in OXT neurons and the development of non-motor features of HD remains to be established.

Due to the modulatory role of OXT in social behaviour, metabolism, depression and anxiety as well as the observed neuronal loss and atrophy of OXT neurons in HD patients, we hypothesise that OXT signalling is impaired in HD. This potential impairment in turn could lead to the development of non-motor symptoms observed in HD. Therefore, this study aimed to examine whether the expression of mHTT in OXT neurons leads to the development of the neuropsychiatric and metabolic phenotype in HD and neuropathology. To investigate this, we conducted two experiments. In the first experiment, we selectively induced mHTT expression only in OXT neurons using a novel flex-switch recombinant adeno-associated viral vector approach. In the second experiment, we selectively deleted mHTT expression in OXT neurons using the BACHD mouse model. We assessed the behavioural phenotype with relevant psychiatric tests (including for anxiety, depression and altered sociability) and body weight measurements followed by post-mortem analyses using biochemical and immunohistochemical methods.

MATERIALS AND METHODS

The complete experimental procedures can be found in Appendix S1.

Animals

All animals had ad libitum access to chow diet and water and were kept in a 12-h light/dark cycle. The mice were housed in cages of four to six animals. The experimental procedures performed in animals were approved by the Regional Ethical Committee in Lund, Sweden (permit numbers: M65-13 and 15499/2017).

Experiment I: Selective induction of mHTT expression in OXT neurons

To induce selective mHTT expression only in OXT neurons, flex-switch recombinant adeno-associated viral (rAAV) vectors were bilaterally injected into the PVN of mice expressing Cre-recombinase (Cre) under the OXT promoter hereafter referred to as OXT-Cre mice (B6;129S-Oxt^{tm1.1(cre)Dolsn}/J, Jackson Laboratories). OXT-Cre mice were originally obtained as a mixed C57BL/6J $\times$ 129S background strain but maintained at the Jackson Laboratory on the C57BL/6J background strain. We designed three different flex-switch rAAV vectors that resulted in transgene expression of green fluorescent protein (GFP) (mCherry/GFP), a truncated N-terminal fragment of 166 amino acids (N166) of human WT HTT with 18Q repeats (N166-18Q) and a truncated N-terminal fragment of 248 amino acids (N248) with 100Q repeats (N248-100Q). The mice were bilaterally injected at 2 months of age (6–8 weeks) and were subjected to behavioural analyses and body weight measurements at 3 months post-injection, and sacrifice was conducted at 4 months post-injection.

Experiment II: Selective deletion of mHTT expression in OXT neurons

A mutant line that ubiquitously expresses mHTT except in OXT neurons was generated by crossing BACHD on the C57BL/6J background mice with OXT-Cre mice (Jackson Laboratories). The BACHD mouse model carries the full-length mHTT gene with 97Q and develops the psychiatric, motor and metabolic disturbances of HD.^{13,14,28} When Cre is present (in OXT neurons) in loxP-flanked BACHD mice, a fragment of exon 1 of the mHTT gene is deleted. Mating of male BACHD mice and female OXT-Cre mice generated the mutant lines, BACHD/OXT-Cre and BACHD, as well as littermate controls, WT and OXT-Cre mice. Mice were subjected to behavioural analyses and body weight measurements at 2 months of age and sacrificed at 3 months of age. To validate the specificity of Cre recombinase under the OXT promoter, we crossed OXT-Cre mice with the B6.129X1-Gt (ROSA) 26-Sortm1(EYFP)Cos/J (ROSA-EYFP) reporter mice (Jackson Laboratories). The ROSA-EYFP reporter line expresses a loxP-flanked stop-codon inserted before the enhanced yellow fluorescent protein (EYFP) gene which allows for the expression of EYFP only in presence of Cre.

rAAV vector production

The flex-switch rAAV2/5 vectors were produced using a method of double transfection as described previously.²⁹ The transgene of interest was flanked by inverted terminal repeats from AAV2 and packaged in a capsid from AAV5. Three flex-switch rAAV vectors were designed expressing GFP (mCherry/GFP, $1.0\text{--}1.9 \times 10^{14}$ GC/mL) or 166 and 248 amino acids of the N-terminal fragments of the human HTT gene for N166-18Q ($1.9\text{--}2.2 \times 10^{14}$ GC/mL) and N248-100Q ($1.8\text{--}2.3 \times 10^{14}$ GC/mL). The transgene sequences were regulated under the human synapsin-1 promoter. A polyadenylation tail was integrated to increase the mRNA half-life by protecting the transcript against endogenous endonuclease activity. The HTT transgenes were coupled with a GFP reporter gene and separated with a viral 2A sequence (58 amino acids long) that mediates cleavage between peptides during translation.

Stereotactic injection of rAAV vectors

Mice were anaesthetised with a mixture of 2% isoflurane and N₂O. rAAV vectors were bilaterally injected into OXT-Cre mice at 2 months of age using the following coordinates with bregma as the origin to target the PVN: 0.6 mm posterior to the bregma, ± 0.45 mm lateral to the bregma and 4.9 mm ventral to the dura. The bilateral injections were performed using a 5- μ L Hamilton (Nevada, USA) syringe attached to a pulled glass capillary with an outer diameter of 80 μ m. A total of 0.5- μ L viral vector solution was injected at the site of coordinates at a rate of 0.05 μ L/15 s. The needle was left in place for an additional 5 min in situ after infusion of the viral vector before being slowly retracted.

Behavioural procedures

The mice underwent a battery of behavioural tests to assess social behaviour as well as anxiety- and depressive-like behaviour at 3 months post-injection in Experiment I and at 2 months of age in Experiment II. The experiments were performed during the light phase of the circadian rhythm.

The social interaction test (SIT) was applied to assess interest in social affiliation (desire to interact with a stranger mouse) and social novelty (desire to interact with a previously unencountered mouse rather than a familiar mouse) as previously described.^{26,30} The test chamber was composed of a clear acrylic box (measuring 68 $\times$ 41 $\times$ 22.5 cm), divided into three chambers with doorway openings to allow for free passage of the mouse. The side chambers each contained a cup (25-cm high $\times$ 19-cm diameter) to enclose stranger/novel mice. The experiment consisted of two testing phases, the first phase tested for social affiliation and the second for interest in social novelty. Before the testing phase, each mouse was allowed to habituate to the testing chamber for 5 min.

In the first phase (social affiliation), the experimental mouse is allowed to freely interact for 10 min, either with an empty cup or a cup containing another mouse of the same sex. In the second phase (social novelty), the experimental mouse was allowed to freely interact either with a cup containing the same familiar mouse that it had interacted with before in the first session or another cup containing a novel mouse (no previous interaction) for 10 min. The number of interactions (defined as the mouse touching the cup with its nose and/or hind paws) with stranger mouse versus empty cup in the first phase, and familiar versus novel mouse in the second phase, was used to calculate the preference of the experimental mouse.

To assess anxiety-like behaviour, the elevated plus maze (EPM) was conducted as previously described by Pellow.^{13,26,31} The maze was elevated 55 cm above its base and consisted of two closed arms and two opposing open arms (each arm measuring 35 × 7.5 cm) with a centre platform connecting the arms (7.5 × 7.5 cm). The walls of the closed arms had a height of 30 cm. The mouse was placed in the centre platform and recorded for 10 min, and the percentage of time spent in the open versus closed arms was recorded during this time interval. Analysis was performed using the Ethovision XT 13 Software system.

Porsolt's forced swim test (FST) was used to assess depressive-like behaviour.^{32,33} The mice were recorded for 6 min; the first 2 min of the recording was regarded as the acclimatisation period, and the total time spent immobile in the last 4 min of the recording was manually scored by an experimenter blinded to the experimental groupings. The scoring is based on immobility, which is a sign of depressive-like behaviour. The criteria for immobility were that no swimming movements can be made and only movements necessary to keep the head above the water were included.

Open field test (OF) is a test that was used to measure the general activity level of mice. The test chamber consisted of an activity box (measuring 40 × 40 × 40.5 cm). The mouse was placed in the centre of an activity box, and the total distance travelled during the 1-h time frame was tracked and recorded using the Ethovision XT 13 Software system.

Fluorescence immunohistochemistry and confocal analyses

Brain tissue was immunohistochemically processed to detect OXT or both OXT and GFP. Free-floating brain sections were incubated with 5% normal donkey serum followed by incubation with a primary antibody for OXT (1:2000, H-051-01, anti-rabbit, Phoenix Pharmaceuticals) and/or GFP (1:10,000, ab13970, anti-chicken, Abcam). The sections were labelled with the respective fluorescent secondary antibodies Alexa 647-conjugated (1:200, anti-rabbit, Jackson Laboratories) and/or the appropriate biotinylated antibody followed by streptavidin-conjugated Alexa 488 (1:200, Jackson Laboratories). Image acquisition and analysis for fluorescence detection were performed with the Nikon Ti-Eclipse confocal microscope equipped with

a Nikon A1RHD laser scanning unit (Nikon Corporation, Japan) comprising 488-nM Sapphire, 543-nM Sapphire and 639-nM cube diode lasers (Coherent Inc, USA). Plan Apochromat 10× N.A. 0.45 and 20× N.A. 0.75 objectives were used to collect Z-stack images (2-μm step) with a 1.2-μm pinhole aperture.

Peroxidase-based immunohistochemistry

Mice brains were cut in the coronal plane into 30-μm sections using a freezing microtome (Microm HM450) and collected in six series. Free-floating sections were processed for immunohistochemistry using primary antibodies: chicken antisera against GFP (1:20,000, ab13970, Abcam), rabbit antisera against OXT (1:4000, H-051-01, Phoenix Pharmaceuticals, Inc.) or goat antisera against HTT (1:500, sc-8767, Santa Cruz, Biotechnology, Inc.). Sections were labelled with an appropriate biotinylated secondary antibody (1:200, anti-rabbit, anti-goat or anti-chicken IgG in Tris-buffered saline containing 1% bovine serum albumin and triton X-100, Vector Laboratories, Inc.). After incubation with the Vectastain Elite avidin-biotin kit, peroxidase labelling was visualised with 3,3'-diaminobenzidine as chromogen using hydrogen peroxidase. Finally, the sections were mounted on chromatin-gelatin-coated glass slides and coverslipped with DPX (Sigma Aldrich) after a series of dehydration steps in ascending concentrations of ethanol and xylene. Ten cases from the N248-100Q HTT-stained sections were also counterstained in haematoxylin for 5 min (Mayer's HTX, 01820, Histolab Products AB) to verify whether the HTT inclusions were expressed in the nuclei.

Quantification of OXT immunopositive cells

To estimate the number of OXT-immunopositive cells, an unbiased quantification method was implemented whereby counting was performed by an observer that was blinded to the sex and treatment groups. The counting was performed with a 60X Plan-Apochromat (1.4 N.A.) oil objective on a Nikon Eclipse 80i microscope, equipped with an X-Y motorised stage (Märzhauser, Wetzlar, Germany). All OXT-immunopositive cells of the PVN were counted unilaterally, and the final cell count was estimated using the equation: $N = \sum Q \times s \times 2$, where Q is the number of counted cells, s is the number of the series and 2 is the correction for both hemispheres.

DNA and RNA sampling and extraction

To prepare the brain tissue for post-mortem analyses of mice from Experiment I, the mice were anaesthetised at 3–4 months of age with a terminal dose of sodium pentobarbital (~150 mg/kg i.p.). The mice were decapitated, and the brain was removed and embedded in Tissue-Tek® O.C.T compound. Three hundred-micrometre-thick sections were cut in the cryostat (Microm HM 560). Micropunches from

the PVN and SON were obtained using a 1-mm biopsy punch from which genomic DNA and RNA were extracted. DNA extraction was performed using the DNeasy Blood and Tissue kit (Qiagen), and RNA extraction was performed with the RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions and their respective concentrations determined with the Nanodrop 2000 (ThermoFisher Scientific, CA, USA). All primers are designed with Beacon Designer (Version 8.02, Premier Biosoft, USA).

Polymerase chain reaction

The primers were designed to detect exon 1 of the mHTT gene, HTT forward primer (5' to 3') ATTCATTGCCCGGTGCTGA and HTT reverse primer (5' to 3') AGCCCTCTTCCTCTCAGACTAGAAGAGG. The expected polymerase chain reaction (PCR) products were 1050 bp for the non-excised HTT fragment and 600 bp for the Cre excised HTT fragment. PCR was performed to validate the excision of mHTT in the PVN and SON of the BACHD/OXT-Cre mutant line. Genomic DNA was amplified using KAPA2G Fast genotyping mix (Kapa Biosystems) according to the manufacturer's protocol. Sixty-eight nanograms of DNA sample was added to each well. SureCycler 8800 (Agilent Technologies) PCR machine was used for the following PCR amplification for 40 cycles: 95.0°C for 3 min, denaturation at 95.0°C for 15 s, annealing at 63°C for 15 s and elongation at 72.0°C for 30 s. Twenty microlitres of PCR product was loaded onto a 1% agarose gel with SYBR[®] Safe DNA gel stain (Invitrogen) for 35 min at 100 V and visualised with the ChemiDoc MP imaging system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Quantitative real-time PCR

Random hexamer primers and SuperScript IV Reverse Transcriptase (Invitrogen) were used to generate cDNA according to the manufacturer's instructions. Analysis of mRNA HTT expression was carried out by quantitative real-time PCR (qRT-PCR). The quantitative PCR was performed with a LightCycler 480 (Roche) in a two-step cycling protocol using SYBR Green I Master mix (Roche, Switzerland) after assessing the specificity of the amplified product by analysing the melting curves. The data were quantified using the $\Delta\Delta CT$ method and normalised to the expression of three house-keeping genes: beta-actin, hypoxanthine-guanine phosphoribosyl transferase (HPRT) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. All fold changes were presented as a ratio to the mean of the control uninjected group. qRT-PCR was performed in uninjected mice ($n = 2$), as well as in mice injected with N166-18Q ($n = 3$) and N248-100Q ($n = 3$). All primers are designed with Beacon Designer (Version 8.02, Premier Biosoft, USA). The sequences of the primers are as follows (5' to 3'): HTT forward GCACCGACCAAGAAAGA, HTT reverse ACCATCTGACATCTGAC, beta-actin forward GCTGTGCTAT GTTGCTCTA, beta-actin reverse TCGTTGCCAATAGTGATGA, GAPDH forward AACCTGCCAAGTATGATGA, GAPDH reverse

GGAGTTGCTGTTGAAGTC, HPRT forward ACCTCTATGAATGT-TACTG and HPRT reverse GATAAGCGACAATCTACC.

Statistical analyses

Statistical analyses were performed using SPSS 18 statistical package (SPSS inc. Chicago IL). All data were first assessed for normal distribution. If the data were not normally distributed, log10 or square root transformation was performed to obtain a normal distribution to allow for the use of parametric tests. Data were assessed by either two-way analyses of variance (ANOVA) followed by post hoc analyses or one-way ANOVA followed by Tukey's multiple-comparison test to analyse the statistical differences between groups. Significant statistical difference was accepted at $p < 0.05$, and the data are expressed as a mean $\pm$ standard deviation (SD). A full report of the statistical analyses is presented in Appendix S2.

RESULTS

Development of mHTT inclusions in OXT neurons of OXT-Cre mice

The debilitating psychiatric and metabolic features seen in early HD may be caused by pathology of the OXT system. Hence, in the first experiment, we wanted to investigate whether selective mHTT expression in OXT neurons of healthy OXT-Cre mice could result in the development of non-motor features of HD. To selectively express mHTT in OXT neurons, OXT-Cre mice were bilaterally injected with a flex-switch N248-100Q viral vector into the PVN. We had two control groups of mice in this present study, one group was injected with the N166-18Q vector (to express WT HTT) and the other rAAV-mCherry/GFP, to verify the functionality of the vector construct and the site of injection, respectively (Figure 1A).

To validate the efficacy of the flex-switch rAAV viral vectors, confocal microscopy images of the PVN were taken in mice injected with the rAAV-mCherry/GFP vector. We confirmed that GFP transgene expression is co-localised with OXT neurons, where Cre was expressed under the OXT promoter. In contrast, mCherry transgene expression was observed in transduced cells not expressing Cre recombinase (Figure 1B). Furthermore, qRT-PCR analysis demonstrated a substantial increase of HTT mRNA expression in mice injected with N248-100Q and N166-18Q compared with uninjected mice (Figure 1C).

Next, immunohistochemical processing of brain tissue with antibodies against HTT and GFP confirmed transgene expression exclusively in the PVN at 4 months post-injection (Figure 2A). Interestingly, in mice injected with the N248-100Q vector, we observed two distinct subsets. Both subsets formed mHTT inclusions; however, one subset formed larger, predominantly intranuclear inclusions and the other formed smaller, more clustered and predominantly extranuclear inclusions. The two subsets were analysed separately as two distinct

(A) Experimental design I

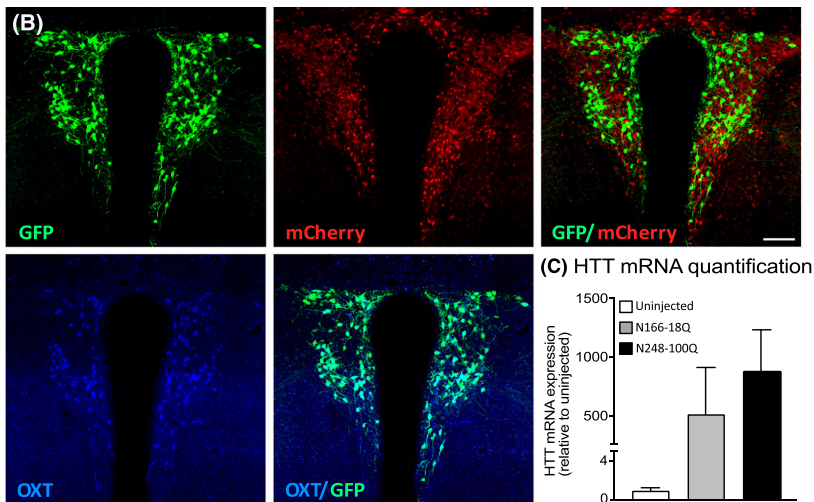
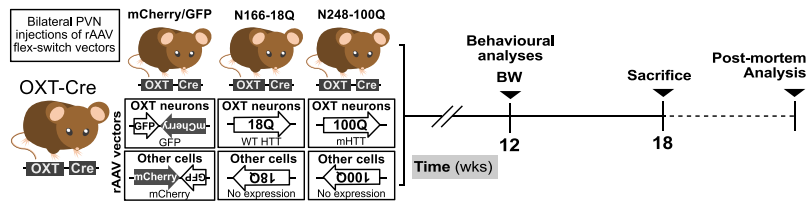


FIGURE 1 Validation of the efficacy of viral vectors in OX2-Cre mice. (A) Schematic representation of Experiment I. (B) Confocal microscopy images of the PVN in OX2-Cre mice with bilateral injections of the flex-switch rAAV-mCherry/GFP vector. Neurons in which Cre is present express the GFP transgene (green), whereas other transduced non-Cre positive cells express mCherry (red). Immunopositive OX2 neurons (blue) are co-localised with GFP, and there is no overlap between GFP and mCherry. (C) mRNA levels of HTT in the PVN of OX2-Cre mice injected with rAAV-HTT-18Q ($n = 3$) and rAAV-HTT-100Q ($n = 3$) relative to uninjected OX2-Cre mice ($n = 2$).

experimental groups throughout the study. Counterstaining with haematoxylin (which stains nuclei) confirmed that the intranuclear inclusions are found within nuclei whereas the clusters of extranuclear inclusions are predominantly found outside the nuclei (Figure 2B).

Reduced numbers of OX2-immunopositive cells in mice that formed mHTT inclusions

Loss of OX2 neurons has previously been well documented in clinical HD.^{11,15} We, therefore, estimated the total number of OX2-immunopositive cells in the PVN (Figure 2C). One-way ANOVA followed by a Tukey's test revealed a significant reduction in the number of OX2-immunopositive numbers in mice that formed intranuclear mHTT inclusions compared with all groups including the mice with extranuclear mHTT inclusions (uninjected

$p < 0.001$, mCherry/GFP $p < 0.001$, N166-18Q $p < 0.01$ and N248-100Q with extranuclear inclusions $p < 0.001$; Figure 2C). The numbers of OX2-immunopositive neurons were 26% lower in mice that formed intranuclear inclusions compared with uninjected mice and 25% lower compared with mice that formed extranuclear inclusions. We observed no loss of OX2-immunopositive cells in mice that formed extranuclear inclusions, indicating that may be a causal link between intranuclear mHTT inclusion formation and OX2 pathology.

Sex-specific body weight gain in mice that formed intranuclear mHTT inclusions compared with uninjected mice

Increased body weight has previously been reported after selective mHTT expression in the hypothalamus of mice.²⁸ We wanted to

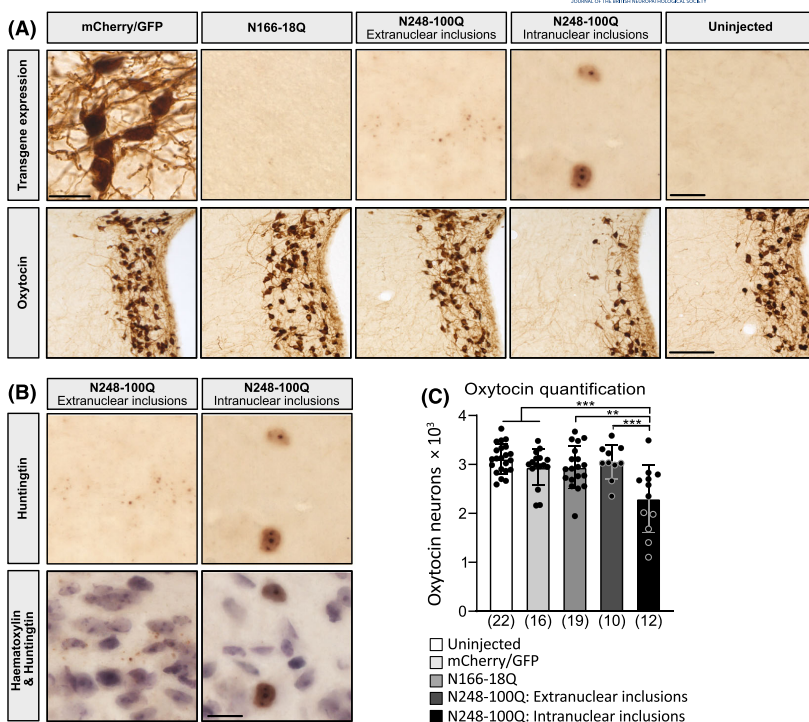


FIGURE 2 Reduced numbers of OXT-immunopositive cells in mice that formed intranuclear mutant huntingtin inclusions. (A) OXT-, GFP- and huntingtin-immunopositive neurons in OXT-Cre-recombinase mice 16 weeks post bilateral injection of rAAV vectors expressing mCherry/GFP, N166-18Q and N248-100Q. (B) Mice that formed intranuclear and extranuclear inclusions were identified as distinct subsets of mice after injection of the N248-100Q vector using haematoxylin which stains nuclei. Images showing huntingtin-immunopositive neurons were taken before haematoxylin counterstaining and are from the same section. (C) Estimated number of neurons expressing OXT of the paraventricular nucleus. The data are expressed as a mean $\pm$ standard deviation. ** = $p < 0.01$ and *** = $p < 0.01$, one-way analysis of variance followed by Tukey's multiple-comparison test. Scale bar in (A): 100, 10 and 100 μ m, respectively, and (B): 10 μ m. The number of animals in each group is indicated in parentheses.

investigate if selectively inducing mHTT expression in OXT neurons of the PVN would be sufficient to affect body weight in a similar manner. Our results showed a significant sex-dependent effect ($p < 0.001$) and an interaction between sex and treatment ($p < 0.001$). In females, mice that formed intranuclear mHTT inclusions developed an increased body weight at 12 weeks post-injection of N248-100Q compared with mice that formed extranuclear inclusions ($p < 0.05$; Figure 3A). Interestingly, male mice injected with the mCherry/GFP vector developed an increased body weight compared with mice from all other groups ($p < 0.05$; Figure 3B). When expressed as percentage body weight gain from 2 to 12 weeks post-injection, we found an interaction effect between sex and treatment ($p < 0.01$). Female mice injected with N248-100Q AAV vector that formed intranuclear inclusions had a significantly higher body weight gain compared with uninjected ($p < 0.05$) and mCherry/GFP injected mice ($p < 0.01$)

(Figure 3C) and no significant differences between the treatment groups in males (Figure 3D).

Normal general locomotor activity in mice with selective mHTT expression in OXT neurons

Motor disturbances in HD are associated with pathology of the basal ganglia and striatum.² As the PVN is not a brain region involved in motor function, we wanted to exclude the possibility that mHTT in OXT neurons would contribute to motor manifestation, and as such, general locomotor activity was evaluated. In addition, we also wanted to verify that the mice were spared from any motoric adverse effects due to the stereotactic surgery. Total locomotor activity was assessed with OF test and revealed that all mice

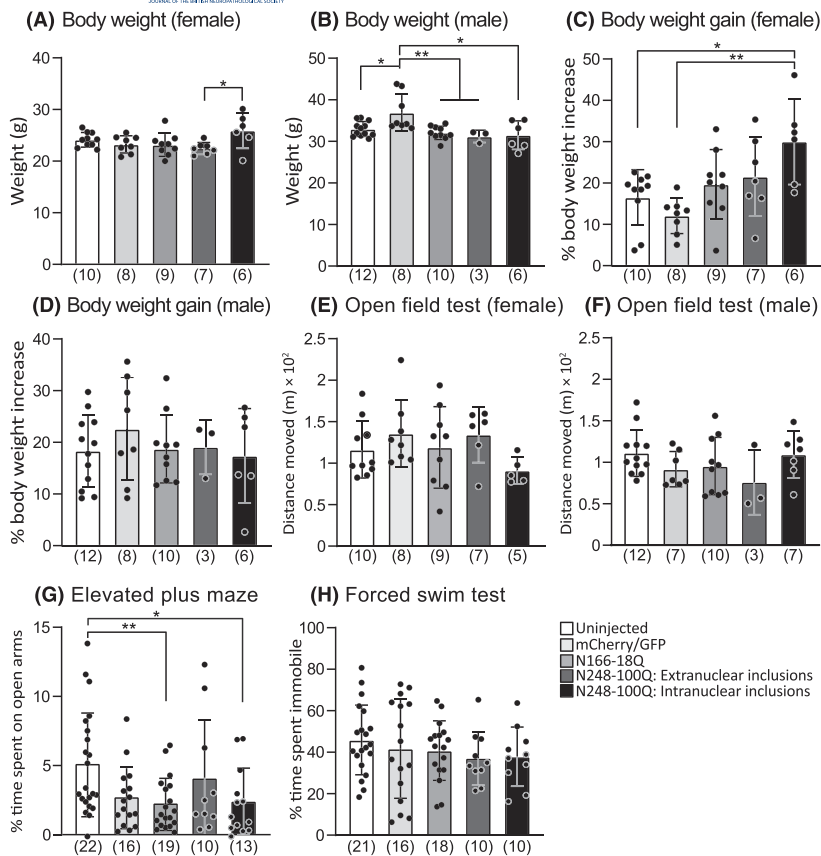


FIGURE 3 Behavioural effects after injections of a flex-switch vector expressing mutant huntingtin in oxytocin-Cre-recombinase mice. Increased body weight was observed in female mice (A) that formed intranuclear inclusions compared with mice that formed extranuclear inclusions 12 weeks after bilateral injections of N248-100Q. (B) Male mice injected with mCherry/GFP rAAV vector developed increased body weight compared with other groups. (C) Percentage body weight gain between 2 and 12 weeks post-injection was significantly larger in female mice that formed intranuclear inclusions compared with uninjected and mCherry/GFP expressing mice. (D) No change in the percentage body weight gain between treatment groups in male mice. (E, F) No difference in activity level between groups, regardless of sex and treatment. (G) Mice that formed intranuclear inclusions as well as N166-18Q mice spent less percentage time in the open arms compared with uninjected mice. (H) We observed no differences in depressive-like behaviour between the groups. The data are expressed as a mean $\pm$ standard deviation. * = $p < 0.05$ and ** = $p < 0.01$, two-way analyses of variance for the influence of sex and treatment, with a subsequent one-way analysis of variance if the effect of sex or treatment was established. The number of animals in each group is indicated in parentheses.

travelled a similar distance, regardless of treatment in females (Figure 3E) and males (Figure 3F).

Increased anxiety-like behaviour in mice that formed intranuclear inclusions compared with uninjected mice

Next, we wanted to investigate if OXT's role in anxiety and depression was disrupted by the expression of mHTT in OXT neurons. We utilised EPM and Porsolt's FST to assess anxiety- and depressive-like

behaviours, respectively. For the EPM, a two-way ANOVA revealed no effect of sex, and thus, the data were collapsed across sex. One-way ANOVA followed by a Tukey's multiple-comparison test revealed that mice in the N248-100Q intranuclear inclusions group spent less percentage of time on open arms compared with uninjected mice ($p < 0.05$; Figure 3G). This effect was not found in the N248-100Q extranuclear inclusions group. Furthermore, mice injected with the N166-18Q vector spent less percentage of time in open arms compared with uninjected mice ($p < 0.01$), but there were no differences between mice injected with the N166-18Q and N248-100Q vectors

or between the N248-100Q mice, which formed intranuclear or extranuclear inclusions. This suggests that mHTT expression in OXT neurons by itself or inclusion formation may not be sufficient to increase anxiety-like behaviour.

In the terms of depressive-like behaviours assessed with the FST, no significant differences were detected between the different groups regardless of sex and treatment, indicating that mHTT expression in OXT neurons does not contribute to the development of HD depressive-like behaviours (Figure 3H).

Effect of selective mHTT expression in OXT neurons on social behaviour

Due to the important role of OXT in social behaviour and cognition, we next investigated the effect of selective mHTT expression in OXT neurons at 3 months post-injection on general sociability and social novelty with the SIT. When assessing social affiliation, we did not observe any changes across treatment groups. Although there is an increased preference for the stranger mouse cup over the empty cup in all mice, there were no significant differences between any of the groups, with no effect of sex and treatment (Figure 4A). Assessment of social novelty indicated that all mice had a preference for the novel mouse cup over the familiar mouse cup except for mice that formed extranuclear HTT inclusions, which preferred the familiar mouse cup. The preference to interact with the novel mouse cup was significantly lower in mice that formed extranuclear inclusions compared with mCherry/GFP-injected mice (Figure 4B; $p < 0.05$). These results indicate that although mHTT expression in OXT neurons does not affect general social behaviour, the formation of extranuclear inclusions may have contributed to the decreased interest to explore and interact with the novel mouse.

Selectively deleting mHTT expression in OXT neurons does not prevent the development of increased body weight in BACHD/OXT-Cre mice

In the next part of the study, we performed a mirror experiment in a well-established HD mouse model to determine if deleting mHTT expression in OXT neurons is sufficient to rescue non-motor features of HD. We crossed OXT-Cre mice with loxP-flanked BACHD mice to generate offspring with excised exon 1 fragment of the HTT gene exclusively in OXT neurons (Figure 5A). First, we validated Cre expression in OXT neurons, by crossing the OXT-Cre mice with the ROSA-EYFP reporter line. Immunohistochemical staining of the generated ROSA-EYFP/OXT-Cre mice revealed selective staining of the PVN and SON, indicating that Cre expression is specific to OXT neurons (Figure 5B). Next, we performed PCR analysis of dissected brain tissue of the PVN and SON and verified excision of the HTT exon 1 fragment only in BACHD/OXT-Cre mutant line (Figure 5C).

It has previously been established that BACHD mice develop a higher body weight compared with WT littermates¹⁴ and that deletion

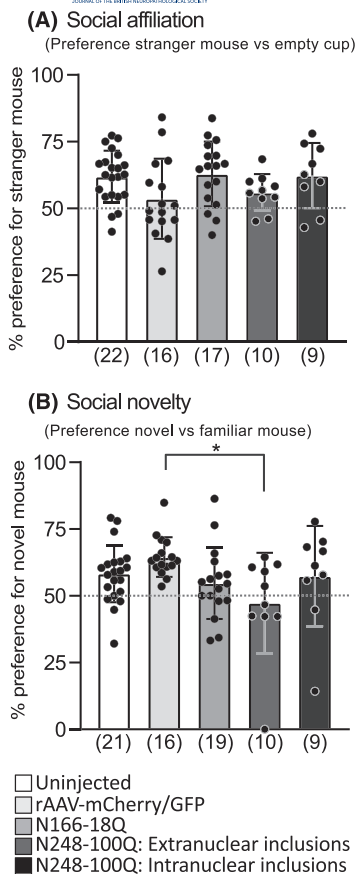
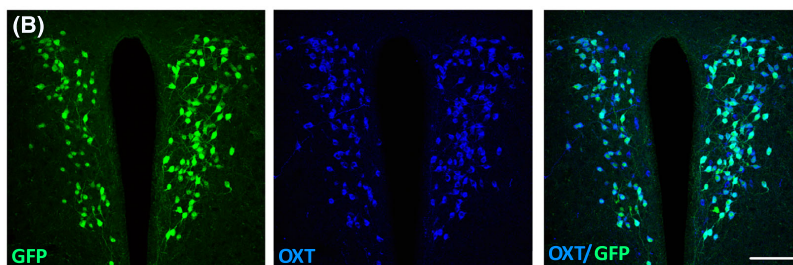
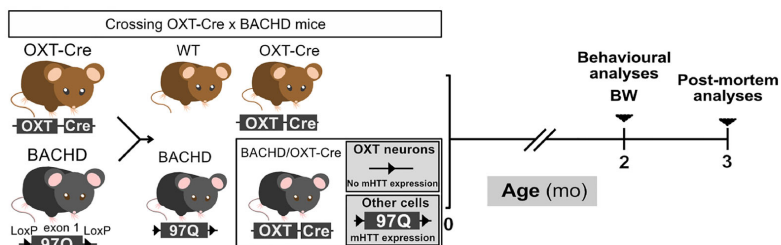


FIGURE 4 Effect of selective mutant huntingtin expression in OXT neuron on social behaviour. (A) Assessment of social affiliation, the percentage preference to interact with the cup enclosed with a stranger mouse over an empty cup. No effect in social affiliation was observed in OXT-Cre-recombinase mice bilaterally injected with rAAV-HTT-100Q. (B) Assessment of social novelty, the percentage preference to interact with the cup enclosed with a novel mouse over the previously encountered mouse cup. No effect on social novelty in OXT-Cre-recombinase mice injected with rAAV-HTT-100Q is detected. The data are expressed as a mean $\pm$ standard deviation. * = $p < 0.05$, two-way analyses of variance for the influence of sex and treatment, with a subsequent one-way analysis of variance if the effect of sex or treatment was established. The number of animals in each group is indicated in parentheses.

of mHTT expression in the hypothalamus of BACHD mice prevents the development of obesity.²⁸ Here, we investigated whether the deletion of mHTT expression exclusively in OXT neurons would be sufficient to alter the metabolic phenotype of the BACHD mice. As we detected a sex-dependent effect ($p < 0.001$), the body weight data are presented separately according to sex. Body weight was

(A) Experimental design II



(C) Cre excision PCR

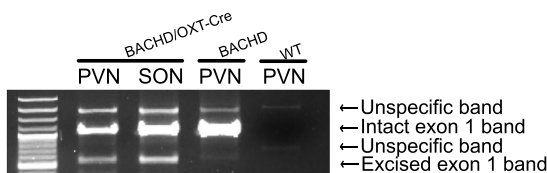


FIGURE 5 Validation of mutant HTT deletion from BACHD/OXT-Cre mice. (A) Schematic representation of Experiment II and the generation of the BACHD/OXT-Cre mutant mouse line. (B) Confocal microscopy images of the PVN in BACHD/OXT-Cre mice that were crossed with the B6.129X1-Gt(ROSA)26-185 Sortm1(EYFP)Cos/J reporter line. Sections were immunohistologically stained with GFP (green) and detected in cells in which Cre is present. Immunopositive OXT neurons (blue) are co-localised with GFP (teal). (C) Validation of Cre excision. Gel electrophoresis of PCR products shows an intact HTT exon 1 band around 1050 bp in BACHD and BACHD/OXT-Cre mice and an excised HTT exon 1 band around 600 bp detected only in BACHD/OXT-Cre mice. No HTT band is detected in WT mice. Scale bar in (B): 200 μ m.

significantly higher in both BACHD and BACHD/OXT-Cre mice compared with WT littermates ($p < 0.001$ in females and $p < 0.05$ in males; Figure 6A,B). Despite this, the increase in body weight observed in BACHD mice was not prevented by deleting mHTT expression in OXT neurons. In female mice, however, there appears to be a trend of increased body weight in BACHD/OXT-Cre mice compared with BACHD mice ($p = 0.063$; Figure 6A).

Effect of selective mHTT expression in OXT neurons on general locomotor activity in BACHD/OXT-Cre mice

General activity levels were assessed with OF test and revealed that all mice travelled a similar distance, regardless of sex and genotype (Figure 6C).

No effect on anxiety-like or depressive-like behaviour in BACHD/OXT-Cre mice with selective deletion of mHTT expression in OXT neurons

We further investigated whether there is a direct link between mHTT expression in OXT neurons and the development of neuropsychiatric behaviour by selective deletion of mHTT expression in OXT neurons of BACHD/OXT-Cre mice. Assessment of anxiety-like behaviour was performed at 2 months of age using the EPM. Two-way ANOVA revealed no effect of sex, and the data were collapsed across sex. One-way ANOVA followed by a Tukey's multiple comparison test revealed a reduction in the percentage of time spent on open arms in BACHD/OXT-Cre mice compared with WT mice ($p < 0.01$; Figure 6D), whereas there was a trend for BACHD mice to spend less time on open arms ($p = 0.077$). However, as there were no differences between BACHD and BACHD/OXT-Cre mice, this suggests

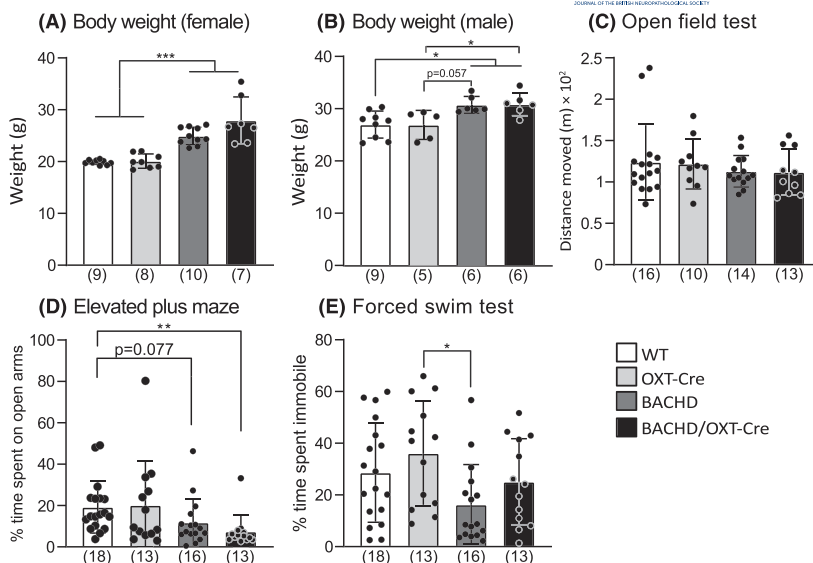


FIGURE 6 Deletion of mutant huntingtin expression in OXT neurons does not prevent the development of increased body weight. (A) BACHD and BACHD/OXT-Cre mice displayed significantly increased body weight, in both females (A) and males (B), compared with WT littermates at 2 months of age, but this effect was not restored by deleting mutant huntingtin expression. (C) No sex- nor genotype-dependent effect on general activity level was detected with the open field test. (D) BACHD/OXT-Cre mice displayed an increase in anxiety-like behaviour with the elevated plus maze. (E) No significant differences in the percentage of time spent immobile with the forced swim test between BACHD and BACHD/OXT-Cre mice. The data are presented as mean $\pm$ standard deviation. *** = $p < 0.001$, ** = $p < 0.001$ and * = $p < 0.05$ two-way analyses of variance for the influence of sex and genotype, with a subsequent one-way analysis of variance if the effect of sex or genotype was established. The number of animals in each group is indicated in parentheses.

that the deletion of mHTT expression in OXT neurons does not contribute to the development of anxiety-like behaviour.

In terms of depressive-like behaviours, the depressive-like phenotype of BACHD mice was not present in this transgenic crossing. An unexpected observation was that BACHD mice showed a significant increase in mobility compared with the OXT-Cre control group (Figure 6E). No differences were observed between BACHD and BACHD/OXT-Cre mice.

BACHD/OXT-Cre mice with selective deletion of mHTT expression in OXT neurons display normal social behaviour

The assessment of social behaviour using SIT revealed no significant differences in social affiliation (Figure 7A) or social novelty (Figure 7B) between the different groups, regardless of sex and treatment.

DISCUSSION

Understanding the effect of mHTT expression in the OXT system on the development of non-motor features and OXT pathology in HD

may be important for unravelling novel targets for early therapeutic interventions. OXT neurons are affected in early HD.^{11,15} Given the function of OXT in social, metabolic and emotional control, any disruption in its function may contribute to the non-motor features seen in HD.^{20,21,23} The present study aimed to investigate whether mHTT expression in OXT neurons contributes to the development of non-motor features and neuropathology. We found that intranuclear mHTT inclusions, formed as a result of overexpression of mHTT selectively in OXT neurons, were associated with reduced numbers of OXT-immunopositive cells. This suggests that mHTT exerts cell-autonomous effects in this population of selectively sensitive neurons in HD. However, we did not detect any significant effects of selective mHTT expression or deletion in OXT neurons on non-motor features examined at relatively early time points in mice in this study.

We have previously reported a 45% loss of OXT neurons in the PVN of post-mortem Grades 2–4 HD cases as well as in a Grade 0 case.^{11,15} In this study, we found that mice that formed intranuclear inclusions in OXT neurons after bilateral rAAV-HTT-100Q injections into the PVN of OXT-Cre mice displayed a significant 22–26% reduction in OXT-immunopositive neurons compared with all groups including the mice that formed extranuclear inclusions. As the formation of intranuclear inclusions is a major hallmark of HD,⁵ there might be a direct link between intranuclear inclusion formation and loss of

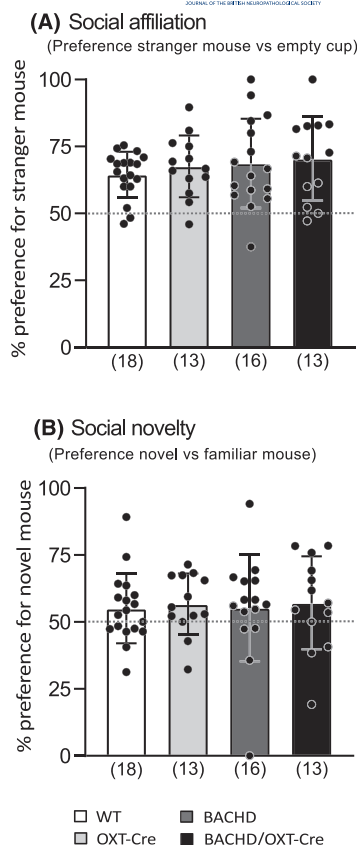


FIGURE 7 Deletion of mutant huntingtin expression in OXT neurons of BACHD/OXT-Cre mice does not affect social behaviour. (A) Assessment of social affiliation, the percentage preference to interact with cup enclosed with stranger mouse over an empty cup. No effect in social affiliation was observed between the different genotypes. Mice in all groups show an increased preference for the stranger mouse over the empty cup. (B) Assessment of social novelty, the percentage preference to interact with the cup enclosed with a novel mouse over the cup enclosing the previously encountered mouse (familiar mouse). No differences between the different genotypes were detected. The data are expressed as a mean $\pm$ standard deviation, two-way analyses of variance for the influence of sex and genotype. The number of animals in each group is indicated in parentheses.

OXT neurons. The observed reduction in the numbers of OXT-immunopositive cells may be caused by the loss of OXT neurons or by reduced neuronal OXT expression due to the presence of intranuclear mHTT inclusions. The question remains as to whether these intranuclear inclusions are formed as a protective mechanism against mHTT toxicity or are themselves cytotoxic and cause cell death. As there was no loss of OXT neurons in mice injected with N248-100Q that

instead formed extranuclear inclusions, this suggests that intranuclear mHTT inclusions may be associated with toxic effects of OXT neurons by other unknown mechanisms. The absence of intranuclear inclusions reported in BACHD mice corresponds to the lack of any evidence showing a loss of OXT-immunopositive neurons in this HD model supports this notion.^{13,14,34} However, further investigation of the OXT system after inclusion formation is required.

OXT has predominantly been described as a neuropeptide with anxiolytic properties and has been of key interest in the search for alternative treatment for anxiety disorders.^{25,35} However, the role of OXT in anxiety does not appear to be definite and has been suggested to also have anxiogenic properties, especially after a chronic state of increased OXT.^{24,25,36,37} Here, we investigated anxiety-like behaviour using EPM in N248-100Q injected mice with induced mHTT expression selectively in OXT neurons and in BACHD/OXT-Cre mice with deleted mHTT expression selectively in OXT neurons. However, we found no major effect on anxiety-like behaviour after the manipulation of mHTT expression in OXT neurons. We did find increased anxiety-like behaviour in mice that formed intranuclear inclusions post-injection of N248-100Q compared with uninjected mice, whereas mice that formed extranuclear inclusions appeared to be unaffected. Furthermore, there was no difference between the N248-100Q intranuclear and extranuclear groups, indicating that intranuclear inclusion formation alone may not contribute to the development of anxiety-like behaviour. In addition, for reasons that are unclear, we also observed increased anxiety-like behaviour in N166-18Q injected mice. This phenotype could be an unexpected adverse effect of WT HTT overexpression in mice or possibly indicate a connection between overexpression of WT HTT and the development of anxiety-like behaviour in mice. There was no difference between the BACHD and BACHD/OXT-Cre mice, suggesting that deleting mHTT expression in OXT neurons does not contribute to anxiety-like behaviour. Importantly, we also acknowledge that mice across all groups displayed higher levels of anxiety in this study. Although sound and lighting within the experimental rooms were controlled during the test, there could be other physical stressors in the housing or testing environment that could have resulted in increased anxiety levels. However, as all mice were housed and tested in a similar way, this aspect is likely not to have significantly impacted the possibility to detect differences between treatment groups.

Depression is highly prevalent in HD.⁴ A number of valid transgenic HD animal models have been developed, and several studies have demonstrated affective symptoms of HD in these models.^{13,38–40} They can therefore serve as important tools to test novel potential therapies against affective symptoms. OXT is a neuromodulator in the amygdala, hypothalamus and nucleus accumbens, regions which are linked to depression (as well as social cognition).⁴¹ However, the effect of intranasal OXT administration on the manifestation of depression in patients appears to be inconsistent and has been linked to both anti-depressive and pro-depressive properties.^{41–43} Previously, acute intranasal OXT administration improved the depressive phenotype in BACHD mice.²⁶ However, this may be due to the intranasal OXT treatments targeting more regions

of the brain than the hypothalamus. OXT receptors (OXTRs) have been found in many other brain regions of the limbic system involved in emotional control and cognition, including the amygdala and prefrontal cortex.^{44,45} Genetic variations of OXTR have recently been shown to influence the prevalence of social impairment in HD patients.⁴⁶ The effects of manipulation of mHTT expression in OXTR-expressing neurons would, therefore, be interesting to explore.

In this study, mHTT expression in OXT neurons did not appear to have any effect on depressive-like behaviour according to the FST, even considering the reduced numbers of OXT-immunopositive cells following mHTT inclusion formation. These results indicate that mHTT expression in OXT neurons is not sufficient to affect the development of the depressive phenotype. It appears that all mice display heightened depressive-like behaviour, regardless of the grouping. It is also important to note that the BACHD mice in the present study did not display increased depressive-like behaviour compared with their WT littermates. This could be due to strain differences.^{13,26,28} Most of the studies using BACHD mice have been largely on the FVB/N background where both anxiety- and depressive-like phenotypes were present as early as 2 months of age. BACHD mice on the C57BL/6J background have previously been reported to have less severe behavioural deficits in HD compared with FVB/N mice.⁴⁷ We had chosen to use BACHD mice on the C57BL/6J mice to avoid strain differences due to a mixed background. Our previous work has reported deficits in psychiatric behaviours in BACHD mice on the C57BL/6J background at 6 months of age.²⁶ Behavioural deficits might be more prominent at a later time point and further studies are warranted.

HD patients experience impairments of social behaviour and social memory.¹⁹ Decreased OXT levels in the cerebrospinal fluid of manifest HD patients have recently been linked to increased social cognitive impairment in HD patients.⁴⁸ Furthermore, OXT knock-out mice show deficits in social memory and recognition.^{49,50} Here, we tested whether mHTT expression or inactivation in OXT neurons affected social behaviour as assessed with SIT in mice. We did not find any effects on the studied parameters at 3 months post-injection or at 2 months of age. However, the manifestation of the social features of HD may be time dependent as impairments of social behaviour have previously been reported in BACHD mice at 6 months of age using this test.²⁶ Thus, an interesting further direction may be to assess these mice at a later stage in time.

Metabolic disturbances have previously been described in HD patients including increased food intake⁵¹ and are generally associated with decreased body weight.⁵² OXT has been linked to metabolism and more specifically food intake,²⁰ where increased OXT is linked to reduced food intake in model systems.⁵³ BACHD mice, contrary to HD patients, are reported to develop increased body weight.^{13,54} The effect on the development of increased body weight is linked to mHTT expression selectively in the hypothalamus caused by increased food intake.²⁸ Therefore, we investigated if weight gain following mHTT expression could be specifically linked to the OXT system but found no support for this notion in the present study.

The present study has investigated the potential effects of mHTT expression in OXT neurons in two different animal models. The results

indicated an association between intranuclear mHTT inclusions and a reduction in the number of OXT-immunopositive neurons, suggesting that the end product of mHTT expression is important in HD OXT pathology. However, selective mHTT expression or inactivation in OXT neurons alone was not sufficient to alter the metabolic and psychiatric phenotype in mice at this early time point. Further studies with different time points are warranted to understand the exact biological mechanisms that mHTT inclusions exert on the OXT system. It is clear that loss of OXT neurons is an early change in HD patients, primarily associated with the onset or presence of affective symptoms, and therefore, targeting the OXT system could provide a good therapeutic strategy for HD affective symptoms. Hence, based on the increasing evidence of the involvement of the OXT system in HD,⁵⁵ it would now be timely to assess the effects of OXT administration on both affective symptoms and altered social cognition in individuals with HD.

AUTHOR CONTRIBUTIONS

ÅP and RYC conceived and designed the experiments. SB, ST and RYC performed the experiments and analysed the data. SG analysed the data. SB, ÅP and RYC wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Björn Anzelius, Anneli Josefsson, Anna Hansen, Ulla Samuelsson and Ulrika Sparrhult-Björk at Lund University for their valuable technical assistance. BACHD mice on the C57BL/6J background were provided by Dr X. William Yang at Semel Institute and David Geffen School of Medicine at the University of California, Los Angeles.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets analysed during the current study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The experimental procedures performed in animals were approved by the Regional Ethical Committee in Lund, Sweden, under permit numbers M65-13 and 15499/2017.

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PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/nan.12891>.

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SUPPORTING INFORMATION

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How to cite this article: Bergh S, Gabery S, Tonetto S, Kirik D, Petersén Å, Cheong RY. Effects of mutant huntingtin in oxytocin neurons on non-motor features of Huntington's disease. *Neuropathol Appl Neurobiol*. 2023;49(2):e12891. doi:[10.1111/nan.12891](https://doi.org/10.1111/nan.12891)

Paper IV



SHORT COMMUNICATION OPEN ACCESS

Analyses of the Effects of Wild-Type TDP-43 Overexpression in Oxytocin Neurons in Mice

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- Selective TDP-43 overexpression in oxytocin neurons in the paraventricular nucleus of the hypothalamus causes a decrease in oxytocin-immunopositive cells compared to uninjected mice.
- AAV-mediated TDP-43 overexpression in oxytocin neurons does not appear to be a major driver of behavioural and metabolic phenotypes in mice.

TAR DNA-binding protein 43 (TDP-43) is the major pathological protein in amyotrophic lateral sclerosis (ALS) [1]. Motor symptoms in ALS are often preceded by psychiatric features, including apathy and depression, as well as metabolic dysfunction characterised by a reduced body mass index [2, 3]. The hypothalamus, a key regulator of metabolism and autonomic and endocrine responses to emotion, is affected in clinical ALS [4]. Recent post-mortem studies on ALS cases have revealed TDP-43 inclusions in and atrophy of the hypothalamus, along with the selective loss of key hypothalamic neuronal populations that express oxytocin, hypocretin (orexin) or melanin-concentrating hormone (MCH). In addition, TDP-43 inclusions are found in oxytocin neurons in ALS cases [5–7]. Because oxytocin is a neuropeptide important for complex social behaviour, metabolism, anxiety and depression, its pathology may be linked to the emotional and metabolic dysregulation seen early in ALS [8]. Also, some effects on apathy have been found after administration of oxytocin to patients with another TDP-43 proteinopathy, frontal temporal dementia (FTD) [9]. A recent study demonstrated

that overexpression of wild-type TDP-43 in the entire hypothalamus of mice replicates clinical hypothalamic pathology in ALS, including loss of neurons expressing oxytocin, hypocretin and MCH as well as the development of metabolic dysfunction and apathy [10]. However, the cell-autonomous effect of overexpression of wild-type TDP-43 in oxytocin neurons on neuropathology, metabolism and behaviour remains to be investigated. Understanding this direct effect could be important for understanding the role of the oxytocin system in neurodegenerative disorders with TDP-43 pathology. In the present study, we therefore aimed to investigate whether selective overexpression of wild-type TDP-43 in oxytocin neurons would have effects on neuropathology as well as metabolic and behavioural features.

We used a flex-switch adeno-associated viral (AAV) vector to express either wild-type human TDP-43 (AAV-TDP43) or GFP (AAV-GFP) in oxytocin neurons in mice. Transgene expression was dependent on the presence of Cre-recombinase (Cre), which was expressed under the oxytocin promoter in

Abbreviations: AAV, adeno-associated virus; AAV-GFP, AAV-vector expressing GFP; AAV-TDP43, AAV-vector expressing TDP-43; ALS, amyotrophic lateral sclerosis; ANOVA, analyses of variance; Cre, Cre-recombinase; DEXA, dual-energy x-ray absorptiometry; EPM, elevated plus maze; FST, forced swim test; FTD, frontal temporal dementia; GFP, green fluorescent protein; MCH, melanin-concentrating hormone; Oxytocin-Cre, mice expressing Cre-recombinase under the oxytocin promoter; PVN, paraventricular nucleus; TDP-43, TAR DNA-binding protein 43.

Sofia Bergh and Oskar Simonsson shared first authorship.

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mice (oxytocin-Cre mice; B6; 129S-Oxt^{tm1.1(Cre)Dolsn}/J, Jackson Laboratories). Flex-switch AAV-vectors (titre: 2.0 E14 genome copies/mL) were bilaterally injected into the paraventricular nucleus (PVN) of the hypothalamus of 6-week-old oxytocin-Cre mice. Housing conditions, AAV vector production and stereotactic delivery were similar to those described previously [11].

For neuropathological analyses, mice were perfused at 24 weeks post-injection. The cohort included 11 AAV-TDP43 (female = 7, male = 4) or 7 AAV-GFP (female = 5, male = 2) injected mice and 9 age-matched uninjected controls (female = 9). One AAV-GFP and one AAV-TDP43 brain were excluded from neuropathological analyses due to unsuccessful sectioning. Tissue preparation and immunohistochemical analyses as well as image acquisition and processing were performed as previously described [10, 11]. For vector targeting and quantification of oxytocin-expressing neurons, images of coronal sections between bregma -0.59 to -0.94 were acquired at 10×/0.25 NA using a ZEISS Primostar microscope and analysed in ImageJ 1.53t software. The oxytocin-expressing cells were quantified manually using ImageJ's multipoint tool, whereas the cells overexpressing TDP-43 were quantified with an automated ImageJ script thresholding for pixel intensity (0–100) and circularity (0.30–1.00). Missing sections were imputed with an average from the same group and bregma. The total number of neurons was estimated by multiplying the observed cell count by the cutting interval of the collected sections (i.e., every sixth section was counted, resulting in a multiplier of 6).

Effects on the behavioural and metabolic phenotype following TDP-43 overexpression in oxytocin neurons were investigated in the same cohort of mice with 11 AAV-TDP43 (female = 7, male = 4) and 8 AAV-GFP (female = 5, male = 3) injected oxytocin-Cre mice. Behavioural experiments were performed at 20–23 weeks post-injection as previously described [10, 11]. These tests included elevated plus maze (anxiety-like behaviour), forced swim test (depressive-like behaviour), social interaction test (social cognition) and rotarod (motor coordination and motivation). Body weight measurements and dual-energy x-ray absorptiometry (DEXA) scans were performed at 23 weeks post-injection.

Statistical analyses were performed in SPSS 29.0. One-way ANOVA with Tukey's post hoc was used for normally distributed data, whereas the Mann-Whitney *U* test and Spearman's ranks correlation were used for non-parametric data. A *p*-value of <0.05 was considered significant.

Immunohistochemical analyses confirmed that TDP-43 and GFP immunoreactivity were limited to the PVN of oxytocin-Cre mice (Figure 1A–C). Immunofluorescent analysis confirmed that TDP-43 overexpression was selective to oxytocin neurons (Figure 1D). The flex-switch AAV-GFP vector has been previously validated [11]. The AAV-TDP43 vector achieved a targeting efficacy of around 60% (range 13%–91%) of oxytocin neurons in the PVN (Figure 1J). A trend towards a negative correlation was found between the number of TDP-43-overexpressing cells and the number of oxytocin-expressing cells ($r(8) = -0.61$, $p = 0.060$; Figure 1K).

To investigate effects on neuropathology, we performed quantitative analyses of the number of oxytocin-expressing neurons. AAV-TDP43-injected mice displayed a 19% reduction in oxytocin-expressing neurons (2000 ± 202) compared to uninjected controls (2465 ± 294 , $p = 0.001$; Figure 1E–G,I). However, this reduction was not significantly different from AAV-GFP mice (2217 ± 236 , $p = 0.177$). There was no difference between uninjected and AAV-GFP mice ($p = 0.132$). This suggests that wild-type TDP-43 overexpression can exert mild cell-autonomous toxicity in oxytocin neurons.

Nuclear-to-cytoplasmic mislocalisation and inclusions of TDP-43 are found in cases with ALS [12, 13] and in our previous pan-hypothalamic TDP-43 overexpression mouse model [10]. We therefore investigated the molecular properties of AAV-mediated TDP-43 overexpression in oxytocin neurons. TDP-43 was detected within the nucleus and did not appear to form nuclear or cytoplasmic inclusions in the remaining cells in this model (Figure 1H). Given that the same wild-type TDP-43 construct was used as in our previous pan-hypothalamic TDP-43 model [10], this finding suggests that oxytocin neurons may be able to manage high levels of wild-type TDP-43 within the nucleus without forming inclusions or inducing cytosolic mislocalisation.

Oxytocin plays a role in anxiety, depression, apathy and complex social behaviour, which are early features of ALS [8] as well as in FTD [9]. We therefore investigated the effects of TDP-43 overexpression in oxytocin neurons on the development of behavioural features. Mice overexpressing TDP-43 in oxytocin neurons spent a similar percentage of time on open arms compared to AAV-GFP control mice in the elevated plus maze (Figure 2A) and time spent immobile in the forced swim test (Figure 2B), suggesting no anxiety- or depressive-like phenotypes. The assessment of social behaviour using the social interaction test revealed no significant differences in social affiliation or social novelty (Figure 2C,D) between the different groups. We found no correlation between the results from the behavioural experiments and either the number of oxytocin-expressing neurons or the number of TDP-43-immunopositive cells (data not shown). The AAV-TDP43-injected mice had an increased latency to fall as assessed with the rotarod test compared to AAV-GFP-injected mice ($p = 0.001$; Figure 2E).

Because hypothalamic atrophy has been associated with reduced body mass index preceding motor symptoms in ALS [14], we investigated the metabolic phenotype in mice overexpressing TDP-43 in oxytocin neurons. We observed no effect on body weight gain and body fat percentage, regardless of sex and treatment (Figure 2F–I). However, we observed a trend of decreased body weight gain in male AAV-TDP43 mice compared to male AAV-GFP mice ($p = 0.057$; Figure 2F).

Given the observed variability of TDP-43 targeting efficacy in oxytocin neurons, we performed Spearman's rank correlation analyses between TDP-43 overexpression targeting efficacy and metabolic and behavioural features. A strong negative correlation was detected in male mice between TDP-43 targeting efficacy and body weight gain ($r(4) = -1.00$, $p = 0.01$; Figure 2J). Conversely, the analysis in female mice showed a trend that did not reach statistical significance towards a

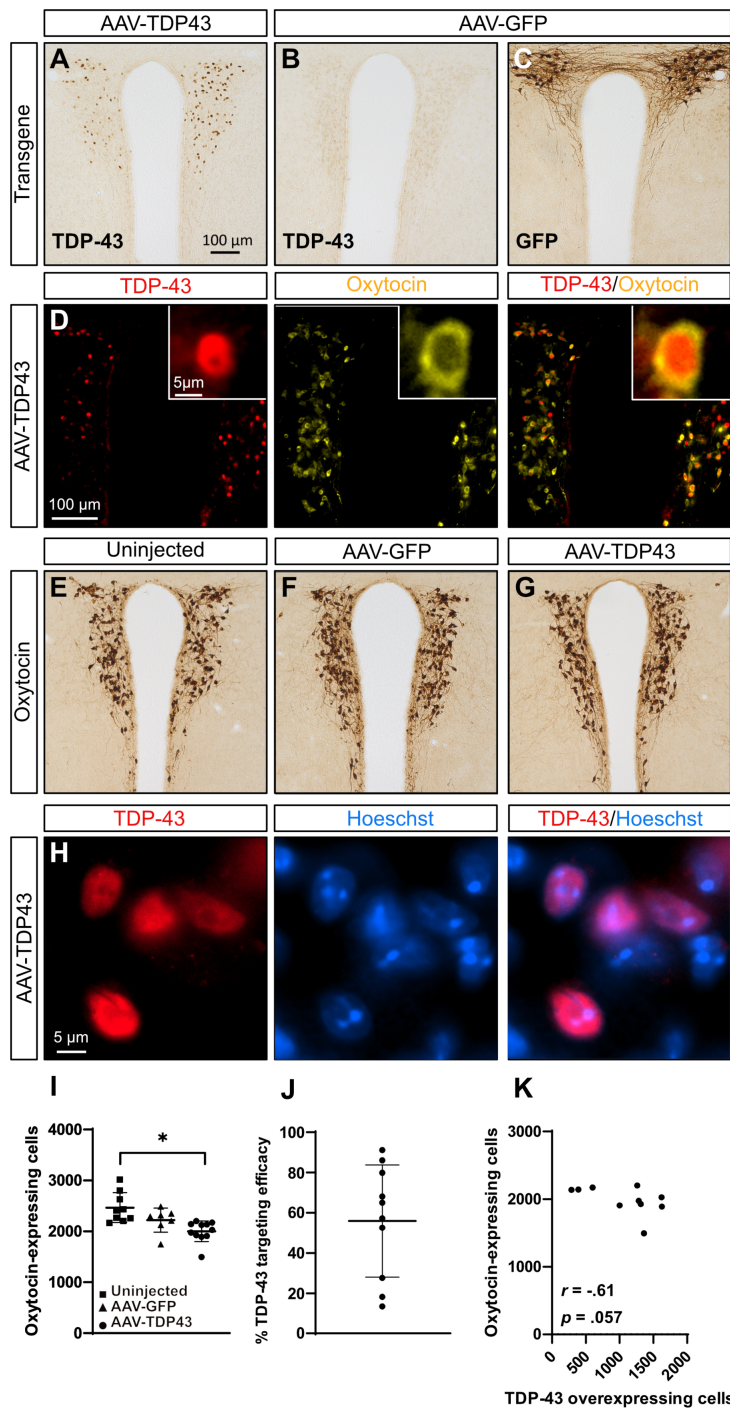


FIGURE 1 | Legend on next page.

FIGURE 1 | Selective TDP-43 overexpression in oxytocin neurons results in loss of oxytocin-expressing neurons compared to uninjected mice. Oxytocin-Cre mice were injected bilaterally with either an AAV-TDP43 or AAV-GFP vector, resulting in selective transgene expression in oxytocin neurons. (A–C) At 24 weeks post-injection, TDP-43 and GFP immunoreactivity were detected, limited to the PVN. (D) TDP-43 overexpression was found in oxytocin-immunopositive neurons. (E–G,I) The number of oxytocin-expressing neurons was reduced in AAV-TDP43 mice compared to uninjected controls ($p=0.001$), but not compared to AAV-GFP mice. (H) Immunofluorescent staining for TDP-43 and Hoechst (nuclear counterstain) revealed TDP-43 immunoreactivity limited to the nucleus, without inclusion formation. (J) Stereotactic injections of AAV-TDP43 led to clear overexpression of TDP-43 in an average of 60% of oxytocin neurons in the PVN. (K) A trend towards a negative correlation was observed between the number of TDP-43-overexpressing cells and the number of oxytocin-expressing cells ($r(8)=-0.61$, $p=0.060$). Data are presented as mean $\pm$ SD. Statistical analyses: one-way ANOVA with Tukey's post hoc (I) and Spearman's rank correlation (K). * $p<0.05$.

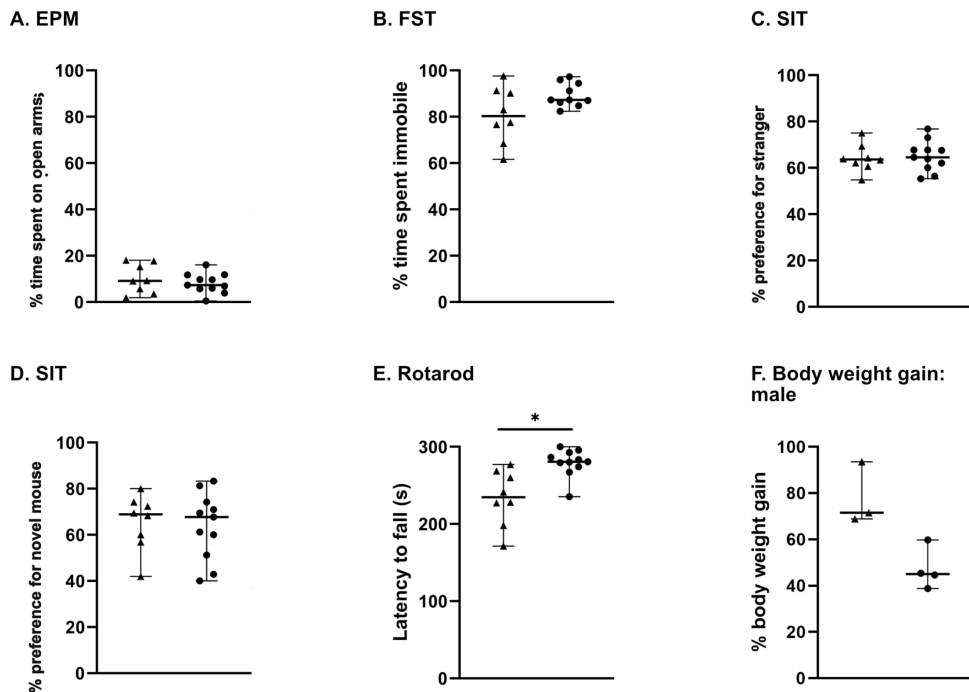


FIGURE 2 | Overexpression of TDP-43 in oxytocin neurons has no major impact on behavioural or metabolic phenotypes in mice. Behavioural and metabolic assessments were performed at 20–23 weeks post-injection. (A,B) No significant differences were observed with elevated plus maze (EPM) or the forced swim test (FST). (C,D) The social interaction test (SIT) revealed no differences in social affiliation or social novelty. (E) AAV-TDP43 mice displayed a significantly increased latency to fall in the rotarod test compared to AAV-GFP mice ($p=0.001$). (F–I) No significant differences were found in body fat percentage or body weight change across sex and treatment groups; however, there was a trend of decreased body weight gain in male AAV-TDP43 mice ($p=0.057$). (J) A strong negative correlation was found in male mice between the TDP-43 targeting efficacy and body weight gain. (K) Female mice displayed a trend towards a positive correlation between TDP-43 targeting efficacy and body weight gain. Data are presented as median with range. Statistical analyses: Mann–Whitney U test (A–I) and Spearman's rank correlation (J–L).

positive correlation between TDP-43 expression efficacy and body weight gain ($r(6)=0.77$, $p=0.072$; Figure 2K). These differences between males and females suggest that TDP-43 may interact with sex-specific metabolic pathways in oxytocin neurons. A recognised limitation of using AAV-mediated expression is the inherent variability in targeting efficacy; however, we did not detect any other significant correlations between the TDP-43 targeting efficacy and the remaining phenotypic

features measured (Figure S1). Taken together, these data suggest that overexpression of wild-type TDP-43 in oxytocin neurons has limited effects on the development of behavioural and metabolic features.

This study reveals a mild cell-autonomous neurotoxic effect of overexpressed wild-type TDP-43 on oxytocin neurons. We observed a decrease in the number of neurons expressing oxytocin

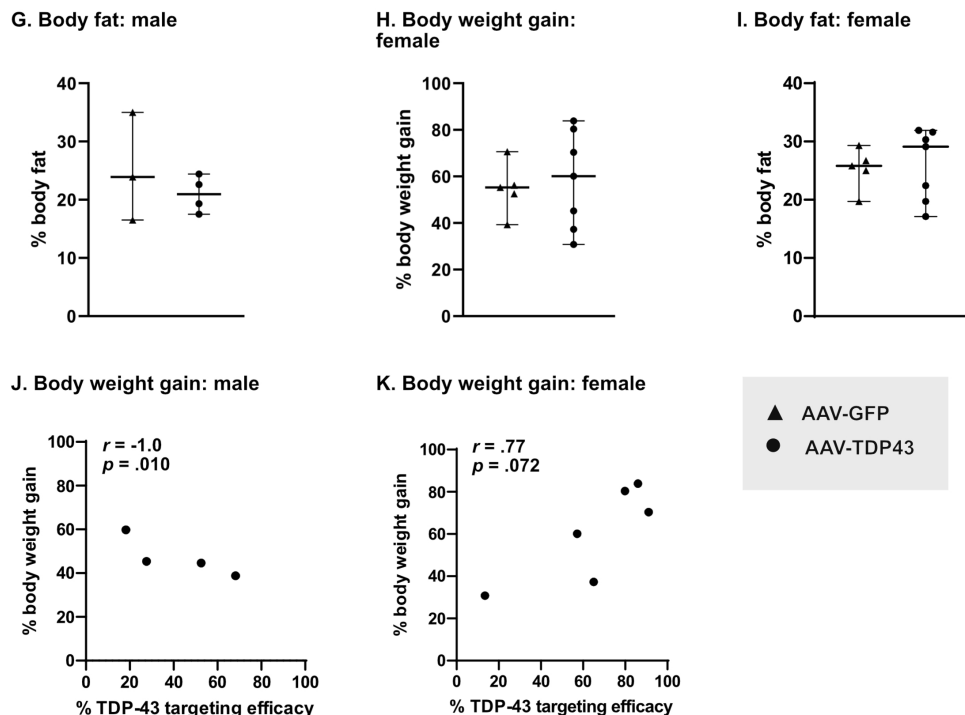


FIGURE 2 | (Continued)

compared to uninjected controls, but not relative to AAV-GFP mice. In our recent study [12], pan-hypothalamic overexpression of wild-type TDP-43 resulted in a 42% loss of oxytocin neurons, downregulation of oxytocin transcripts and clear metabolic and behavioural impairment. The difference in oxytocin system pathology between the two models may imply that the effect on oxytocin is not solely cell autonomous. Our data also suggest that metabolic and behavioural phenotypes observed after overexpression of TDP-43 in the hypothalamus are likely not a result of TDP-43 dysfunction in oxytocin neurons. Hence, further studies investigating the effects of TDP-43 on other hypothalamic neuronal populations may shed light on the relationship between specific hypothalamic circuitry dysfunction and the development of non-motor manifestations in TDP-43 proteinopathies.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and data analysis were performed by all authors. The first draft of the manuscript was written by Sofia Bergh, Oskar Simonsson and Åsa Petersén. All authors read and commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Ulrika Sparrhult-Björk, Björn Anzelius and Anneli Josefsson for excellent technical assistance.

Funding

This study was funded by research grants to Åsa Petersén from the Swedish Research Council (grant number 2022/01092), the Swedish Brain Foundation, the Swedish governmental funding of clinical research (ALF) at Region Skåne and the Knut and Alice Wallenberg Foundation (# 2019.0467).

Ethics Statement

All animal procedures within this project have been approved by the Malmö/Lund ethical committee according to permit number 17113/2022.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Correlation analysis of TDP-43 targeting efficacy and phenotypic outcomes in mice. Spearman's rank correlation analyses examining the relationship between the percentage of TDP-43 expression oxytocin neurons and results from (A) elevated plus

maze (EPM), (B) forced swim test (FST), (C,D) social interaction test (SIT), (E) rotarod, body fat percentage in male (F) and female (G) mice. **Data S1:** Supporting Information.

Review article





OPEN ACCESS

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SPECIALTY SECTION

This article was submitted to
Molecular Signaling and Pathways,
a section of the journal
Frontiers in Molecular Neuroscience

RECEIVED 01 July 2022

ACCEPTED 19 August 2022

PUBLISHED 14 September 2022

CITATION

Bergh S, Cheong RY, Petersén Å and
Gabery S (2022) Oxytocin
in Huntington's disease and
the spectrum of amyotrophic lateral
sclerosis-frontotemporal dementia.
Front. Mol. Neurosci. 15:984317.
doi: 10.3389/fnmol.2022.984317

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Oxytocin in Huntington's disease and the spectrum of amyotrophic lateral sclerosis-frontotemporal dementia

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Neurodegenerative disorders (NDDs) such as Huntington's disease (HD) and the spectrum of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are characterized by progressive loss of selectively vulnerable populations of neurons. Although often associated with motor impairments, these NDDs share several commonalities in early symptoms and signs that extend beyond motor dysfunction. These include impairments in social cognition and psychiatric symptoms. Oxytocin (OXT) is a neuropeptide known to play a pivotal role in the regulation of social cognition as well as in emotional behaviors such as anxiety and depression. Here, we present an overview of key results implicating OXT in the pathology of HD, ALS and FTD and seek to identify commonalities across these NDDs. OXT is produced in the hypothalamus, a region in the brain that during the past decade has been shown to be affected in HD, ALS, and FTD. Several studies using human post-mortem neuropathological analyses, measurements of cerebrospinal fluid, experimental treatments with OXT as well as genetic animal models have collectively implicated an important role of central OXT in the development of altered social cognition and psychiatric features across these diseases. Understanding central OXT signaling may unveil the underlying mechanisms of early signs of the social cognitive impairment and the psychiatric features in NDDs. It is therefore possible that OXT might have potential therapeutic value for early disease intervention and better symptomatic treatment in NDDs.

KEYWORDS

oxytocin, Huntington's disease, amyotrophic lateral sclerosis, frontotemporal dementia, neurodegenerative diseases, huntingtin, TDP-43

Introduction

Neurodegenerative disorders (NDDs) are a group of diseases caused by progressive and irreversible deterioration of neurons within the central nervous system (CNS). Current treatment is only symptomatic but does not modify disease progression or reverse the neuronal dysfunction. These disorders are characterized by selective cellular vulnerability to the pathogenic process that often involve the accumulation of disease-associated proteins. Important research questions are centered on understanding the underlying mechanisms of selective vulnerability as well as what changes occur early and could be targeted for therapeutic disease-modifying interventions. Recent work has indicated interesting similarities between Huntington's disease (HD) and the spectrum of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (Vercruysse et al., 2018). The concept of an ALS and FTD disease spectrum continuum has emerged, largely from an overlap in pathological and genetic associations between the two conditions (Neumann et al., 2006; Renton et al., 2011; Strong et al., 2017). Although previous research has mainly focused on the well-known disturbances in motor function and accompanying neuropathology of movement-regulating neurons for HD and ALS, recent work show early manifestation of psychiatric symptoms and altered social cognition as well as selective vulnerability of hypothalamic neurons in both HD and the spectrum of ALS and FTD disorders (Vercruysse et al., 2018; Gabery et al., 2021). Understanding the underlying mechanisms of selective vulnerability of hypothalamic neurons and the link to early psychiatric symptoms in these disorders may open up for novel avenues for therapeutic interventions for these disorders.

Several genes have been identified in the familial forms of these NDDs. HD is caused by an expansion of the trinucleotide CAG within the *huntingtin* (*HTT*) gene (HDCRG, 1993), while there are several known genetic mutations in ALS such as *superoxide dismutase 1* (*SOD1*), *chromosome 9 open reading frame 72* (*C9ORF72*) and, *trans-activation responsive RNA-binding protein* (*TARBP*). The latter two genes are also affected in FTD as well as *progranulin* (*GRN*) and *microtubule associated protein tau* (*MAPT*) (Taylor et al., 2016; Greaves and Rohrer, 2019). Also, CAG expansions within the *ataxin-2* have also been shown to be associated with ALS (Elden et al., 2010).

Cognitive and social behavioral alterations are key early features that are present in HD, ALS, and FTD (Craufurd et al., 2001; Phukan et al., 2007; Paulsen et al., 2008; Steenland et al., 2010; Ahmed et al., 2014; Bott et al., 2014; McCarter et al., 2016; Herzig-Krzywoszanska and Krzywoszanski, 2019; Blasco et al., 2020; Boentert, 2020; Singh and Agrawal, 2021). In particular, social cognitive impairment (e.g., processing of facial expression of emotions, theory of mind, and empathy) in NDDs has the potential to disrupt interpersonal relationships,

thereby eliminating the benefits that social interactions may bring for patients suffering from these debilitating conditions (Christidi et al., 2018). The underlying biological mechanisms mediating these features in NDDs are not well understood. However, given that both altered social cognition as well as psychiatric features constitute a common denominator in the early phases across all three diseases, it is likely that there could also be common pathologies in HD, ALS, and FTD.

Hypothalamic alterations can be observed in all three NDDs, including loss of different hypothalamic neurons (Gabery et al., 2010, 2015, 2021; Vercruysse et al., 2018; Ahmed et al., 2021c). The neuropeptide oxytocin (OXT) has long been known to play a pivotal role in the regulation of complex social cognition and behaviors, including prosocial behavior and pair-bonding (Heinrichs et al., 2009; Galbally et al., 2011; Odent, 2013) as well as in emotional behavior including anxiety and depression (Neumann and Landgraf, 2012; Jurek and Neumann, 2018; Onaka and Takayanagi, 2019; Yoon and Kim, 2020). OXT is synthesized in the paraventricular nucleus (PVN) and supraoptic nucleus of the hypothalamus (Gimpl and Fahrenholz, 2001). OXT binds to OXT-receptors (OXTR) that are located throughout the brain most prominently within the limbic structures (Jurek and Neumann, 2018). Interestingly, in a recent study, distinct OXTR expression patterns have been shown in psychiatric disorders as well as in metabolic regulation processes across development in humans (Rokicki et al., 2022). Furthermore, genetic variation of OXTR has also been shown to be associated with social impairment in HD (Saiz-Rodríguez et al., 2022). When exogenously administered, OXT facilitates social encounters, improved social cognitive outcomes as well as emotion recognition in healthy and clinical groups characterized by social deficits, such as autism and social anxiety disorder (Heinrichs et al., 2009; Keech et al., 2018).

Other hypothalamic specific neuropeptides that have been studied in NDDs include hypocretin (orexin) which has been shown to be reduced in both HD and ALS (Gabery et al., 2010, 2021) as well as in Parkinson's disease (Fronczek et al., 2007). Like OXT, hypocretin is exclusively produced in the hypothalamus and is involved in the regulation of sleep, emotion, and metabolism (Tyree et al., 2018). Other hypothalamic specific neurons such as vasopressin has also been shown to be reduced in HD (Gabery et al., 2010), but not in ALS or FTD (Piguet et al., 2011; Gabery et al., 2021). Hypothalamic expression of neuropeptide Y (NPY), a neuropeptide involved mostly in appetite regulation has also been studied and is preserved in HD, ALS, and FTD (Gabery et al., 2010; Piguet et al., 2011).

Hence, several studies have indicated that the OXT system may be implicated across both HD and the spectrum of ALS/FTD and therefore constitute an interesting common denominator (Gabery et al., 2010, 2015, 2021; Jesso et al., 2011;

Finger et al., 2015). Given the role OXT has on social cognition and emotional regulation, there may be a link between the pathology in the OXT system identified in these disorders and some of the early social cognitive and psychiatric features. OXT system may provide a common therapeutic target for disease intervention. Understanding similarities and differences of these disorders including of how the OXT system is affected may provide important information in that direction. This review therefore focuses on the current state of knowledge regarding changes in the OXT system in these particular NDDs and highlights the major results obtained so far in this emerging field.

Clinical features and neuropathology of Huntington's disease

The clinical diagnosis of HD is based on a positive gene test in combination with the manifestation of overt motor disturbances including chorea (exaggerated involuntary movements), rigidity and gait imbalance (Novak and Tabrizi, 2010; Ross et al., 2014). The onset of motor disturbances usually occurs in midlife (30–55 years of age) followed by 20 years of disease progression. Individuals with HD also experience a range of non-motor symptoms and signs. These include cognitive changes, such as executive dysfunction and altered social cognition, as well as psychiatric symptoms such as anxiety, depression, irritability, and apathy (Duff et al., 2007; Paulsen et al., 2008). The disease-causing mutation is an expansion of a CAG trinucleotide repeat in the *HTT* gene which encodes an extended polyglutamine (Q) of the HTT protein (HD-CRG, 1993; Ross et al., 2014). Individuals carrying more than 36Q will develop HD, but there is reduced penetrance if the patient is carrying between 36 and 39Q (Duyao et al., 1993). Despite a major hallmark of HD pathology being the formation of intraneuronal aggregates of the mutant HTT protein, the role of these aggregates in the pathogenesis is not well understood (DiFiglia et al., 1997; Ross and Shoulson, 2009; Cisbani and Cicchetti, 2012). In HD, the most pronounced neuropathology is observed in the striatum of the basal ganglia and the cerebral cortex which are regions associated with motor function (Vonsattel et al., 1985). The site of pathology for psychiatric signs and symptoms as well as altered social cognition is still not established but may in part be explained by dysfunctional neural circuitries and neuronal cell death in the hypothalamus. Hypothalamic alterations have been observed decades before onset of motor disturbances (Politis et al., 2008; Sonesson et al., 2010; Cheong et al., 2019). Recent studies have aimed to increase the understanding of the non-motor features of HD and in particular the role of OXT in this paradigm. A summary of the main results for the role of OXT in HD pathology can be found in Table 1.

Changes in the oxytocin system in Huntington's disease

The first clinical study to investigate OXT pathology in clinical HD was conducted by Gabery et al. (2010). Immunohistochemically processed post-mortem brain tissue from HD patients of different Vonsattel grades (grades 2–4) revealed a selective 45% OXT neuronal loss as well as a reduced cell size of the remaining OXT neurons. The Vonsattel grading system is a five-step grading system (0–4) for neurodegeneration in HD focusing on the striatum, the most affected brain region (Vonsattel et al., 1985). Furthermore, a case report based on one HD patient with Vonsattel grade 0 showed the same low number of OXT-expressing neurons as late-stage HD patients with Vonsattel grade 2–4 (Gabery et al., 2015). The same patient had deceased before the onset of any motor signs and symptoms, however, had developed anxiety and sleep disturbances (Gabery et al., 2015). These results suggest that early changes in hypothalamic neuronal populations expressing emotion-regulating neuropeptides could contribute to the early behavioral phenotype of HD. However, one study reported no changes in number of OXT neurons within the PVN (van Wamelen et al., 2012). This discrepancy in results may stem from different quantification approaches used. In the study from Gabery et al. stereological quantification with the physical disector principle was applied while this was not the case in the study by van Wamelen et al. (2012).

In several studies, OXT has been measured in both blood and cerebrospinal fluid (CSF) samples from individuals with HD. A recent study showed a significant 38% reduction in OXT CSF levels in individuals carrying the mutant *HTT* gene (Hellem et al., 2022). No changes in OXT plasma levels in HD patients have been found, highlighting that changes in OXT levels occur centrally and not in the periphery (Unti et al., 2018; Fisher et al., 2021; Hellem et al., 2022).

There are a number of different transgenic mouse models for HD (Pouladi et al., 2013). A decrease in OXT mRNA levels in the CNS of both the R6/2 mouse expressing a short fragment of mutant *HTT* as well as HD190QG expressing a longer fragment of mutant *HTT* gene was observed (Kotliarova et al., 2005). A decrease of OXT mRNA levels has also been reported in a viral vector model with overexpression of mutant HTT selectively in the hypothalamus (Hult et al., 2011). Furthermore, R6/2 mice displayed loss of OXT-expressing neurons, which was not found in two other HD animal models; the BACHD and the HD190Q transgenic HD mice (Kotliarova et al., 2005; Soyly-Kucharz et al., 2016; Henningsen et al., 2021). In the PVN of HD190QG mice, lower levels of OXT mRNA were associated with a high frequency of mutant HTT aggregates (Kotliarova et al., 2005). Furthermore, pretreatment with OXT before intracerebroventricular (icv) injection 3-nitropropionic acid (3-NP)-induced HD mouse model prevented the development

TABLE 1 Summary of oxytocin (OXT) results in Huntington’s disease (HD).

Tissue	HD stage	Analysis	Treatment		Results	References
Clinical HD	Premanifest and manifest	IHC, stereology		↓	Number of OXT neurons	Gabery et al., 2010; Gabery et al., 2015
		IHC		=	Number of OXT neurons	van Wamelen et al., 2012
		IHC, stereology		↑	Atrophic OXT neurons	Gabery et al., 2010
		EIA, ELISA, RIA		=	OXT plasma level	Unti et al., 2018; Fisher et al., 2021; Hellem et al., 2022
	Premanifest	ELISA, SDMT, VDT		↑	OXT plasma levels correlated with ↑ executive function	Fisher et al., 2021
	Premanifest and manifest	ELISA, PBA		↑	OXT plasma levels correlated with ↓ depressive-symptoms	Fisher et al., 2021
	Manifest	EIA, faux-pas		↑	OXT correlated with ↑ social cognition	Unti et al., 2018
	Premanifest	fMRI, emotional face matching task	Intranasal OXT adm.	↑	Ability to process disgust stimuli	Labuschagne et al., 2018
	Manifest	RIA		↓	OXT CSF levels	Hellem et al., 2022
		RIA, MMSE, MoCA, TASIT, EHt, RME		↓	OXT CSF levels correlated with ↑ cognitive impairment	Hellem et al., 2022
HD190QG (Mouse)		RT-PCR		↓	OXT mRNA	Kotliarova et al., 2005
BACHD (Mouse)		IHC		=	Number of OXT neurons	Kotliarova et al., 2005
		RIA		↓	OXT plasma level	Cheong et al., 2020
		RIA, EPM, FST, SIT		↓	OXT plasma levels with ↑ depressive-, anxiety-like and altered social behavior	Cheong et al., 2020
		FST	Intranasal OXT adm.	↓	Depressive-like symptoms	Cheong et al., 2020
		IHC, stereology		=	Number of OXT neurons	Soylu-Kucharz et al., 2016
		IHC, stereology	QA injection	=	Number of OXT neurons	Henningsen et al., 2021
R6/2 (MOUSE)		RT-PCR		↓	OXT mRNA levels	Kotliarova et al., 2005
AAV-MHTT (Mouse)		IHC, stereology		↓	Number of OXT neurons	Henningsen et al., 2021
		IHC, stereology	QA injection	=	Number of OXT neurons	Henningsen et al., 2021
		RT-PCR	mHTT AAV vector injection	↓	OXT mRNA	Hult et al., 2011
NP-3 (Rat)		OFT, EPM, FST	icv OXT injection	↓	Anxiety- and depressive-like symptoms	Khodaghohi et al., 2022
		Western blot, ellman method	icv OXT injection	↑	OXTR, mGluR2, GSH levels	Khodaghohi et al., 2022
		Western blot	icv OXT injection	↓	mGluR5 levels	Khodaghohi et al., 2022

↓: decrease in symptomatic or abundance, ↑: increase in abundance, =: no change in neuronal population. adm, administration; AAV, adeno-associated virus; EHT, emotion hexagon test; EIA, enzyme inhibition assay; ELISA, enzyme-linked immunosorbent assay; EPM, elevated plus maze; FST, forced swim test; IHC, immunohistochemistry; icv, intracerebroventricular injection; mHTT, mutant huntingtin; MoCA, Montreal cognitive assessment; MMSE, mini mental state examination; OFT, open field test; QA, quinolinic acid; RIA, radioimmunoassay; RME, reading the mind of the eyes; RT-PCR, real-time polymerase chain reaction; PBA, problem behaviors assessment; SDMT, symbol digit modalities test; SIT, social interaction test; TASIT, the awareness of social interference test; VDT, verbal fluency test.

of several changes including decreased levels of the OXT receptor, mGluR2 and glutathione as well as increased mGluR5 levels in the striatum, hippocampus, prefrontal cortex, and amygdala (Khodaghohi et al., 2022). These results indicate that OXT might have a protective effect on these molecular changes in HD. However, the OXT neuronal population appear to be resistant to quinolinic acid induced toxicity

(Henningsen et al., 2021), which is an excitotoxin that has been linked to a loss of medium spiny neurons in the striatum of HD (Beal et al., 1986, 1991; Ferrante et al., 1993). This suggests that the vulnerability of OXT expressing neurons in HD is not caused by excitotoxicity. The underlying mechanisms of OXT loss in HD are not known and need further study.

Effects of oxytocin on social cognition and psychiatric features in Huntington's disease

Oxytocin has previously been established to have an important role in social cognition. Clinical studies have revealed a link between OXT and social cognition in HD. In HD patients, the ability to process emotions such as disgust, fear, anger, sad, surprise, and happiness is reduced (Labuschagne et al., 2018). Interestingly, a higher baseline level of OXT in the plasma was associated with a better recognition of emotion facial expression in HD gene carriers at an early disease stage before any onset of motor signs and symptoms onset (premanifest HD) (Unti et al., 2018). Moreover, intranasal OXT treatment normalized the ability of premanifest patients to process disgust stimuli (Labuschagne et al., 2018). More recently, Hellem et al. (2022) reported that HD patients with social cognitive impairment had significantly lower OXT CSF levels, suggesting a correlation between OXT CSF levels and social cognitive function. OXT may also be associated with executive dysfunction in HD. A clinical pilot study revealed that premanifest HD patients with higher OXT plasma levels performed better at cognitive tasks including verbal functioning, visual spatial attention, processing speed and working memory (Fisher et al., 2021). With an unmet clinical need for HD biomarkers, both OXT CSF and plasma levels may give some indication of the status of social cognitive deficits in HD.

Oxytocin could also play a role for the neuropsychiatric features of HD. In clinical HD, a positive correlation between OXT plasma levels and depression in both motor manifest and premanifest HD patients has been observed (Fisher et al., 2021). In the BACHD mouse model, OXT plasma level is lower than in wild-type littermates with increased depressive-, anxiety-like and social behavior (Cheong et al., 2020). Moreover, acute intranasal OXT administration reduced depressive-like behavior in this mouse model with no effect on anxiety-like behavior (Cheong et al., 2020). Furthermore, pretreatment with OXT injections prior to icv NP-3 injection in rats prevented the development of anxiety- and depressive-like behavior (Khodaghali et al., 2022). These results suggest that OXT might have protective effects and should be further investigated as a potential treatment in preventing the development of depressive and/or anxious phenotype in HD.

Clinical features and neuropathology of frontotemporal dementia

Frontotemporal dementia is a group of NDDs that are characterized by progressive altered behavior and decline in executive functions. FTD is the second most common dementia after Alzheimer's disease (Ahmed et al., 2021b). Clinically it is subdivided into three main types including behavioral-variant

frontotemporal dementia (bv-FTD), semantic dementia and progressive non-fluent aphasia (Neary et al., 1998; Hodges and Patterson, 2007). bv-FTD is the most common form and comprises over 50% of all FTD cases. The syndrome has an early age of onset with a mean around 50 years of age and disease duration of approximately 8 years. Affected individuals have a range of clinical symptoms such as behavioral disinhibition, hallucinations, apathy, executive dysfunction as well as changes in eating behavior with hyperorality (Woolley et al., 2007; Rascovsky et al., 2011). However, a central feature in this condition is the early progressive loss of empathy and social cognition (Rankin et al., 2005). As such, considerable amount of research has during the past two decades been devoted to this topic. Impaired recognition of the facial expression of emotions occurs at an early stage, which in turn complicates the engagement or response to social cues (Keane et al., 2002; Rosen et al., 2004). Regions in the brain that are thought to be involved in social cognition include frontal, temporal and parietal lobes, which are the regions with predisposition to neuropathological changes and atrophy in FTD (Kennedy and Adolphs, 2012; Ahmed et al., 2021b).

Psychiatric symptoms can often initially mask an FTD diagnosis, as several of the symptoms overlap with other psychiatric syndromes such as obsessive-compulsive disorder, bipolar disorder and major depressive disorder (MDD). Around 50% of patients with bv-FTD receive initially a psychiatric diagnosis (Woolley et al., 2011). Neuroimaging studies have revealed a loss of gray matter in both MDD and bipolar disease that overlap with the affected regions in FTD (Peet et al., 2021). This, therefore, poses a diagnostic difficulty in the clinical setting (Ducharme et al., 2020). Recently, post-mortem analysis on brain tissue from FTD cases have shown a correlation between psychiatric symptoms and a higher abundance of the transactive response DNA-binding protein 43 kDa (TDP-43) inclusion pathology (Scarioni et al., 2020).

Clinical features and neuropathology of amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is a fast-progressing NDD associated with both upper and lower motor neuron dysfunction leading to muscle weakness and bulbar dysfunction (Taylor et al., 2016). The disease onset occurs commonly in mid-adulthood (at a mean age of 55 years) with death typically 3–5 years after diagnosis usually due to respiratory failure (Zarei et al., 2015; Taylor et al., 2016). ALS patients also exhibit a range of non-motor symptoms including altered energy metabolism and eating behavior (Dupuis et al., 2011; Ahmed et al., 2016, 2021b). Pathological findings have been observed in the frontal and temporal cortices as well as in the hypothalamus that may underlie some of these changes (Neumann et al., 2006; Gabery et al., 2021).

Psychiatric symptoms such as apathy and depression have also been described in ALS, which have been shown to precede the onset of motor symptoms (Mioshi et al., 2014; Caga et al., 2018, 2021). In particular, depression is reported before and after diagnosis (Turner et al., 2016). Recent studies indicate that social cognitive impairment and emotion facial expression processing deficits is present in ALS patients. Also, an increased atrophy of the fornix, the main white matter tract of the limbic system, has shown to correlate with increased behavioral changes in ALS patients (Gabery et al., 2021). A recent report has shown that ALS patients have more difficulty with recognition of facial expression of emotions such as disgust, anger, fear and sadness (Palumbo et al., 2022). These findings suggest that the OXT pathway might be affected in ALS.

Overlap between amyotrophic lateral sclerosis and frontotemporal dementia

In recent years, a growing amount of evidence point toward that ALS and a large proportion of FTD are part of a disease spectrum continuum (Clark and Forman, 2006). Approximately 15% of FTD patients develop ALS-associated motor signs and symptoms and 15–18% of ALS patients exhibit FTD-like symptoms (Burrell et al., 2011; Lattante et al., 2015; Taylor et al., 2016). This concept was potentiated further with the discovery of the C9ORF72 expansion causing both ALS and FTD (DeJesus-Hernandez et al., 2011; Hodges, 2012). Both conditions share overlap at the neuropathological level including cytoplasmic TDP-43 inclusions in both neurons and glia cells (Buratti and Baralle, 2008, 2012).

Changes in the oxytocin system in amyotrophic lateral sclerosis and frontotemporal dementia

For FTD and ALS, the symptomatology and the clinical presentation suggest involvement of key physiological functions of the hypothalamus, which might even precede the onset of the cognitive and motor symptoms development (Vercruysse et al., 2018; Ahmed et al., 2021a). Significant atrophy of the hypothalamus is present on structural magnetic resonance imaging (MRI) as well as on post-mortem analyses in patients with bv-FTD and ALS (Piguet et al., 2011; Gorges et al., 2017; Gabery et al., 2021). So far, only a few studies have investigated OXT in ALS and FTD.

Recently, a 33% loss of OXT-expressing neurons in post-mortem tissue from ALS patients was reported together with the presence of TDP-43 inclusions in OXT-expressing neurons (Gabery et al., 2021). In bv-FTD, promising results have been observed during pharmacological treatments with

OXT mainly targeting loss of empathy and the ability to process facial expression of emotions. So far, two small randomized controlled trials with intranasal OXT treatment have shown safety and tolerability as well as significant improvement in neuropsychiatric inventory scores assessing agitation, depression, apathy and irritability (Table 2; Jesso et al., 2011; Finger et al., 2015). However, larger randomized control trials are required before more definitive conclusions of the potential positive therapeutic effects of OXT can be made.

Furthermore, using blood oxygen level dependent signal during functional MRI, intranasal OXT treatment showed enhanced activity in limbic regions associated with processing of facial expression of emotions (Oliver et al., 2020). However, to date, no studies have investigated OXT in animal models of ALS or FTD. Nevertheless, these findings together highlight the potential of OXT as symptomatic treatment for deficits.

Similarities across Huntington's disease and the spectrum of amyotrophic lateral sclerosis and frontotemporal dementia disorders

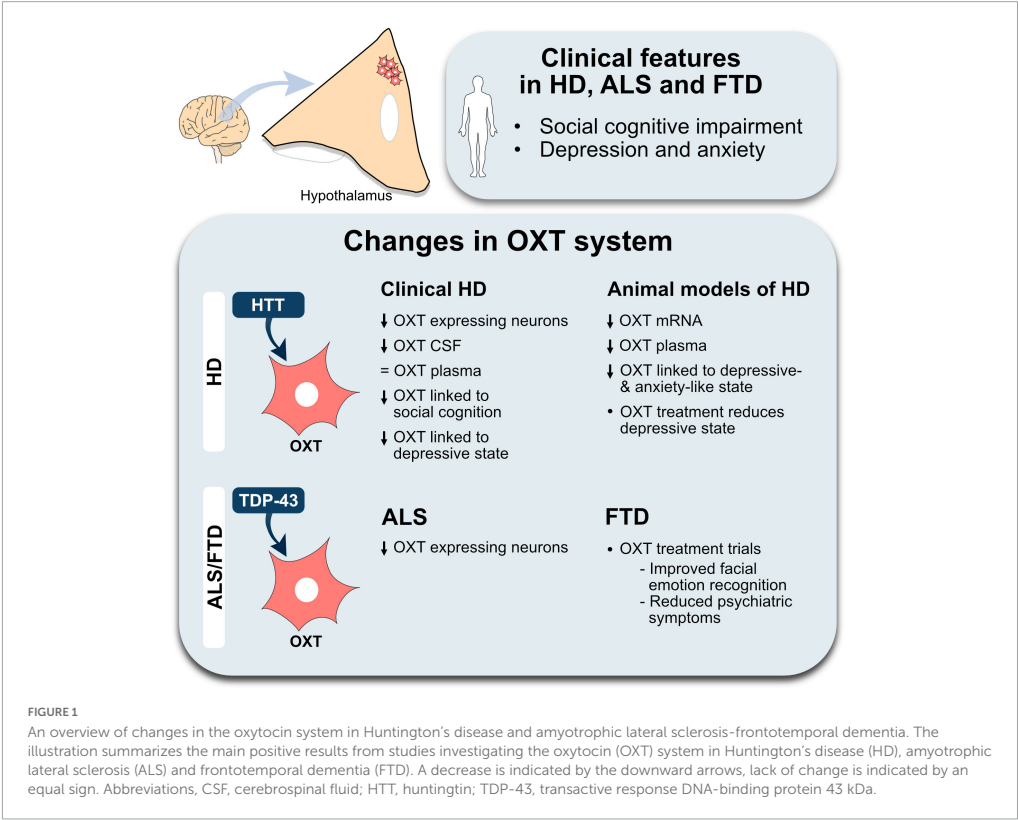
The role of OXT has evolved from solely being related to parturition and breastfeeding to be able to modulate aspects of social behavior as well as emotional regulation. As these functions are affected early on in HD and the spectrum of ALS/FTD, OXT may play an important role (Figure 1). Impairments in social cognition as well as a reduced ability to recognize facial expression of emotions in all three conditions have been established (Craufurd et al., 2001; Christidi et al., 2018; Keech et al., 2018; Palumbo et al., 2022). This could be associated with OXT, in particular in HD and FTD in which direct correlations of OXT levels has been found (Jesso et al., 2011; Labuschagne et al., 2018; Unti et al., 2018; Hellem et al., 2022). Moreover, hypothalamic pathology has been identified in all three conditions with a selective OXT loss in HD and ALS. Histopathological findings such as HTT inclusions in HD as well as presence of TDP-43 inclusions in OXT-expressing neurons suggest a selective vulnerability of this neuronal population to the presence of mutant HTT and TDP-43 (Gabery et al., 2010, 2021). To date, no studies have investigated the number of OXT cells in FTD. Collectively, these findings could indicate a mechanistic overlap across all three NDDs.

Furthermore, pharmacological administration with exogenous OXT improved processing of facial expression of emotions in both HD and FTD, thus supporting the possibility of a therapeutic application of OXT in the future. However, chronic administration of OXT at least in rodents lead to anxiety, via alternative splicing of *Crf2a* (Winter et al., 2021), which needs to be considered in the development of clinical applications.

TABLE 2 Summary of oxytocin (OXT) results in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).

Tissue	Condition	Analysis	Treatment	Results	References
Clinical ALS/FTD	ALS	IHC, stereology		↓ Number of OXT neurons	Gabery et al., 2021
	FTD		Intranasal OXT adm.	Safe and well tolerated	Finger et al., 2015
	FTD	Facial expression recognition task, neuropsychiatric inventory scale	Intranasal OXT adm.	↑ Emotion recognition Improvement in neuropsychiatric inventory score	Jesso et al., 2011
	FTD	fMRI, Bold	Intranasal OXT adm.	↑ Increased activity in limbic regions	Oliver et al., 2020

↓: decrease in neuronal population, ↑: improvement in capacity. IHC, immunohistochemistry; fMRI, functional magnetic resonance imaging; BOLD, blood oxygenation level dependent.



Conclusion

In this review, we provided a summary of the main results implicating changes in the OXT system related to HD, ALS and FTD in the literature. Across all three conditions, the impairments in social cognition and neuropsychiatric behavior

occur early in the disease progression, prior to the onset of motor disturbances. OXT neuropathology may at least in part explain the development of these early features. There is an unmet need for biomarkers to track early disease progression in HD, ALS, and FTD. Levels of OXT in both CSF and plasma have been shown to track certain social cognitive

features, rendering OXT a potential biomarker candidate. Also, initial pharmacological intervention with OXT shows promising results. However, more experimental studies are needed to further determine the causative role of OXT in the development of the social and psychiatric impairments in HD, ALS, and FTD.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Funding

ÅP was supported by the Swedish Research Council (grant number #2018/02559), the ALF system at Region Skåne, and Swedish Brain Foundation (#2021-0076) as well as the Knut and

Alice Wallenberg Foundation (#2019.0467). SG was a Swedish Brain Foundation Post-doctoral Fellow (#PS2018-0002).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The hypothalamus as a key mediator of neurodegenerative disorders

Each neurodegenerative disorder has a distinct clinical presentation and primary affected brain region, but many share a surprising commonality – pathology of the hypothalamus. By bridging the gap between Huntington's disease, ALS/frontotemporal dementia, Alzheimer's disease and Parkinson's disease, the hypothalamus may hold the key to a unifying biological mechanism.

This thesis characterises dysfunction of the hypothalamus and links it to the psychiatric and metabolic disturbances observed in neurodegenerative disorders, integrating human, animal and in vitro data to propose novel disease mechanisms.

