# Mechanisms underlying metabolic alterations in Huntington's disease. Beyond the brain and back.

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## Beyond the brain and back

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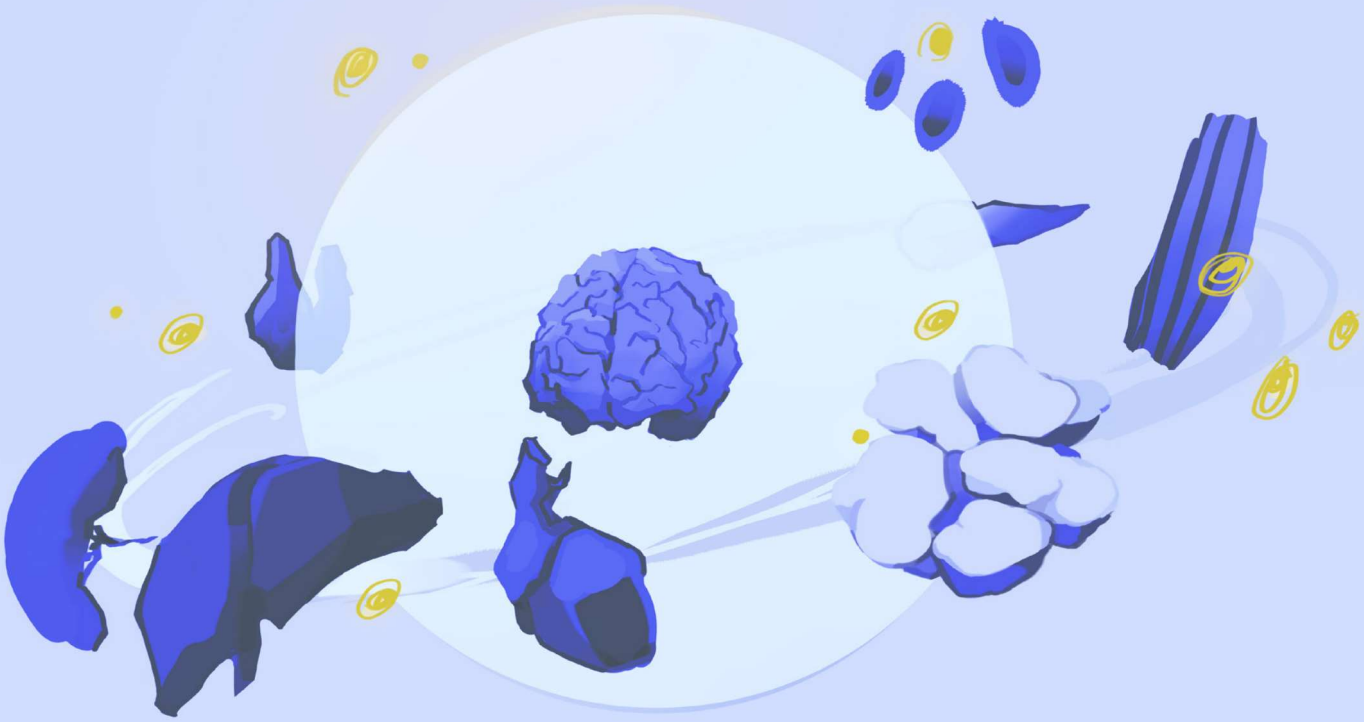

# Mechanisms underlying metabolic alterations in Huntington's disease

## Beyond the brain and back

Elna Dickson

LUND
UNIVERSITY

DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden.
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Cover photo by Elna Dickson. The illustration is inspired by the geocentric model of the Universe with Earth at the center, as retold by Stephen Hawking in *A Brief History of Time.*



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*Till mamma och pappa*

*Do the best you can until you know better.*
*Then when you know better, do better*
Maya Angelou

*I can fix it all - but hey - nay*
*Let's just do it some other day*
Pressure by MARINA & THE KATS

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I. **Dickson, E.**, Soylu-Kucharz, K., Petersén, Å. & Björkqvist, M. (2022). Hypothalamic expression of huntingtin causes distinct metabolic changes in Huntington's disease mice. Molecular metabolism 57: 101439.

II. **Dickson, E.**, Dwijesha, A., Andersson, N., Lundh, S., Björkqvist, M., Petersén, Å. & Soylu-Kucharz, K. (2022). Microarray profiling of hypothalamic gene expression changes in Huntington's disease mouse models. Frontiers in Neuroscience 16.

III. **Dickson, E.**, Fryklund, C., Soylu-Kucharz, R., Sjögren, M., Stenkula, K. & Björkqvist, M. Altered adipocyte function prior to weight loss in the R6/2 model of Huntington's disease. (Manuscript)

# Popular scientific summary

Huntington's disease is caused by a mutation in the gene that codes for the protein huntingtin (HTT). A therapeutic strategy for Huntington's disease is to lower the levels of huntingtin in the brain. However, multiple clinical trials have been stopped due to adverse effects. This indicates that there is still a lot that we do not understand about huntingtin.

Similarly to other neurodegenerative diseases like Parkinson's disease, Huntington's disease is caused by cell death in the basal ganglia in the brain. This results in symptoms of the motor systems that facilitate our movements. A hallmark of Huntington's disease is chorea, which are involuntary movements that worsen over time. However, Huntington's disease differs from many other neurodegenerative diseases in that huntingtin is expressed throughout the whole body. Therefore, the mutation in huntingtin not only affects the brain but the whole body. What we know is that when our normal huntingtin protein is mutated it becomes harmful to cells and tissues. In some cases, mutant huntingtin causes cell death. In other cases, huntingtin disturbs or changes the function of the cells; the cells loose important functions or gain new functions that can be harmful. In Huntington's disease there are also memory problems, psychiatric- and metabolic symptoms ("non-motor symptoms"). Several changes occur in the peripheral tissue, for example there is muscle wasting over time (muscle atrophy), heart problems, the production of insulin can deteriorate and there is weight loss that occurs regardless of high caloric intake and/or high appetite. By investigating how body weight and metabolism are affected by the change in huntingtin, new treatment alternatives can be found. A candidate region to study is the hypothalamus in the brain that is central for communication between the brain and periphery. The hypothalamus controls functions such as energy balance and the endocrine system.

Mouse models can be used to study Huntington's disease by reproducing certain features of the disease. We use mice in which a normal copy of the huntingtin gene has been replaced with a mutant copy. In these mouse models, we can see that both tissues in the brain and periphery involved in the control of metabolism are affected by mutant huntingtin, such as the hypothalamus and the adipose tissue. The metabolic symptoms also differ between different mouse models of Huntington's disease.

The thesis consists of three projects, where we have shown that:

- By increasing the levels of mutant huntingtin in the hypothalamus, we can cause early body weight gain
- Higher levels of huntingtin in the hypothalamus causes changes in gene expression
- Certain types of cells in the hypothalamus are more sensitive than others to elevated levels of huntingtin. Brain cells that produce orexin (regulates appetite, sleep, and metabolism) and enzymes that act on histamine and dopamine synthesis are affected early
- Before weight loss, there are changes in body fat, for example a redistribution in cell size from larger to smaller fat cells

# Populärvetenskaplig sammanfattning

Huntingtons sjukdom orsakas av en mutation i genen som kodar för proteinet huntingtin (förkortat HTT). En behandlingsstrategi för att lindra sjukdomsförloppet vid Huntingtons sjukdom är att sänka nivåerna av huntingtin i hjärnan. Dock har flera kliniska prövningar fått stoppas på grund av biverkningar. Detta visar på att där fortfarande finns mycket vi inte förstår kring huntingtin.

Likt andra neurodegenerativa sjukdomar som exempelvis Parkinsons sjukdom, orsakas Huntingtons sjukdom av celldöd i basala ganglierna i hjärnan. Detta resulterar i symptom på det motoriska systemet som styr våra rörelser. Vid Huntingtons sjukdom förekommer korea (engelska: chorea), vilket är ofrivilliga rörelser som blir värre över tid. Huntingtons sjukdom skiljer sig dock från många andra neurodegenerativa sjukdomar i det att huntingtin uttrycks i alla kroppens celler. Därmed påverkas inte bara hjärnan utan hela kroppen av om huntingtin förändras. Vad vi vet är att när vårt normala huntingtin muteras så blir det skadligt för kroppens celler och vävnader. I vissa fall så orsakar muterat huntingtin celldöd, i andra så stör det cellerna, antingen så förlorar de viktiga funktioner eller så kan nya skadliga funktioner uppkomma. I Huntingtons sjukdom förekommer även minnesproblem, psykiatriska och metabola symptom (”icke-motoriska symtom”). Flera förändringar sker i den perifera vävnaden, exempelvis så förtvinar musklerna med tiden (muskelatrofi), man kan få problem med hjärtat, produktionen av insulin kan försämras och man kan drabbas av en viktnedgång som sker oavsett om kaloriintaget och aptiten är hög. Genom att undersöka hur kroppsvikten och metabolismen påverkas av förändringen i huntingtin kan nya behandlingsvägar hittas. En kandidatregion är hypotalamus i hjärnan som utgör vår kommunikationscentral mellan periferi och hjärna. Hypotalamus kontrollerar funktioner såsom energibalans och det endokrina systemet.

För att studera Huntingtons sjukdom kan man använda sig av musmodeller som återskapar vissa delar av sjukdomsförloppet. Vi använder möss där en normal kopia av genen som kodar för huntingtin har ersatts med en muterad kopia. I dessa musmodeller kan vi se att både vävnader i hjärnan och periferin som styr metabolismen påverkas av muterat huntingtin, exempel är hypotalamus och kroppsfettet. De metabola symptomen kan också skilja sig mellan olika musmodeller.

Avhandlingen består av tre delarbeten där vi visat att:

- Genom att höja nivåerna av muterat huntingtin i hypotalamus kan vi orsaka tidig kroppsviktsökning
- Höjd nivå av huntingtin i hypotalamus leder till förändringar i genuttryck
- Vissa typer av celler i hypotalamus är känsligare än andra för höjda nivåer av huntingtin, exempelvis nervceller som producerar orexin (reglerar aptit, sömn och metabolism) samt enzym som verkar vid histamin- och dopaminsyntesen påverkas tidigt
- Innan viktnedgång hos musmodeller så sker förändringar i kroppsfettet, tex sker en omfördelning i cellstorlek från större till mindre fettceller

# Abbreviations

| | |
|---|---|
| AD | Alzheimer's disease |
| ASO | Antisense oligonucleotide |
| ATP | Adenosine triphosphate |
| B3AR | Beta-3-adrenergic receptor |
| BAC | Bacterial artificial chromosome |
| BAT | Brown adipose tissue |
| BBB | Blood brain barrier |
| BDNF | Brain-derived neurotrophic factor |
| BMI | Body mass index |
| CAG | Cytosine-adenine-guanine |
| CART | Cocaine- and amphetamine regulated transcript |
| CRH | Corticotropin releasing hormone |
| DDC | Dopa decarboxylase |
| ER | Estrogen receptor |
| FGF21 | Fibroblast growth factor 21 |
| FSH | Follicle-stimulating hormone |
| GLUT4 | Glucose transporter type 4 |
| GnRH | Gonadotropin-releasing hormone |
| GO | Gene ontology |
| GSEA | Gene Set Enrichment Analysis |
| gWAT | Gonadal white adipose tissue |
| HCRT | Hypocretin neuropeptide precursor |
| HD | Huntington's disease |

| | |
|---|---|
| HDC | Histidine decarboxylase |
| HMGCS1 | 3-hydroxy-3-methylglutaryl-CoA synthase 1 |
| HPA | Hypothalamic-pituitary-adrenal axis |
| HPG | Hypothalamic-pituitary-gonadal axis |
| HTT | huntingtin |
| IGFBP2 | Insulin-like growth factor binding protein 2 |
| IL-6 | Interleukin-6 |
| iWAT | Inguinal white adipose tissue |
| LDLR | Low-density lipoprotein receptor |
| LH | Luteinizing hormone |
| limma | linear models for microarray data |
| MSMO1 | Methylsterol monooxygenase 1 |
| MSN | Medium spiny neuron |
| mHTT | Mutant huntingtin |
| NKB | Neurokinin B |
| PD | Parkinson's disease |
| PGC-1α | Peroxisome proliferator-activated receptor gamma coactivator 1-alpha |
| polyQ | poly-glutamine |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| RISC | RNA-induced silencing complex |
| RMR | Resting metabolic rate |
| siRNA | Small interfering RNA |
| Slc17a7 | Vesicular glutamate transporter 1 |
| SNS | Sympathetic nervous system |
| T2D | Type 2 diabetes |
| TACR3 | Tachykinin receptor 3 |
| UCP-1 | Uncoupling protein 1 |
| WAT | White adipose tissue |

| | |
|---|---|
| wtHTT | Wild-type huntingtin |
| YAC | Yeast artificial chromosome |

# Introduction

## Huntington's disease (HD)

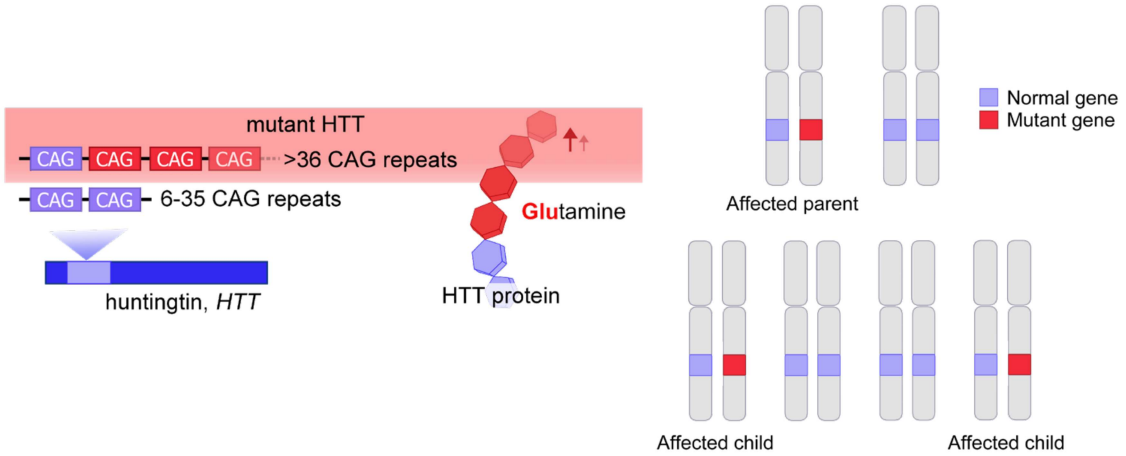


**Figure 1. The mutant HTT (mHTT) gene is inherited through an autosominal dominant pattern.** A CAG repeat expansion of >36 repeats in exon 1 of *HTT* leads to the production of mHTT that contains an abnormally long poly-glutamine (polyQ) tract. HD is an autosomal dominant disease, meaning that inheriting one copy of the mutant gene is sufficient to cause the disease.

HD is a rare neurodegenerative disorder caused by an autosomal dominant mutation in the huntingtin (HTT) gene (Figure 1). A systematic review published by Rawlins and colleagues in 2016 [1] showed a heterogenicity in HD prevalence between geographical locations. The average prevalence per 100 000 was found to be 2.17 in Central and Eastern Europe, 3.60 in Western Europe, 6.68 in the UK and 7.33 in North America. In Asia the average prevalence was calculated to 0.40 per 100000. [1]. In Sweden there are an estimated 1000 people living with HD [2]. However, HD affects more people than the individuals who inherit the mutant copy of HTT. Charlotte, Maria and many other strong and admirable individuals have openly shared their personal stories and reflections of growing up in a family affected by HD [3, 4].

Motor-, cognitive-, psychiatric- and metabolic symptoms are all found in HD and manifest at different disease stages [5]. Pre-manifest HD is characterized by non-motor symptoms and subtle motor symptoms [6-9]. Hyperkinetic involuntary movements (chorea) is the hallmark motor feature of HD and basis for clinical

diagnosis, while impairment of voluntary movements such as bradykinesia and rigidity become more prominent towards the late, manifest stages [10]. Accompanied with cognitive changes, neuropsychiatric- and behavioural symptoms, speech- and communication problems, the motor symptoms severely debilitate the autonomy of the individual over the course of disease [10, 11].

There is currently no cure for HD, nor are there disease-modifying treatments that can slow the disease progression. Symptomatic treatments can be used to manage chorea, depression, and other mood disturbances. However, what is critical to keep in mind is that the impact from HD does not start at the time of recognizing the first symptoms, clinical diagnosis, nor genetic testing.

A foundation for effective disease management is through multidisciplinary teams that can offer care, symptomatic treatment and long-term follow up. Here in Lund at the Skånes universitetssjukhus (SUS) Neurologimottagningen, there is Huntingtoncentrum (https://huntingtoncentrum.se/) led by Prof. Åsa Petersén. Huntingtoncentrum consists of doctors specialized in Psychiatry and Neurology backed up by a multi-professional team of therapists, counsellors, dieticians, nurses, and neuropsychologists.

## Huntingtin (HTT)

*Structure*

HTT is a large protein of size 347 kDa [12]. To compare, hemoglobin, a tetrameric protein that mediates our oxygen transport in the blood, is approximately 64.5 kDa [13]. The mutation in *HTT* that is causative for HD causes the number of cytosine-adenine-guanine (CAG) repeats in exon 1 to expand beyond a certain threshold for pathogenesis [14] (Figure 1). CAG repeats of 40 or more is associated with full penetrance, while 36-39 repeats are considered ‘reduced penetrance’ alleles [15, 16]. The number of CAG repeats is inversely correlated with age of onset [17]. The mutant HTT gene in turn produces a mutant HTT (mHTT) protein that contains an abnormally long poly-glutamine (polyQ) domain.

*Expression*

*HTT* is expressed throughout the whole body and in both neuronal and non-neuronal cells. The highest expression of *HTT* is found in the brain and testis. (Human Protein Atlas, proteinatlas.org, [18])

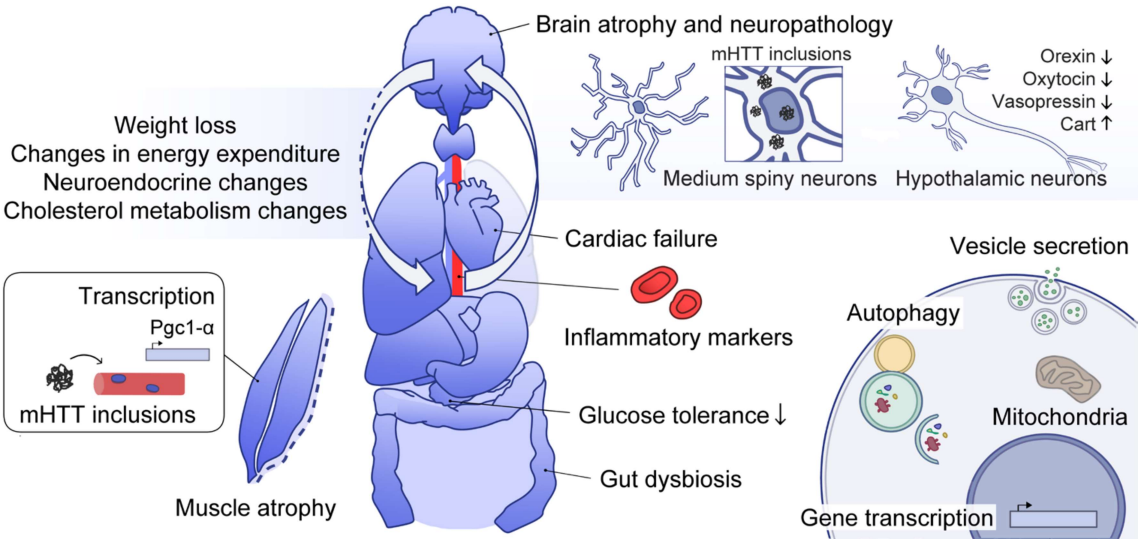


**Figure 2. mHTT causes both central- and peripheral pathology.** Shown are examples of tissues and processes affected. mHTT is prone to aggregate and forms inclusions in neuronal and non-neuronal cells. In clinical HD cases inclusions have been found in skeletal muscle. Sparse inclusions can also be found in different hypothalamic nuclei. Normal HTT (wtHTT) participates in numerous essential cellular functions and in the lower right, a few intracellular functions of HTT is highlighted. Adapted/referenced from [19-23]. mHTT = mutant HTT, wtHTT = wild-type HTT.

*Function: wild-type versus mutant HTT*

Normal HTT (wild-type HTT) has more than 350 different interaction partners [21] and partakes in cellular functions such as vesicle trafficking [24-26], transcriptional regulation [27, 28], autophagy [29, 30], neuronal survival [31-33] and the cell cycle [34, 35]. If you knock out *HTT* during the embryonal stage it is fatal [36], indicating that the gene and protein is involved in vital functions.

Since normal HTT has a vast interaction network and partakes in numerous vital functions of both neuronal and non-neuronal cells (Figure 2), mHTT is associated with both gain- and loss of function mechanisms of pathology [20, 37-42]. Similarly to other neurodegenerative diseases there is selective vulnerability of cells in HD. The medium spiny neurons (MSNs) of the striatum have shown to be particularly vulnerable to mHTT [43-48]. In the hypothalamus that is central for neuroendocrine regulation and responsible for control of metabolism, there is selective loss in neuronal populations (Figure 2) [49-53]. Peripheral tissues also undergo pathological changes in response to the expression of mHTT, an example is skeletal muscle that is characterized by loss in muscle strength, inclusion formation, mitochondrial dysfunction, and transcriptional dysregulation [39, 54-57]. There may be similar mechanisms of mHTT-mediated pathology; genes that are expressed in both the brain and periphery are affected by mHTT, such as Brain-derived neurotrophic factor (BDNF) and Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [31, 58-72]. Furthermore, studies of clinical HD and HD animal models show that mHTT can form intracellular aggregates in both central and peripheral tissues [73-76] (Figure 2). Apart from direct effects of mHTT, several symptoms of HD, such as weight loss despite an increased appetite and

impairment in cholesterol metabolism may originate from a disrupted central-peripheral crosstalk (Figure 2) [77-83].

The loss of normal HTT function and direct effects from mHTT expression could therefore both be key factors in the central- and peripheral pathogenesis of HD. The studies on HTT function can be summarized as: 1) directly investigating normal, wild-type HTT in model systems, and/or 2) investigating how a system changes based on mHTT expression and comparing it to models that express normal wild-type HTT.

However, despite extensive studies, we yet do not fully understand the function and roles of HTT in the body, and this has had particularly detrimental effects in recent clinical trials.

## Silencing HTT

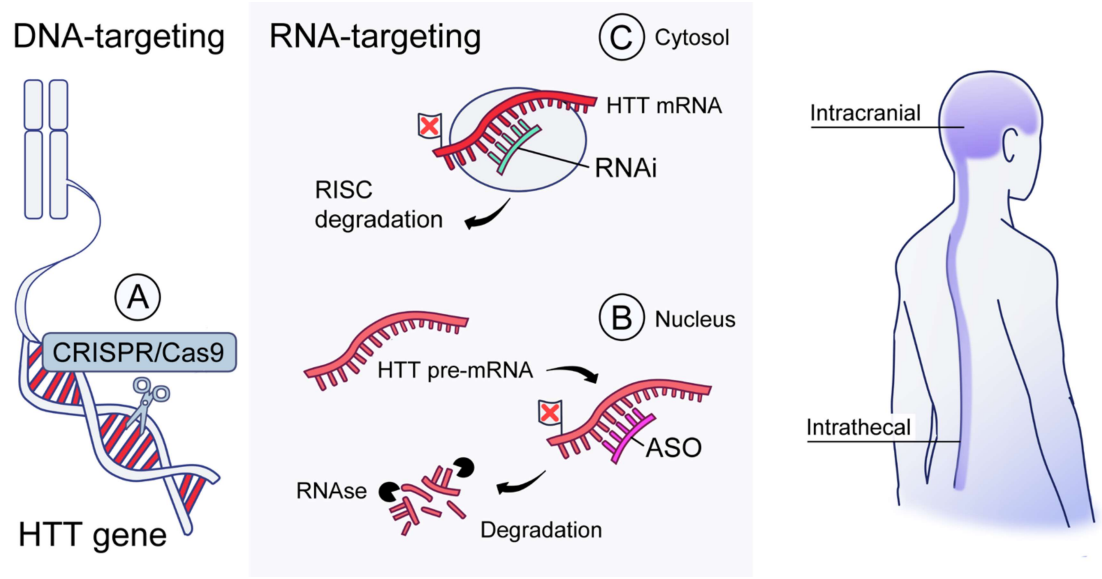


**Figure 3. Strategies of HTT silencing in clinical trials.** A) DNA-targeting therapies include CRISPR/Cas9 that can recognize and bind DNA to either modify transcription or directly edit the HTT gene. B) Antisense oligonucleotides (ASOs) are small, artificial single-stranded DNA sequences designed to target the HTT pre-mRNAs for degradation by endogenous RNAses. C) RNAis such as the small interfering RNA (siRNA) binds to the cytosolic HTT mRNA transcripts and acts as a template for the RISC (RNA-induced silencing complex) to recognize and cleave the mRNA [84]. Common delivery routes for the HTT-lowering therapies are intracranial or intrathecal deliveries. Adapted/referenced from [85].

As shown in Figure 1, HD is a "one gene one disease"; where one copy of the mutant gene inherited from an affected parent causes development of HD. One of the main aims of clinical trials has been to lower levels of HTT, either by targeting the mutant protein or non-specifically (both wtHTT and mHTT) [86-88]. The HTT-lowering therapies target the HTT DNA or RNA [85, 89, 90] (Figure 3). The DNA-targeting compounds either directly target the HTT gene to edit the pathogenic sequences, or directly affect the transcription of HTT. One of the tools used is the CRISPR/Cas9

system, the “gene scissor” where the Cas9 nuclease binds and cuts a DNA sequence specified by guide RNAs [91]. The RNA-targeting therapies include Antisense oligonucleotides (ASOs) and RNA interference (RNAi) that selectively target HTT pre-mRNA or spliced HTT mRNAs. To deliver the HTT-lowering compounds, intracranial (“within the skull”) or intrathecal (injection into the spinal canal) administration are common.

Unfortunately, multiple clinical trials have until today been cancelled or suspended. In light of this and for ongoing clinical trials and studies in HD, there is a great need for further knowledge regarding the function of HTT in the body.

One limitation in the HD research field is that the number of studies on the peripheral tissues are much lower than studies focusing on the brain. A quick search on NCBI PubMed (https://pubmed.ncbi.nlm.nih.gov/) of the number of results since the HTT gene was discovered in 1993 [12] finds 9274 results for the search terms “huntington” and “brain” while “huntington” and “periphery” or “peripheral” yields 847 results. A similar search but replacing “huntington” with “huntingtin” finds respectively 2953 and 189. Furthermore, there are less studies on the non-motor symptoms in HD and how the associated tissues are affected by mHTT.

# Metabolic alterations in HD

Studying metabolic alterations in HD is important for understanding the disease progression and to improve disease management. To understand how mHTT can cause metabolic alterations through both central- and peripheral pathology I will in the following section go through the very basics of metabolism and energy balance, then summarize what we know of altered energy metabolism in HD and finally present experimental findings on mHTT pathology in tissues key for metabolic regulation.

## Metabolism and energy homeostasis

Metabolism is an umbrella term for all the chemical and physical reactions in our body that supply and maintain our constant demand for energy. Energy balance is the state achieved when the amount of energy consumption (food intake) and the amount of energy expended are equalized, commonly illustrated as opposing weights on a scale (Figure 4). Changes in body weight occur when this equilibrium is shifted. A positive energy balance (excess energy in the body) will lead to weight gain while a negative energy balance (deficiency of energy in the body) leads to weight loss. To maintain one’s energy balance in reality is complex due to significant variability between individuals. Approximately 50-70% of our daily energy expenditure comes from the resting metabolic rate (RMR); the amount of

energy that the body needs to maintain all physiological metabolic processes at a basal level, or at rest [92]. RMR is affected by several factors including gender and age; where physiological differences such as body composition and hormones account for some of the differences [92]. The energy balance is further influenced by the capacity of the body to efficiently process nutrients to energy (Figure 4).

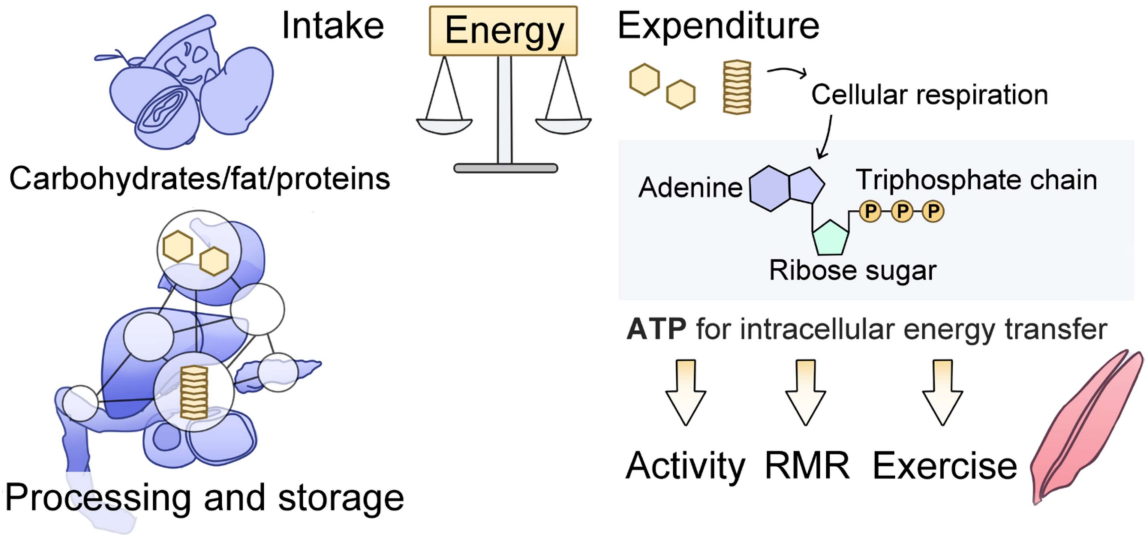


**Figure 4. The basics of energy balance.** Energy consumption can be defined as the energy we retrieve from ingested food, where glucose is the primary source of energy. Glucose is converted to energy through a series of catabolic chemical reactions that are collectively termed cellular respiration. Most of the cellular respiration occurs in the mitochondria of a cell and produces Adenosine triphosphate (ATP). The ATP molecule releases energy when the phosphodiester bonds are broken [93]. Energy expenditure is the combined effect from the resting metabolic rate (RMR), activity, and exercise.

So how does the body monitor the energy demand of all the various tissues and maintain a steady state of energy balance, in practice? First and foremost: it is not a "one-way street" from the brain to body and back. Peripheral tissues such as adipose tissue (fat), muscle, liver and pancreas all maintain whole-body energy balance through diverse and specialized functions. The metabolic reactions can be separated into catabolism ('break down') and anabolism ('build-up'). As an example, insulin is one of the main anabolic hormones in the body and is secreted by the beta cells in the pancreas [94]. After binding to its receptor, insulin promotes recruitment of the Glucose transporter type 4 (GLUT4) to the cell membrane which facilitates the uptake of glucose from the blood into the cells. The glucose can then be stored by converting it to glycogen (glycogenesis) or fat (lipogenesis) [94]. Peripheral tissues also closely communicate the energy state with the brain through discrete signalling mechanisms. The leptin hormone is produced and secreted by adipocytes and acts to suppress appetite via the hypothalamus in the brain [95, 96].

Dysfunction in one or more such tissues can therefore contribute to metabolic dysregulation and disease. Even subtle changes in multiple tissues can be detrimental. Metabolic syndrome, a group of medical conditions, puts people at risk

for developing cardiovascular disease and other metabolic diseases such as Type 2 diabetes (T2D) [97]. Metabolic syndrome is associated with complex pathology involving multiple tissues; it is still debated whether metabolic syndrome results from a common pathological mechanism or distinct mechanisms across tissues [98].

## Altered metabolic control in HD

*Does HTT induce a hypercatabolic state?*

Individuals with HD have been suggested to be in a catabolic state. Calorimetric studies on HD individuals compared with controls have reported higher energy expenditure [99-102] but there are also studies that have reported no change or lower energy expenditure [103, 104].

*BMI and body weight changes*

In 2017, van der Burg and colleagues found an association between body mass index (BMI) and disease progression in clinical HD, in which a higher BMI was associated with slower disease progression [105]. Weight loss is a prominent feature of HD [39, 77-79]. However, several studies show that weight loss is not a consistent feature of HD, nor is weight loss directly causal for disease onset; this suggests that it is not body weight *per se*, but the mechanisms underlying these metabolic changes that contribute to the disease progression [106, 107]. This can further explain the findings of that weight changes occur in HD despite increased appetite and higher caloric intake, which we would normally associate with weight gain [81, 108]. Further, nutritional oral supplements to diet have been tested but do not cause a significant change in a majority of cases [109], and patients in late stages reported to be malnourished [83].

## Pathology in peripheral tissues

HD mouse models show many similarities in pathophysiology to metabolic syndrome, such as body weight changes [110-112], visceral adiposity [111, 113], alterations in adipokine levels and neuroendocrine axes [53, 111, 114, 115], pancreatic insulin deficiency [39, 116, 117]. The R6/2 mouse model exhibit widespread formation of intracellular mHTT inclusions; this includes the peripheral tissues with a role in metabolism [75]. Furthermore, R6/2 mice similarly to clinical HD develop weight loss despite normal or higher caloric intakes, even induced hyperphagic obesity by genetic leptin-deficiency results in weight loss in the R6/2 mice [83, 109, 118, 119]. One tissue that is closely associated with body weight, body composition changes and energy expenditure is the adipose tissue [120-126] which will be elaborated on in the following section.

*The adipose tissue is dysfunctional in HD*

Adipose tissue serves as one of the major energy reservoirs in the body [127]. The cellular structure of white adipocytes has been optimized to facilitate the storage of energy: a unilocular "storage organelle" constitute the majority of the white adipocyte, termed lipid droplets (synonymous "lipid bodies", "adiposomes") [128] (Figure 5). The lipid droplets can store lipids as triacylglycerides and sterol esters [129]. When there is an increasing demand of energy in the body, lipids are released into the bloodstream through a process of hydrolysis (lipolysis). Adipocytes are highly dynamic and can change in size (hypertrophy) or number (hyperplasia) to optimize the storage capacity [130]. This occurs during prolonged states of altered energy balance, e.g. during excessive food intake and obesity. The adipose tissue was long thought as only a site for excess energy storage but in recent years research has established a more complex physiology. Adipocytes take part in endocrine signalling including synthetizing their own hormones that regulate food intake, inflammation, lipid metabolism, and glucose metabolism [131, 132].

Adipose tissue is distributed in distinct locations throughout the body. In terms of white adipose tissue (WAT), the visceral depots are located inside the abdominal cavity while the subcutaneous, "non-visceral" depots are located below the skin [133-136] (Figure 5). There are functional and morphological differences between adipose depots, such as storage capacity [121, 123, 137, 138]. Fat accumulation in the visceral WAT depots is more strongly correlated with risk of metabolic diseases such as T2D [139-141].

In contrast to the structure of WAT, adipocytes of the brown adipose tissue (BAT) have a high number of mitochondria and small lipid droplets [134, 136] (Figure 5). BAT adipocytes express Uncoupling protein 1 (UCP-1) that is located on the inner mitochondrial membrane. UCP-1 facilitates one of the main functions of the BAT, thermogenesis, a process acting to maintain body temperature. UCP-1 uncouples oxidative phosphorylation from the ATP production (Figure 4) and releases the energy as heat [142]. In other words, BAT burns calories and promotes energy expenditure and a negative energy balance, which makes it an attractive target to counteract obesity [143]. Like WAT, BAT can release endocrine factors that may functionally influence tissues in both the periphery and the brain, such as the Fibroblast growth factor 21 (FGF21), Interleukin-6 (IL-6) and Insulin-like growth factor binding protein 2 (IFGBP2) [144].

A second type of thermogenic adipocyte are the beige/brite adipocytes that localize within WAT depots [145]. Despite this, beige adipocytes are structurally more similar to BAT with the multilocular lipid droplets, moderate mitochondrial density and expression of UCP-1 (Figure 5). Expansion of beige adipocytes, a process termed "browning" of WAT can be triggered by cold acclimatization or through stimulation with agonists for Peroxisome proliferator-activated receptor γ (PPARγ) or the β3-adrenergic receptor (B3AR)[145]. "Browning capacity" is also a region-

specific difference for WAT depots, where the subcutaneous depots are considered to have a strong capacity [135].

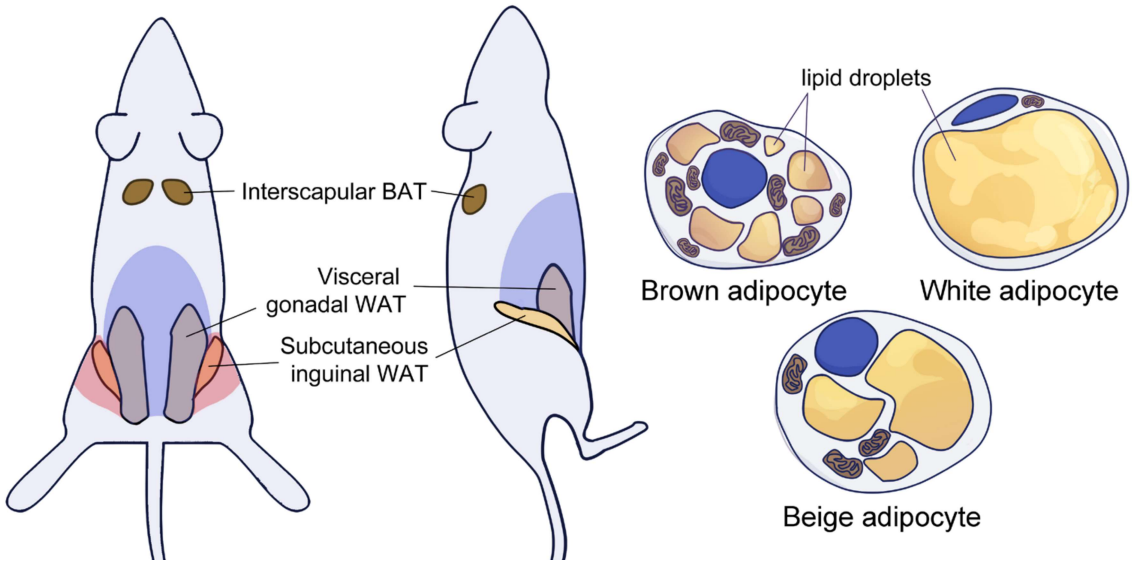


**Figure 5. A simplified illustration of adipose (fat) depots in the mouse.** Displayed on the left are WAT and BAT depots that were the focus of the thesis work. Gonadal WAT (gWAT) is a visceral depot located in the abdominal cavity and is one of the largest WAT depots in the mouse. The inguinal WAT (iWAT) is a subcutaneous depot (located below the skin). The interscapular brown adipose tissue (BAT) is the largest BAT depot in the mouse and situated between the shoulderblades [146]. On the right, the three types of adipocytes are shown. Brown, beige and white adipocytes are distinguishable based on structural features such as mitochondrial content, lipid droplet number and size, and Uncoupling protein 1 (UCP-1) expression. Referenced/adapted from [134-136].

Studies in animal models indicate that mHTT expression has a deleterious effect on the adipose tissue [113, 147-149]. Transgenic Drosophila models that express mHTT exon 1 in the fat body or exclusively in neurons exhibit progressively smaller size of- and decline in lipid droplets and lipid levels, an effect that follows the pattern of weight loss [147, 148]. Mouse models show progressive and distinct alterations in WAT mass and adipokine levels [111, 114]. The R6/2 mice that are prone to weight loss exhibit several changes in the adipose tissue, such as an altered response to isoprenaline to stimulate lipolysis, indications of WAT browning, higher adiposity relative to their body size, and transcriptional dysregulation of key genes involved in adipocyte differentiation and function, [113, 114, 119, 150]. Moreover, even when the R6/2 mice are on a normal chow diet, their adipocytes become larger in size than WT mice [113].

## Pathology in brain regions related to energy metabolism

*Early changes in hypothalamus precede clinical diagnosis*

The hypothalamus is the main region in the brain responsible for monitoring several homeostatic networks [151]. In whole-body energy homeostasis, it actively

coordinates the energy state by integrating signals from the periphery, such as assessing levels of glucose and fatty acids in the circulation, and signalling molecules secreted by peripheral tissues such as leptin from adipocytes (Figure 6) [151, 152]. The hypothalamus is a neuroendocrine organ that together with the pituitary gland provides key signalling for several endocrine systems acting on target tissues (Figure 6), such as the hypothalamic-pituitary-adrenal axis (HPA) that controls the release of cortisol, and the hypothalamic-pituitary-gonadal (HPG) axis for the release of Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH). These axes are essential for control of the immune system, cardiovascular system, metabolic system, reproductive system and central nervous system [153]. Hypothalamic neurons are also tightly innervated with other brain areas [154, 155], and peripheral tissues [156, 157] (Figure 6). The hypothalamus consists of multiple nuclei that can be grouped based on their location [151]. Multiple hypothalamic populations coordinate the control of whole-body metabolism and can be grouped according to whether they are anorexigenic (appetite-suppressing) or orexigenic (appetite-increasing) [158].

One key region for metabolic regulation in the hypothalamus is the arcuate nucleus (ARC). The ARC is located closely to the median eminence, where the blood brain barrier (BBB) is more "leaky" and therefore facilitates transport of metabolic, nutritional and hormonal signals from peripheral tissues [159]. In the ARC there are two distinct and functionally antagonistic populations of neurons (Figure 6). The orexigenic Neuropeptide Y (NPY)- and Agouti-related peptide (AgRP)-expressing neurons stimulate food intake and reduce energy expenditure [156, 159-163]. The anorexigenic Pro-opiomelanocortin (POMC) and Cocaine and amphetamine regulated transcript (CART) neurons decrease food intake and elevate energy expenditure [156, 159, 163-165]. These two populations (NPY/AgRP and POMC/CART) exert their effects by projecting to second-order neurons in other hypothalamic areas, such as the paraventricular nucleus (PVN) that is strongly associated with relaying the sympathetic outflow of the ARC neurons to other neuronal circuits outside the hypothalamus and peripheral tissues [159]. Another population of neurons that is involved in regulating feeding behavior are the orexin neurons in the lateral hypothalamus (LH) [166]. Orexin neurons are involved in a wide variety of processes such as the sleep/wake cycle but has also been shown to interact with the NPY/AgRP and POMC/CART neurons to regulate feeding behavior and energy expenditure [166-169]. Recent studies have also implicated orexin in adipose tissue thermogenesis [157, 163, 170, 171].

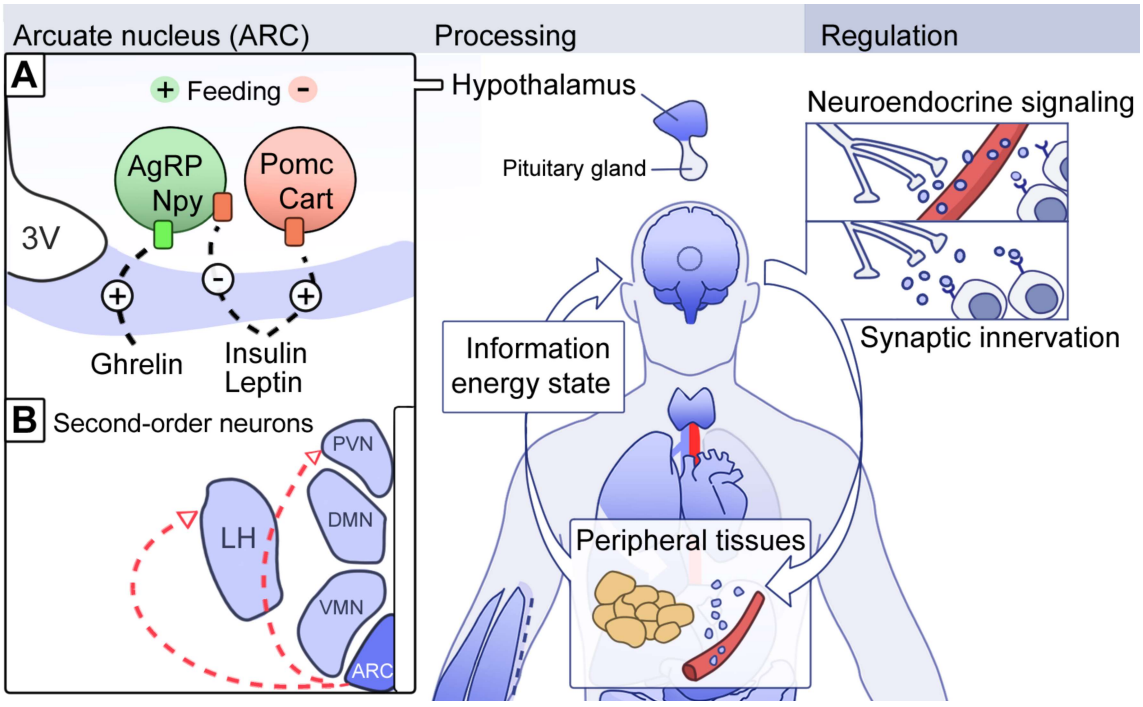


**Figure 6. The hypothalamus regulates whole-body energy homeostasis through a close crosstalk with the periphery.** Peripheral tissues release factors to communicate regarding the energy state back to the hypothalamus. A) Two antagonistic neuronal populations in the arcuate nucleus (ARC) of the hypothalamus regulate feeding behavior and energy expenditure. The AgRP/NPY neurons are stimulated by the gut peptide ghrelin to promote feeding. In contrast, insulin released by the beta cells of the pancreas and leptin released by the adipose tissue inhibits AgRP/NPY neurons and instead stimulates the POMC/CART neurons to suppress feeding. B) The signal from the ARC is then relayed through second order neurons in the hypothalamus. Both neuroendocrine signaling (hypothalamic-pituitary input to endocrine glands for the release of hormones) and synaptic innervation is utilized by the hypothalamus to regulate whole-body energy metabolism. Adapted/referenced from [152, 159, 172]. PVN = paraventricular nucleus, VMN = ventromedial nucleus, DMN = dorsomedial hypothalamic nucleus, LH = lateral hypothalamus.

The hypothalamus is central for the regulation of multiple processes that are altered in HD. In particular the non-motor symptoms could be the result of mHTT-induced pathology in the hypothalamus [7]. In the HD brain, changes in the hypothalamus can be detected before the onset of motor features and clinical diagnosis, and there is selective loss of neuropeptide-expressing neurons (Figure 2) [49, 50, 115, 173-178]. Furthermore, studies in clinical HD and HD animal models show alterations in the HPG and HPA neuroendocrine axes [51, 53, 115, 153, 179-185]. Hypothalamic dysfunction is also present in other neurodegenerative diseases such as Alzheimer's disease (AD) [186]. As will be further elaborated on in a following section, common pathological features of HD can be recapitulated in mice by selectively overexpressing mHTT in hypothalamic neurons [111, 182]. Moreover, in the transgenic BACHD mouse model of HD, silencing of mHTT in hypothalamus can prevent development of certain disease phenotypes [111, 182].

## Evidence for disrupted central-peripheral crosstalk

While we can divide studies of metabolic alterations in HD into two perspectives: 1) Studying effects of mHTT in energy-controlling regions of the brain, and 2) studying the direct effects of mHTT in peripheral tissues important for metabolic control, it does not fully take into account the core principle of the metabolic system. As seen for the hypothalamus, the brain and periphery are closely communicating with one another to coordinate energy homeostasis. Whole-body energy metabolism changes in HD could therefore be a result from disturbed crosstalk due to dysfunction in both CNS and non-CNS tissues. Therefore, in addition to more studies on peripheral tissues that was discussed further above, there is also a need for more studies on the role of HTT and mHTT in candidate areas involved in central-peripheral crosstalk, such as the hypothalamus. This is especially important to consider in light of the HTT-lowering therapies as the current state of the art for HD therapeutic intervention and the unfortunate events leading to multiple suspensions. One example discussed by my colleagues [7] is that the preclinical studies of HTT-lowering therapies have not focused on target engagement in the hypothalamus nor how this area responds to HTT lowering [7]. Not only are non-motor symptoms crucial to consider for disease burden and management, but there may also be several important underlying mechanisms that are being missed out, especially considering the associations between BMI and disease progression in HD [105, 106]

## Transgenic mouse models develop diverse metabolic phenotypes

Studies in animal models of HD suggest that both wtHTT and mHTT play a role in metabolism. Transgenic mouse models of HD can be divided into two groups based on the HTT transgene: short-fragment and full-length models. The ubiquitous expression of different fragment lengths of HTT result in diverse metabolic phenotypes. More details regarding each HD model and HTT constructs can be found in *Key methodology*.

*Full length models show weight gain*

Transgenic mouse models with expression of a full-length HTT fragment are associated with body weight gain and body fat gain.

The BACHD mouse model develops obesity characterized by progressive, early weight gain and resistance to leptin, a hormone released by adipocytes that acts to reduce appetite [187]. Silencing mHTT exon 1 in only hypothalamus during the early stages in BACHD mice is sufficient to prevent their obese phenotype [111]. While there is significant late-onset neuropathology in striatum and cortex [110], the BACHD hypothalamus does not develop any significant atrophy, microglial

activation (Iba-1 reactivity) or neuronal loss [111]. However, there is early transcriptional dysregulation of hypothalamic neuropeptides [7, 182].

Another full-length model of HD are the YAC ("yeast artificial chromosome")-mediated transgenic mice [188]. The YAC18 mice express a full-length wtHTT transgene (18 CAG repeats) while the YAC128 mice express a full-length mHTT transgene (128 CAG repeats) [188-190]. Studies in YAC mice showed that both wtHTT and mHTT can cause body weight gain and that there is a dose-response effect for the levels of HTT [191].

*Short-fragment expressing R6/2 mice exhibit progressive weight loss*

R6/2 mice develop progressive motor dysfunction and neuropathology reminiscent of clinical HD [112, 191-193]. R6/2 mice have further shown formation of mHTT inclusions in the brain but also in the periphery, including in skeletal muscle, cardiac muscle, liver, the stomach wall and pancreas [75]. The metabolic phenotype in R6/2 mice changes progressively; early in disease there are observations of higher adiposity, and R6/2 adipocytes become larger than WT adipocytes when the mice are on a high fat diet [113]. Towards the mid-end stage of disease there is onset of weight loss and diabetes [194-196]. Furthermore, R6/2 can exhibit increased metabolism and white adipose tissue browning [119, 150] which promote higher energy expenditure and negative energy balance. Varying the number of CAG repeats in the mHTT transgene of R6/2 mice modifies the rate of disease progression [194]. The R6/2 cohorts used for this thesis have a longer repeat compared to (CAG)115-(CAG)150 and have a slower rate of disease progression and onset of weight loss [118, 194].

## HTT overexpression in hypothalamus causes weight gain

It has been previously shown that WT mice with selective overexpression of mHTT in hypothalamus exhibit a metabolic phenotype characterized by hyperphagic obesity with higher adiposity that is reminiscent of BACHD mice [111]. Overexpression of mHTT in hypothalamus also causes early downregulation of hypothalamic neuropeptides [111]. In contrast, the effect from wtHTT overexpression in hypothalamus of WT mice and body weight is more variable; either the effect is more delayed and less pronounced than mHTT, or there is no effect [111, 197].

# Experimental outline

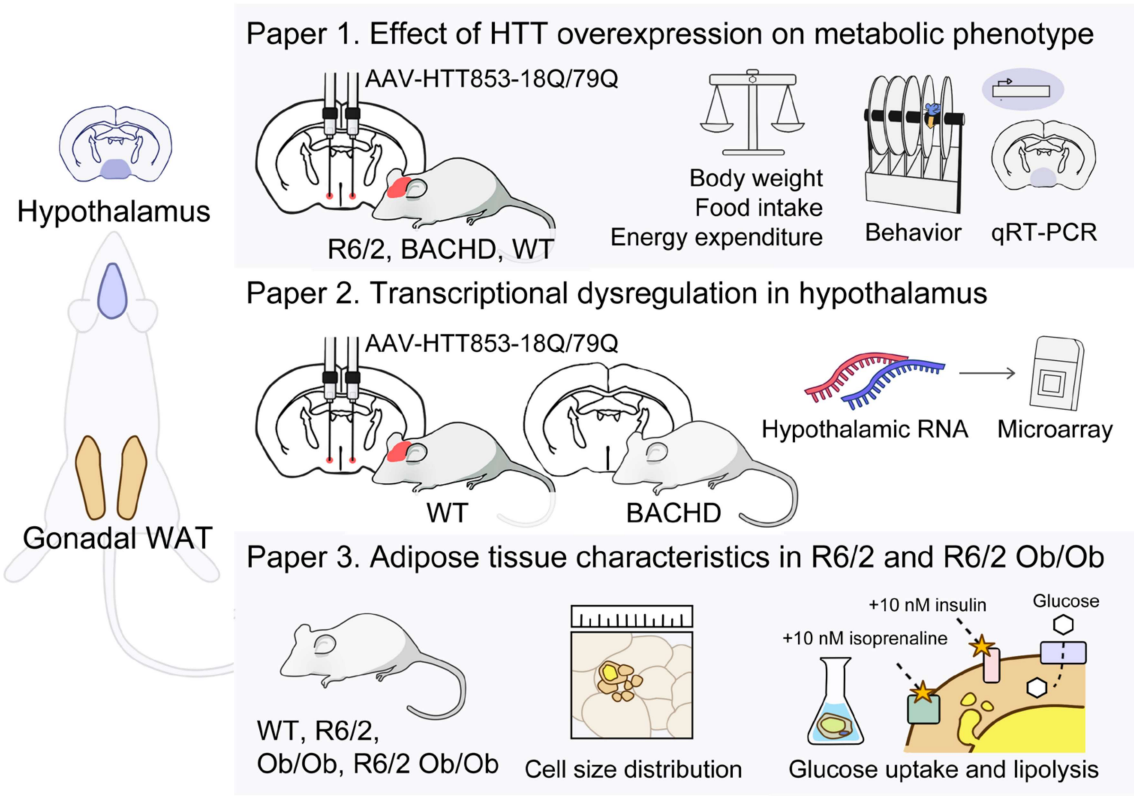


**Figure 7. Overview of Paper 1-3.** In Paper 1, R6/2 and BACHD mice of both genders had their HTT levels in hypothalamus increased through targeted viral-vector mediated overexpression of wtHTT (AAV-HTT853-18Q vector) or mHTT (AAV-HTT853-79Q vector). We then assessed the effect on metabolic and behavioral phenotypes and mRNA levels of hypothalamic neuropeptides. WT littermates that unlike the HD models have no transgenic mHTT expression throughout the body, were used as control groups. In Paper 2, we performed transcriptomics of hypothalamus in two HD models with weight gain (WT mice with mHTT overexpression and BACHD mice). In Paper 3 the visceral gonadal WAT (gWAT) depot was collected and processed for analysis of fixed tissue (cell size distribution) or in cell suspensions (insulin-stimulated glucose uptake and isoprenaline-stimulated lipolysis). For further details regarding the methods, see *Key methodology*. WAT = white adipose tissue.

We hypothesize that HTT, both wtHTT and mHTT, play a role in metabolism, and that mechanisms underlying metabolic changes contribute to the disease progression in HD. As described in *Introduction*, in HD there are limited studies on tissues involved in metabolic control. This is despite that candidate areas involved in whole-body metabolic regulation, such as the hypothalamus, are shown to be affected early [7, 177].

Paper 1 and 2 focuses on the role of HTT in hypothalamus and how mHTT-induced hypothalamic changes affect central-peripheral crosstalk and disease features. It has previously been observed that overexpression of mHTT in hypothalamus of WT mice caused a change in the metabolic phenotype [111]. In Paper 1 we used the same strategy of HTT overexpression using HD mouse models. Paper 2 specifically focused on transcriptional dysregulation as a key mechanism of pathology by mHTT, where we used transcriptomics to perform in-depth assessment of transcriptional changes in hypothalamus of HD models (Figure 7).

In Paper 3 we elaborated on peripheral changes that occur in R6/2 mice just prior to expected weight loss, focusing on mechanisms that underly the switch from a metabolic state in balance to a catabolic state. We focused on characterizing changes in the adipose tissue given its role in maintaining the whole-body energy supply and findings of adipose tissue dysfunction in R6/2 mice [113, 114, 198]. Paper 3 was the first step in characterizing how the R6/2 adipose tissue responds to hyperphagia and morbid obesity through crossbreeding with the Ob/Ob model of obesity (see Key *methodology*) (Figure 7) [118].

# Aims

Paper 1.
To characterize how the disease phenotype changes when levels of wild-type HTT (wtHTT) and mutant HTT (mHTT) are significantly increased in hypothalamus

Paper 2.
To delineate transcriptional changes in hypothalamus in mouse models with the shared feature of weight gain and increased food intake

Paper 3.
To characterize changes in adipose tissue that precede weight loss in the R6/2 mouse model

# Key results and discussion

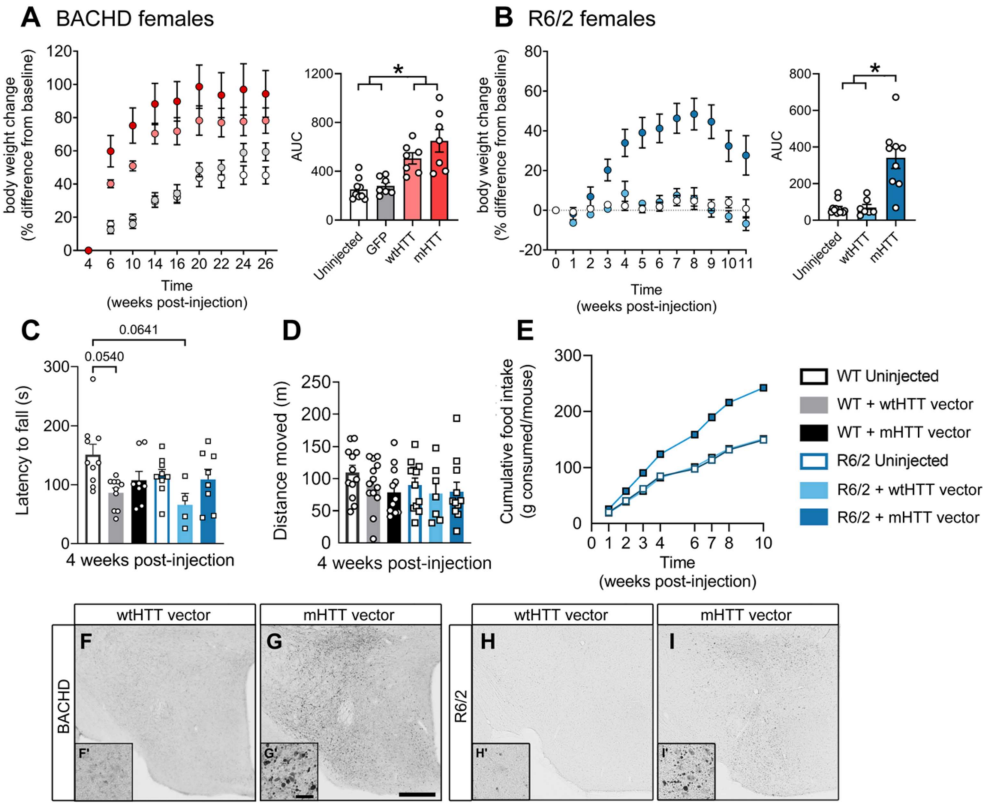


**Figure 8. Viral vector-mediated overexpression of mHTT in hypothalamus causes weight gain, increased food intake and inclusion formation in the hypothalamus.** Based on data from Paper 1 [199]. A-B) time-course body weight data expressed as the percentage (%) difference from baseline. Baseline is defined by the first timepoint of body weight measurement, which was at week 4 post-injection for BACHD (A) and week 0 post-injection for R6/2 (B). The area under curve (AUC) was calculated from the respective time-course body weight graphs. Statistical significance for the AUC was determined using a parametric one-way ANOVA (A) or a non-parametric Kruskal-Wallis test (B). *p < 0.05. C-D) Behavioral analyses were performed in the R6/2 experimental groups at 4 and 8 weeks post-injection, shown are the results from 4 weeks post-injection (period of early weight gain) compared to the WT experimental groups. C) The rotarod test that assesses motor coordination. D) The open field test, where the total distance moved during 60 min was used as a measure of activity. Mean ± SEM. E) Food intake was measured in the R6/2 groups using a home-cage setup (see *Key methodology*). F-I) Histological assessment of the hypothalamus after viral vector overexpression. Scale bars indicate 200 µm and 20 µm. F) BACHD + wtHTT vector, G) BACHD + mHTT vector, H) R6/2 + wtHTT vector and I) R6/2 + mHTT vector. wtHTT and mHTT refer to the wild-type HTT overexpression vector (18 CAG repeats) and mutant HTT overexpression vector (79 CAG repeats).

## HTT overexpression in hypothalamus causes weight gain

In transgenic models of HD, there are differences in body weight phenotypes; short-fragment models are associated with weight loss and full-length models with weight

gain (see Introduction). This diversity may result from that full-length mHTT and short fragment mHTT elicit different effects in tissues involved in metabolic control. Paper 1 is the first study examining metabolic effects of HTT overexpression in hypothalamus in both the short-fragment model, R6/2, and the full-length model BACHD [199]. Interestingly, overexpression of mHTT in hypothalamus caused early body weight gain in both BACHD and R6/2 mice (Figure 8A-B). In contrast, hypothalamic wtHTT overexpression had different impacts in the models. In BACHD wtHTT overexpression induced weight gain, although to a lesser extent than mHTT overexpression (Figure 8A). In R6/2 mice there was no effect of wtHTT overexpression (Figure 8B). For the R6/2 mice we performed follow-up analyses to find factors contributing to the early weight gain phenotype after mHTT overexpression. Reduced activity could affect body weight, especially considering that R6/2 mice exhibit a progressive decline in motor capacity [112]. However, during the period of early weight gain at 4 weeks post-injection, we found no significant difference in motor coordination or activity between R6/2 mice with mHTT overexpression and uninjected R6/2 or uninjected WT mice (Figure 8C-D). Upon food intake analyses in home cages we found that the cage with R6/2 mice overexpressing mHTT had a ~68% higher cumulative food intake than the cage with uninjected R6/2 mice (Figure 8E). In contrast, the cage with R6/2 mice overexpressing wtHTT had a ~1% difference in cumulative food intake compared with uninjected mice.

In line with previous studies [111, 197], the effect of mHTT overexpression on body weight is more pronounced than that of wtHTT. One factor that could explain the difference between the effects of wtHTT and mHTT in hypothalamus is that overexpression of mHTT causes inclusion formation in hypothalamic neurons [111]. HTT inclusions are a pathological hallmark of clinical HD [76, 200, 201]. Histological assessment showed that while wtHTT overexpression is associated with diffuse cytoplasmic staining in hypothalamus, mHTT overexpression in HD models causes widespread formation of HTT inclusions (Figure 8F-I). Conducting a control experiment using the same methodology but instead overexpressing GFP in the hypothalamus did not result in an effect on body weight in the BACHD mice (Figure 8A).

Taken together, results indicate that normal HTT exert an effect on body weight through hypothalamic pathways. One hypothesis for the metabolic phenotypes in HD models is that specifically full-length HTT, both wtHTT and mHTT, play a role in body weight. This is based on that the full-length models, such as BACHD, express two endogenous copies of full-length wtHTT and then additional copies of transgenic full-length mHTT (to generate the disease phenotype); thus increasing the total levels of full-length HTT in the body [110, 191]. The function of HTT in body weight regulation may be lost in the short-fragment models that, in contrast to full-length models like BACHD, express a truncated fragment of HTT. In Paper 1 we reported that the effect of hypothalamic wtHTT overexpression in R6/2 mice

was inadequate to significantly affect their body weight (Figure 8B). R6/2 mice similarly to clinical cases of HD are suggested to be in a hypermetabolic state [119], and therefore the effects from wtHTT overexpression may have been masked by peripheral mHTT effects. In Paper 1 we further found that all R6/2 groups, regardless of body weight and food intake patterns, there was an onset of characteristic weight loss (Figure 8B). This could result from that the influence of HTT overexpression in hypothalamus, including mHTT, become masked by the peripheral mHTT effects as they become increasingly more dominant as the disease progresses.

## While decreasing in body weight, R6/2 females with mHTT overexpression in hypothalamus have higher body fat composition

In Paper 1, while hypothalamic overexpression of mHTT in R6/2 females was insufficient to prevent the onset of weight loss, we found distinct effects in the adipose tissue [199]. Compared to uninjected R6/2 and R6/2 with overexpression of wtHTT, the composition of body fat was ~12% higher in R6/2s overexpressing mHTT (Figure 9A). In a smaller group of female R6/2 mice, we therefore decided to weigh the visceral gWAT and the subcutaneous iWAT to see whether there were regional differences. While both gWAT and iWAT weights increased in the R6/2 overexpressing mHTT, the gWAT had the highest change and this was accompanied with gene expression changes (Figure 9B). There was downregulation of *Pparγ* that plays a key role in the adipocyte function, including the upregulation of genes involved in fatty acid metabolism and triglyceride storage [202] (Figure 9C). We further found that the *B3ar* with roles in adipose lipolysis and thermogenesis [203] was downregulated in gWAT, while no change in *B3ar* was found in BAT (Figure 9C-D). By looking into gene expression changes in the hypothalamus we could see that specifically mHTT overexpression reduced levels of *Bdnf* and appears to accelerate the loss of orexin (Figure 9E). Other hypothalamic neuropeptides such as *Pomc* were similar to the R6/2 uninjected mice (Figure 9E). A study published in Nature 2020 by Wang and colleagues [204] found that sympathetic nervous system (SNS) innervation of adipose tissue is regulated by leptin signalling through AgRP and Pomc neurons in the ARC and downstream Bdnf-expressing neurons in the PVN [204]. Not only did the study implicate a role for Bdnf in energy expenditure but also a functional link to the periphery. Bdnf has also been implicated in satiety and feeding and interact with other anorexigenic factors [205, 206]. However, these effects could either be due to HTT overexpression alone or the manifested obesity phenotype, or a combination of the two. Nonetheless, it is an interesting finding as it would implicate that mHTT-induced dysregulation of Bdnf may elicit additional pathological effects in HD due to its depletion in hypothalamus.

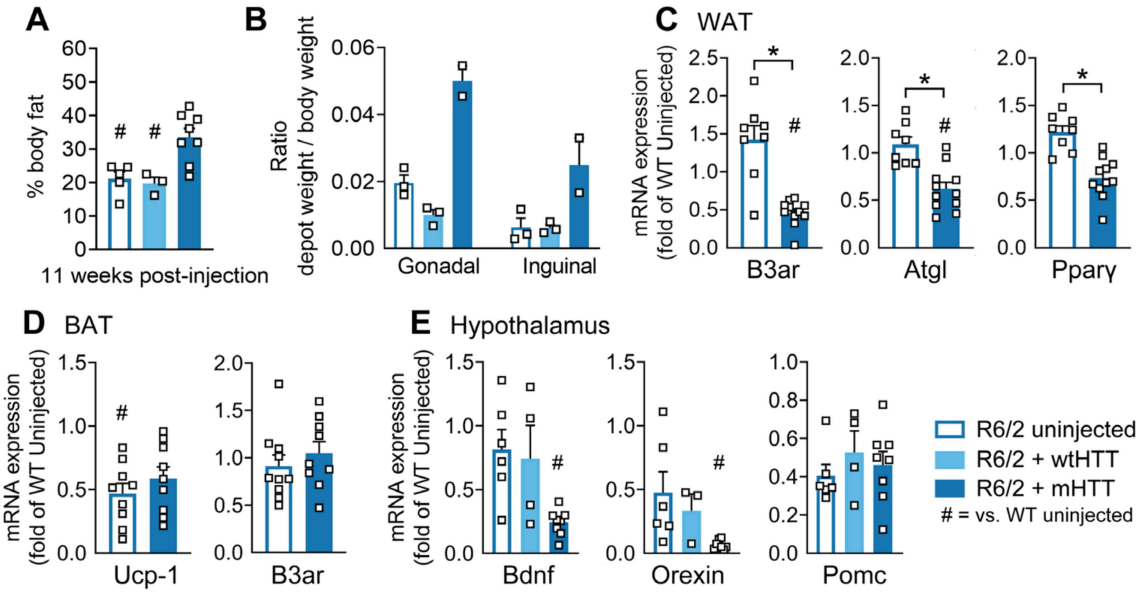


**Figure 9. R6/2 mice with hypothalamic mHTT overexpression display increased adiposity and distinct gene expression changes in adipose tissue and hypothalamus.** Analyses were performed at 11 weeks post-injection, a timepoint when body weights were decreasing in the R6/2 mice. Graphs are based on data from Paper 1 [199], where statistical comparisons were performed including the WT groups, while only the R6/2 groups are shown in the graphs above. Therefore, # denotes a significant ($p < 0.05$) difference versus WT uninjected mice. A) Percentage (%) body fat measured by DEXA. B) WAT depot weights in female R6/2, expressed as the ratio of depot weight to body weight. C) qRT-PCR analysis of the gWAT and D) BAT, shown are uninjected R6/2 and R6/2 with overexpression of mHTT. E) qRT-PCR analysis in hypothalamus. Mean ± SEM. DEXA = Dual-energy X-ray absorptiometry. gWAT = gonadal white adipose tissue, a visceral depot. iWAT = inguinal white adipose tissue, a subcutaneous depot. BAT = brown adipose tissue.

As we in Paper 1 found that HD mice with hypothalamic wtHTT and mHTT overexpression develop distinct metabolic phenotypes and pattern in gene expression, we next aimed to elaborate on this in more detail:

- Is the effect of wtHTT and mHTT overexpression similar in the hypothalamus, or are there distinct gain-or-loss of function mechanisms from mHTT?
- What happens in hypothalamus during the early stages of overexpression?
- Can we delineate the hypothalamic networks that contribute to the weight gain phenotype in full-length transgenic models of HD (BACHD)?

## HTT overexpression in hypothalamus affects sterol- and cholesterol genes

Transcriptional dysregulation by mHTT is one key pathological mechanism that has been extensively studied in both the striatum of HD mice and caudate in clinical HD [27, 207-213]. Dysregulation of distinct genes in hypothalamus has also been found in HD mouse models [111, 182, 214]. However, unlike the striatum there were no

large-scale gene expression analyses performed in the hypothalamus. This is despite that changes in hypothalamus and transcriptional dysregulation are both found to be early hallmarks of HD [7, 53, 177]. Comparing mice with HTT overexpression in hypothalamus to age-matched uninjected mice is complicated in that the transcriptome could be affected by several factors: the intracranial injections, the transgene expression itself, and effects related to HTT. One key analysis in Paper 2 was therefore to identify shared- and unique significant genes between the mHTT vs WT and wtHTT vs WT datasets (see *Key methodology*). In the shared gene list, 410 genes out of the total 449 had a positive fold change, indicative of upregulation [215]. Included with multiple immune system/inflammation clusters in the top 10 output was a cluster consisting of sterol- and cholesterol-related terms (Figure 10). Thirteen genes in this cluster, such as Low-density lipoprotein receptor (*Ldlr*) were significantly downregulated. Alterations in cholesterol metabolism is found in HD, and mHTT-induced transcriptional dysregulation in the striatum of HD mice affects genes involved in cholesterol metabolism, including *Ldlr* [80, 216-219].

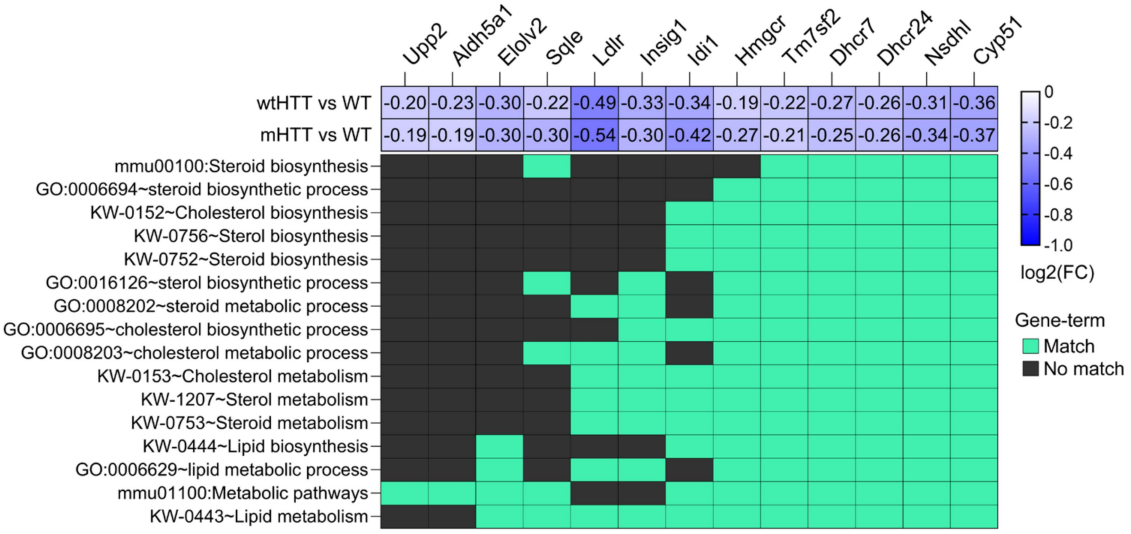


**Figure 10. A set of 37 differentially regulated genes related to cholesterol and sterol metabolism are found in mice with overexpression of HTT in hypothalamus.** Data from Paper 2 [215] showing the 13 genes with log2(FC) < 0 (indicating downregulation) in the sterol- and cholesterol cluster identified by DAVID functional annotation clustering. For each gene, the respective log2(FC) values from each dataset are provided as well as the distribution of gene-term matches. DAVID = Database for Annotation, Visualization and Integrated Discovery. wtHTT and mHTT refer to the wild-type HTT overexpression vector (18 CAG repeats) and mutant HTT overexpression vector (79 CAG repeats).

Our results show that vulnerability to HTT-induced transcriptional dysregulation of cholesterol-related genes is not confined to cells in the striatum [80, 216, 217]. Cholesterol is a central component of the myelin sheath and required for synapse development and neurotransmission [220-225]. Because of the BBB, most of the cholesterol in the brain is generated through local synthesis [226]. Moreover, the brain can synthetize steroids, termed neurosteroids that regulate the development and function of neurons and glia [227], and there is evidence of interactions between peripheral steroids with neurosteroid synthesis in hypothalamus (reviewed in [228]).

Therapies aiming to restore cholesterol metabolism in the brain are being evaluated as potential disease-modifying strategies in HD, and cholesterol-derived compounds are being explored as complementary biomarkers for early neurodegenerative changes [229-235]. An interesting study published by Benraiss et al in 2021 [236] showed that there are differences in the glial transcriptome between the R6/2 mice and the full-length model zQ175 where specifically the truncated HTT inhibits the glial cholesterol pathway [236]. As discussed by the authors, there is regional heterogeneity in the astrocyte population which was hypothesized to be a contributing factor to the selective vulnerability of brain regions such as the striatum to mHTT [219, 237]. More work will be needed to elaborate on the functional implications of altered sterol- and cholesterol genes in hypothalamus in HD, and whether there are similar mechanisms and shared targets of mHTT between the striatum and hypothalamus.

## Selective neuropathology after mutant HTT overexpression in the hypothalamus

In Paper 2 we next considered whether there are effects on the hypothalamic transcriptome that is specific to mHTT overexpression. For the mHTT vs. wtHTT comparison we found zero genes that passed an adj.p < 0.05 (adjusted p-value based on multiple testing). However, using Gene Set Enrichment Analysis (GSEA) [238] to interpret gene expression changes in the context of biological processes and/or pathways found that overexpression of mHTT had a more pronounced suppressive effect on the neuroendocrine system (Figure 11A). Using qRT-PCR we could confirm that during an early stage (4 weeks post-injection) of mHTT overexpression the orexinergic, anorexinergic and histaminergic systems are affected. *Hcrt* and *Cart* neuropeptides were significantly downregulated as well as enzymes involved in catecholamine synthesis: Histidine decarboxylase (*Hdc*) and Dopa decarboxylase (*Ddc*) (Figure 11B-C). However, since both orexinergic and anorexinergic systems are affected, the question is whether specific hypothalamic neurons drive the metabolic phenotype. While there is a complex integration between multiple hypothalamic regions, discrete hypothalamic populations can orchestrate coordinated control of physiological states, including feeding [239, 240]. While activation of Pomc neurons alone can rapidly inhibit feeding behavior in mice, simultaneous stimulation of Pomc and AgRP-expressing neurons instead triggers pronounced feeding behavior [241]. With this in mind, despite the widespread suppression of hypothalamic genes that we see in mice overexpressing mHTT, the summation of the transcriptional changes appears to be the early increase in body weight and food intake that we saw in Paper 1 (Figure 11D). By using a combination of viral and transgenic strategies, Stanley and colleagues [242] mapped a hypothalamic circuitry consisting of neurons from multiple regions of the hypothalamus that all project to both the liver and adipose tissue. Neuronal

subpopulations of the PVN that express oxytocin and Corticotropin releasing hormone (Crh) were suggested to be the point of integration of the circuitry to modulate metabolic activity in these tissues. As discussed by the authors, further work should be aimed to investigate whether activation of exclusively these upstream "hierarchical"/"command-like" neurons is sufficient to regulate metabolic control [242]. Silencing or inducing mHTT expression to delineate distinct hypothalamic populations responsible for the metabolic phenotype in HD models has been performed in leptin-expressing neurons and Sim-1 neurons [183, 243]. By using novel tools such as optogenetics and chemogenetics that allow for specific activation or suppression of cells, we may further delineate contributions of distinct hypothalamic neurons to metabolic control and link to metabolic dysfunction in HD [244, 245].

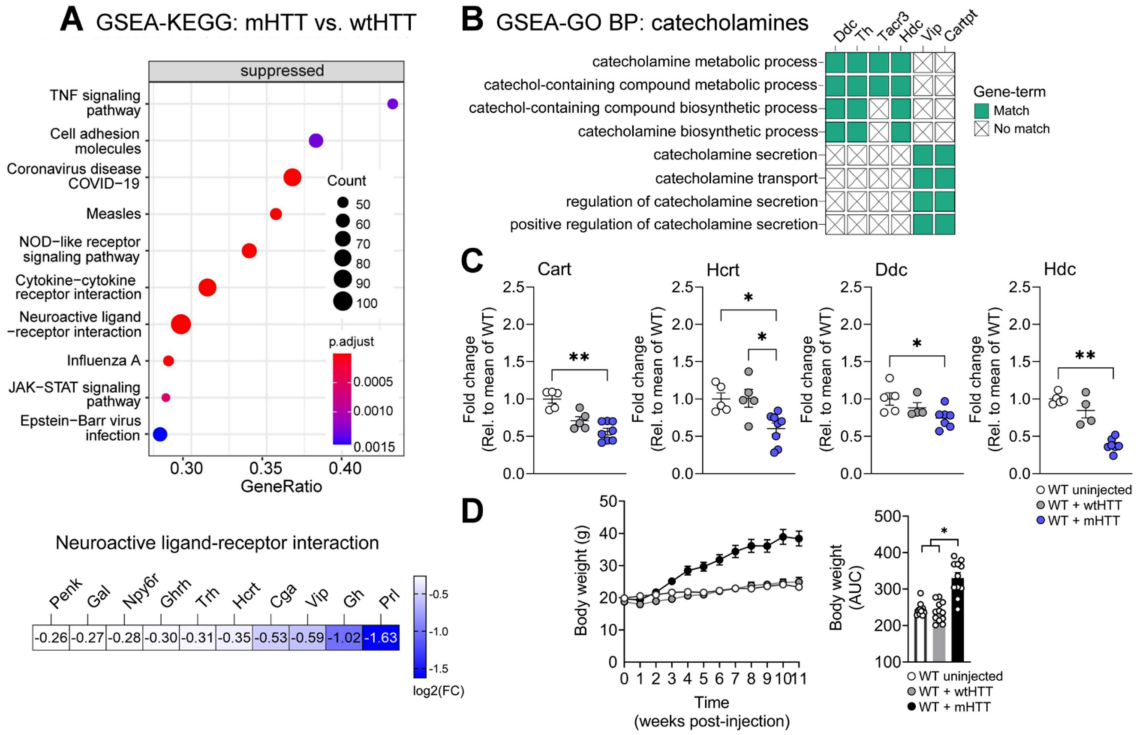


**Figure 11. mHTT overexpression in hypothalamus causes downregulation of enzymes involved in histamine and dopamine synthesis.** A) GSEA of KEGG pathways indicated a number of suppressed pathways that were differentially altered in mHTT-overexpressing mice compared to wtHTT-overexpressing mice, including "Neuroactive ligand-receptor interaction" (shown are log2(FC) values for the top 10 genes with the highest change) B) Catecholamine-related processes were enriched in the GSEA-GO BP (biological process) analysis. In the leading edge gene sets, a few candidate genes were found. C) qRT-PCR validation of candidate genes showing a widespread deleterious effect of mHTT on enzymes involved in neurotransmitter and neuropeptide synthesis. Kruskal-Wallis test followed by Dunn's multiple comparisons. *p < 0.05 and **p < 0.01. D) Body weight graphs from Paper 1, showing that mHTT overexpression in hypothalamus of WT mice causes progressive weight gain. GSEA = Gene Set Enrichment Analysis. wtHTT and mHTT refer to the wild-type HTT overexpression vector (18 CAG repeats) and mutant HTT overexpression vector (79 CAG repeats).

## The weight phenotypes in R6/2 and leptin-deficient R6/2 female mice are associated with proportional weights of iWAT but not gWAT

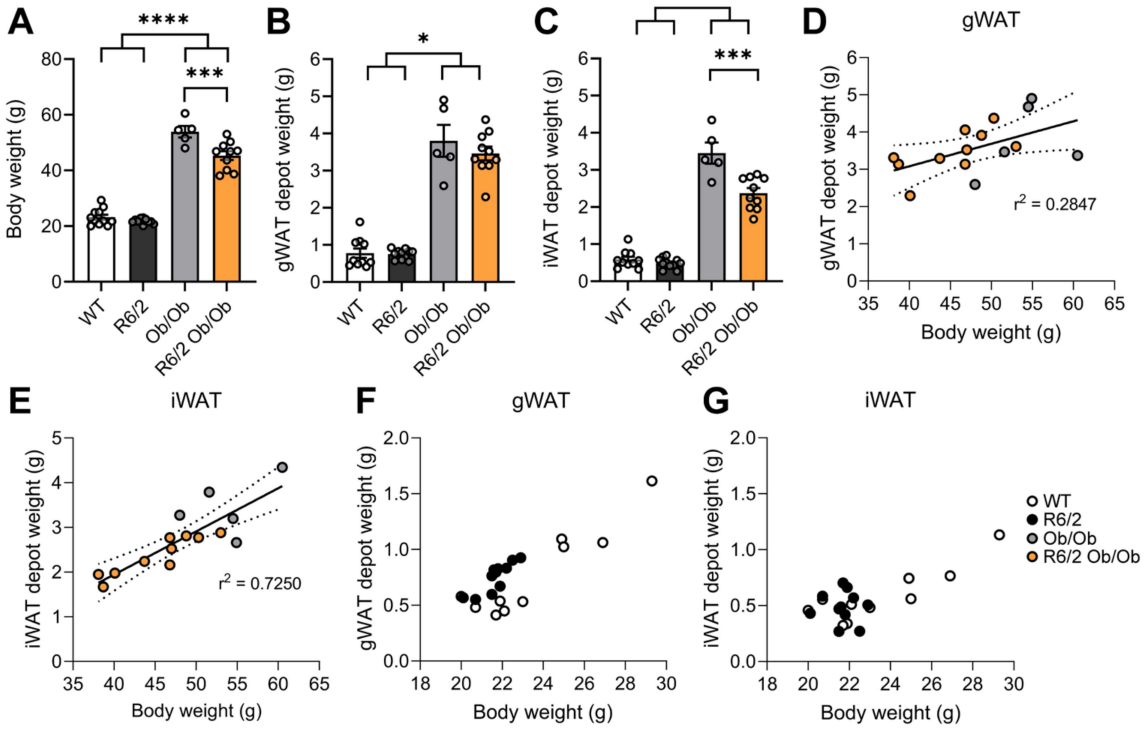


**Figure 12. R6/2 Ob/Ob female mice display reduced body weight and iWAT depot weight.** A) Body weights in R6/2 and leptin-deficient R6/2 (R6/2 Ob/Ob) female mice at 12 weeks of age. Depot weights of the B) gWAT and C) iWAT. Comparison of WAT depot weight to body weight in D) gWAT of Ob/Ob and R6/2 Ob/Ob, E) iWAT of Ob/Ob and R6/2 Ob/Ob, F) gWAT of WT and R6/2, G) iWAT of WT and R6/2. gWAT = gonadal white adipose tissue, iWAT = inguinal white adipose tissue.

One particularly interesting finding from the original paper on the R6/2 Ob/Ob mice was that while R6/2 Ob/Ob mice become obese, their adipose tissue exhibit notable differences compared to the Ob/Ob mice [118]. This would suggest that the mHTT transgene could influence adipose tissue characteristics. Moreover, the R6/2 Ob/Ob mice initially display a body weight pattern that is similar to Ob/Ob mice (rapid early weight gain) but towards a later stage the R6/2 Ob/Ob start to loose weight [118]. Importantly, the onset of weight loss in R6/2 Ob/Ob mice was earlier than the R6/2 mice. Did we make the metabolic dysfunction more apparent in R6/2 mice through the crossbreed with genetically obese mice (Ob/Ob) that due to their hyperphagia (lack of leptin inhibiting hunger) are in a chronic, positive state of energy balance? Somehow, early onset morbid obesity, hyperphagia and reduced energy expenditure [246] is insufficient to oppose pathology in R6/2. What mechanisms are really in play here?

Paper 3 was founded from discussions regarding the pilot results in Paper 1. Why, despite normal or lower body weight in the R6/2 mice, do they consistently exhibit

higher adiposity, even in advanced disease? In theory, weight loss is the result of negative energy balance, so shouldn't the adipose tissue supply the body with energy, rather than the opposite? We've previously also seen that in R6/2 male mice that exhibit weight loss, the iWAT exhibits upregulation of pro-browning genes and BAT-like features [150]. Functionally, this would promote higher energy expenditure, that again is counteractive to weight loss. In Paper 3 we therefore wanted to characterize changes in the R6/2 adipose tissue in more detail. In Paper 3 we found that while body weights and depot weights in R6/2 females are similar to age-matched WTs (Figure 12A-C). For the R6/2 Ob/Ob mice, the females were the first to lose weight and this was accompanied with a reduced mass of the iWAT but not the gWAT (Figure 12A-C). Using linear regression analysis we could see that the iWAT more closely followed the reduction in body weight (Figure 12D-E). In a mouse model of diet-induced obesity, while the iWAT expanded progressively with body weight, the expansion of gWAT became saturated at a body weight around 40 g, indicating that there may be limited expansion and fat storage of gWAT adipocytes [247]. This was accompanied with ectopic fat storage in the liver. This further highlights that while impaired energy storage in itself has a detrimental effect on the body, the adipose tissue also protects the body by neutralizing levels of nutrients in the circulation, preventing development of ectopic fat storage and other pathological states such as peripheral insulin resistance [248].

When looking at depot-specific differences between the female WT and R6/2 mice, while subtle, the gWAT weights of some R6/2 mice are higher for WT mice with similar body weights (Figure 12F). The same pattern is not there for the iWAT weights (Figure 12G). As elaborated on in *Introduction* there are depot-specific differences and evidence of depot-dependent pathophysiology in metabolic diseases such as T2D. To further illustrate, using various studies in mice with diet-induced obesity and hyperphagia, it has been shown that there is rapid proliferation and differentiation of adipocytes specifically in the visceral WAT depots, and in the response to this marked weight gain the gWAT exhibits a high prevalence of adipocyte death, while the iWAT is relatively resistant [249, 250]. Adipocyte death was accompanied with extensive macrophage-mediated adipose tissue remodelling, suggested to be closely associated with increased inflammation and development of whole-body insulin resistance. Therefore, the gWAT and iWAT adipose depots may have contributed differently to the weight loss phenotype resulting from the combination of leptin-deficiency (Ob/Ob) and mHTT expression in the R6/2 Ob/Ob mice [251].

## Changes in adipocyte population dynamics precede weight loss in R6/2 mice

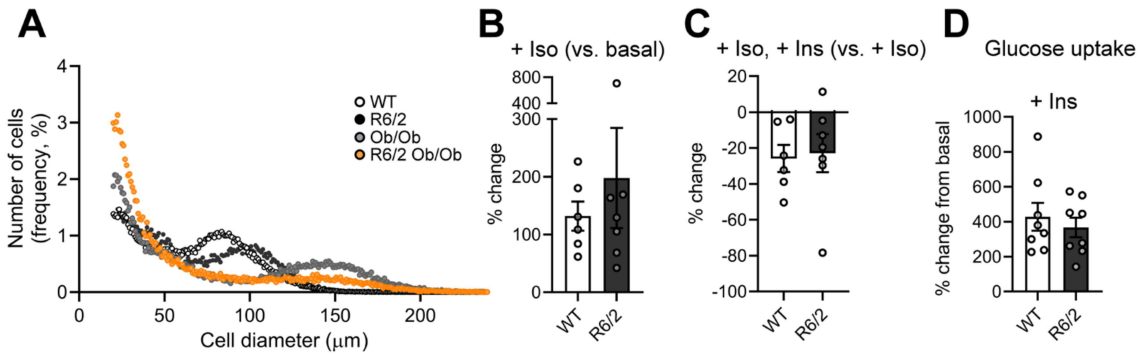


**Figure 13. Changes in the cell size distribution of gWAT in R6/2 mice is not accompanied with changes in lipolysis and glucose uptake.** A) The bimodal cell size distribution of gWAT in female groups, indicating that mice with leptin-deficiency exhibit a higher proportion of large adipocytes. However, notably, R6/2 Ob/Ob females exhibited a larger proportion of small adipocytes compared to Ob/Ob females. For R6/2 females compared to WT females, the cell size distribution was shifted to the right, indicating hypertrophy. B) gWAT adipocytes from female R6/2 mice were treated with isoprenaline, an agonist of the beta-adrenergic pathway that stimulates lipolysis. However, the % change from basal glycerol uptake was similar to WT mice. Furthermore, as shown in C) the combined treatment with isoprenaline and insulin (inhibits lipolysis) showed a similar reduction in the response of R6/2 gWAT and WT gWAT compared to treatment with isoprenaline alone. D) Insulin-stimulated glucose uptake was similar in WT and R6/2 gWAT. gWAT = gonadal white adipose tissue. Iso = isoprenaline (10 nM), Ins = insulin (10 nM).

While looking at the adipose depot weights that indicated a possible link to weight changes in R6/2 and R6/2 Ob/Ob mice, it was important to follow up to see what these changes were really reflecting in terms of adipose tissue dynamics. To maintain control of the energy balance in the body during states of energy surplus or deficiency, the adipocytes can dynamically adapt by changing their number and size. Therefore, visualizing the adipose population dynamics, i.e. the proportion of small and large adipocytes, can indicate pathological mechanisms and metabolic disease [252-255]. Assessing the cell size distribution in the gWAT showed that R6/2 Ob/Ob females had a higher proportion of small adipocytes (Figure 13A). Despite that the large adipocytes were reduced in numbers; their peak size was unaffected. As adipocytes become larger to be able to store more fat, this could indicate that the storage capacity of the gWAT in R6/2 Ob/Ob is impaired due to depleted large adipocytes. However, we also should consider the now larger proportion of small adipocytes in R6/2 Ob/Ob. For example, while some studies on the subject insulin resistance have associated this condition to impaired function in the large, hypertrophic adipocytes, there are also studies showing that the condition is associated with a larger proportion of small adipocytes (reviewed in [252] and [256]). Several studies have shown that size change in adipocytes is accompanied with functional changes, such as differences in the lipolytic response to beta-adrenergic agonists and hormones, and the expression of lipolytic genes (e.g. Hormone-sensitive lipase, HSL and Adipose triglyceride lipase, ATGL) [256-258]. To note, it has previously been shown that beta-adrenergic stimuli of white adipocytes is affected in R6/2 mice [113, 150]. We also performed cell size

distribution analysis in the gWAT of R6/2 females, where we found a shift in the cell size distribution towards larger cells, indicating hypertrophy (Figure 13A). However, despite the changes in cell size distribution, the response to insulin (to regulate glucose uptake and lipolysis) and isoprenaline (to stimulate lipolysis) was preserved in gWAT of R6/2 mice (Figure 13B-C). Follow-up analysis by qRT-PCR showed that there was no change in mRNA levels for B3ar in R6/2 mice compared to WT mice, suggesting that there is no apparent difference in beta-adrenergic signalling.

To conclude, further studies will be needed to link the mechanisms behind these changes, e.g. what causes the shifts in cell size distributions in R6/2 and R6/2 Ob/Ob mice. Delineating the contribution of the adipose tissue to weight loss will be complex in that multiple depots could play a role, especially given that mHTT is ubiquitously expressed. Expanding our analyses to the iWAT will give a clearer picture of this, and whether there are depot-specific vulnerabilities to mHTT expression. We will also need to consider that in Paper 3 we assessed the adipose tissue at a time just before expected weight loss. There are most likely effects from both the early obesity (Ob/Ob) and mutant HTT expression (R6/2) in the R6/2 Ob/Ob mice. Moreover, the pattern in hypertrophy we observe in the R6/2 females could reflect what occurred in the adipose tissue during an even earlier timepoint. Therefore, investigating adipose tissue dynamics during earlier and/or later timepoints may further aid in delineating the progression in adipose tissue dysfunction in R6/2 mice.

## Subtle changes in hypothalamic transcription are found in BACHD

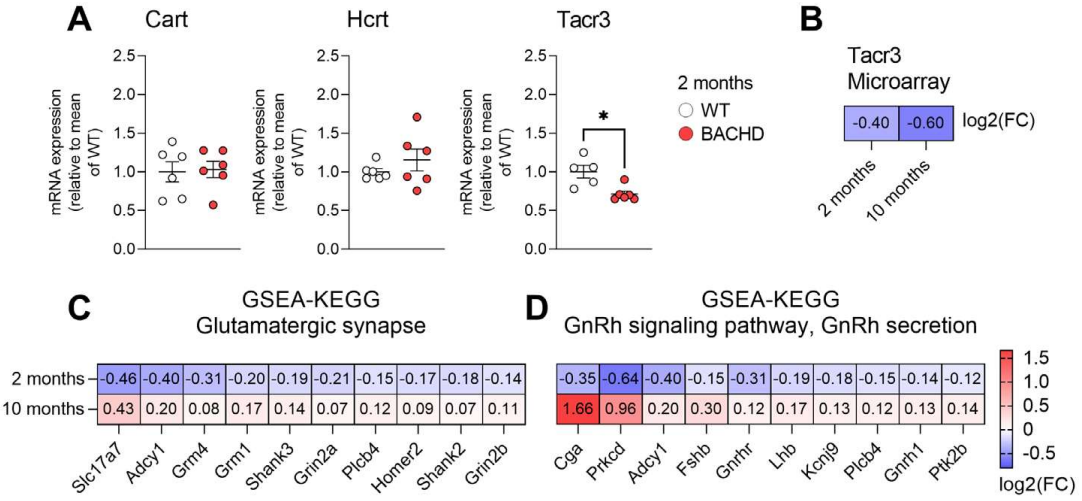


**Figure 14. Transcriptional changes in BACHD mice.** Based on data from Paper 2 [215]. A) qRT-PCR analysis of hypothalamic neuropeptides in 2-month-old BACHD to validate candidate genes from the transcriptomics datasets. *Tacr3* was significantly downregulated compared to age-matched WT mice. Mean ± SEM. Kruskal-Wallis test followed by Dunn's multiple comparisons. *p < 0.05. B) log2(FC) of *Tacr3* from the limma analysis of microarray datasets where 2-month-old BACHD and 10-month-old BACHD were compared to their respective age-matched WT littermates. C)

"Glutamatergic synapse" was one out of the three significantly enriched KEGG pathways in the 2-month-old BACHD dataset (BACHD 2 months vs WT 2 months). Shown are the 10 genes with the highest log2(FC) difference between the age groups. D) For the 10 month-old BACHD dataset (BACHD 10 months vs WT 10 months), there was enrichment of multiple GnRh-related pathways. Shown are the 10 genes with the highest log2(FC) difference between the age groups. FC = fold change. GSEA = Gene set enrichment analysis.

In BACHD mice there is no significant cellular loss in hypothalamus even at later stages [111], but there is early transcriptional dysregulation of discrete hypothalamic neuropeptides [182]. In Paper 2 we used transcriptomics to delineate gene expression changes in the BACHD hypothalamus in more depth. We assessed two age groups: BACHD mice at 2 months of age (early stage of disease) and at 10 months of age (late stage of disease). Compared to their respective age-matched WT littermates, BACHD had no genes that passed significance when adjusted for multiple testing. However, our assessment of candidate genes using qRT-PCR in 2-month-old BACHD showed that Tachykinin receptor 3 (*Tacr3*) was significantly downregulated, while no change was found for the *Hcrt* and *Cart* neuropeptides (Figure 14A) [152, 164, 167, 259-262]. In line with previously published qRT-PCR data from 4 months old BACHD [182], *Tacr3* may be progressively declining (Figure 14B) [182]. Changes in Tacr3 and other tachykinins are associated with glutamate hyperactivity and glutamate toxicity [263, 264]. In the 2-month-old BACHD dataset, "Glutamatergic synapse" was one of the three significantly enriched KEGG pathways. The gene with the highest change in the core gene set was Vesicular glutamate transporter 1 (*Slc17a7*) with a log2(FC) of - 0.46 in 2-month-old BACHD and 0.43 in 10-month-old BACHD compared to their respective WT littermates (Figure 14C).

In the GSEA analyses, there were no exact matches between the 2 months and 10 months datasets. However, gonadotropin-releasing hormone (GnRh) terms were enriched in 10-month-old BACHD (Figure 14D), processes that relate to Neurokinin B (Nkb, encoded by *Tac3*) that binds to Tacr3: Nkb in the arcuate nucleus of the hypothalamus colocalizes with kisspeptin and dynorphin to control GnRh secretion [265-267]. GnRh controls the production and release of Fsh and Lh in the HPG axis. Familial loss-of-function mutations in *TAC3* or *TACR3* results in severe congenital gonadotropin deficiency [268]. Previous studies have shown that BACHD male mice have a reduction in GnRh cells in hypothalamus and alterations in the HPG axis are found in both mouse models of HD and clinical HD [178, 179, 181, 183]. Moreover, there are interactions implicated between the metabolic system and reproductive system (reviewed in [269]). In mice with overexpression of mHTT, *Tacr3* was part of the core gene set in the significantly enriched GO term "feeding behavior" (GO:0007631) [215]. Considering that BACHD in addition to a metabolic phenotype develop anxiety- and depressive-like behaviors [182], Tacr3 has been further implicated in anxiety-like behaviors in mice, mood disorders, schizophrenia, addiction and in other neurodegenerative diseases such as PD [270-273]. It would be interesting to further explore a potential impairment of the systems presented

here, the role of mHTT expression versus obesity, and the implications of early *Tacr3* dysregulation in the BACHD mice.

## The effect of HTT on body weight and body fat is more pronounced in female mice

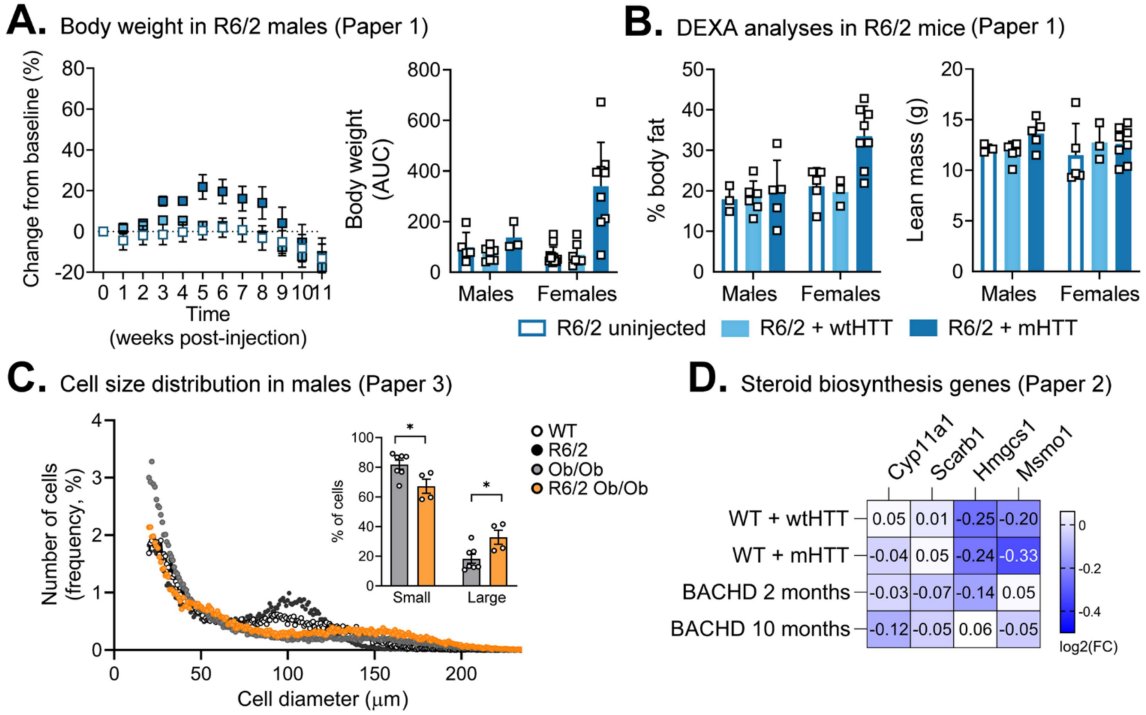


**Figure 15. Implications for gender-dependent differences in HD models.** Data from Paper 1 [199] showing the time-course body weight (A) for R6/2 males with overexpression of HTT in hypothalamus. The AUC compares the graph in A) of R6/2 males to R6/2 females from the graph in Figure 8B. B) DEXA analyses from Paper 1 [199], showing the difference in % body fat and gram lean mass between R6/2 males and R6/2 females with overexpression of HTT in hypothalamus. C) Cell size distribution data from Paper 3, where R6/2 Ob/Ob males exhibit a larger proportion of large adipocytes compared to Ob/Ob males. D) Transcriptomics data from Paper 2 (hypothalamus of female mice) showing log2(FC) data from genes involved in steroid biosynthesis, shown to be significantly altered in the testes of BACHD mice in a separate study [274]. 3-hydroxy-3-methylglutaryl-CoA synthase 1 (Hmgcs1) and Methylsterol monooxygenase 1 (Msmo1) were significantly altered in the mHTT vs. WT dataset. wtHTT and mHTT refer to the wild-type HTT overexpression vector (18 CAG repeats) and mutant HTT overexpression vector (79 CAG repeats).

Thus far in the *Key Results* I have presented results from female mice. Gender-differences on disease phenotypes have been found in previous studies using the HTT overexpression mouse model and the BACHD mice, such as that BACHD female mice display the most pronounced increase in body fat composition, and weights of WAT and BAT [111]. Therefore, we focused analyses on female mice while male mice were analysed in smaller groups in Paper 1 and 3. In Paper 1 we found an effect of increased body weight and body fat composition in female R6/2 mice with hypothalamic overexpression of mHTT but not for male R6/2 (Figure 15A-B, Figure 8 and Figure 9). In Paper 3 the cell size distribution in male R6/2 Ob/Ob showed a significant difference in the proportion of small versus large cells,

in which R6/2 Ob/Ob males showed higher proportion of large cells with no change in the peak cell diameter (Figure 15C). This is despite that the gWAT weight is not different compared to Ob/Ob mice. In Paper 3, whereas the R6/2 Ob/Ob females displayed decreased body weight at the timepoint we assessed the changes in gWAT (Figure 12), body weights between R6/2 Ob/Ob and Ob/Ob males were comparable. Based on the previous study characterizing the R6/2 Ob/Ob we would expect the male R6/2 Ob/Ob to also start decreasing in body weight [118]. Whether the present results in cell size distribution fully reflects a gender-difference or events affected by the present body weight (R6/2 Ob/Ob males: comparable to Ob/Ob females, R6/2 Ob/Ob females: decreased body weight compared to Ob/Ob females) is unclear.

Since you could write another thesis based only on gender-differences in adipose tissue and obesity, I have here chosen to highlight a few hallmarks that may be of relevance for present results from Paper 3. As discussed previously in this thesis, in the adipose tissue there are depot-specific differences, and the visceral WAT is more closely associated with increased risk of developing metabolic disease like T2D [139-141]. Removing the visceral WAT depots or relocating subcutaneous WAT depots into the abdominal cavity decreases body weight and total fat mass, improves insulin resistance and glucose uptake, and reduces lipid accumulation in the liver [275-278]. There are differences in the adipose tissue distribution: men tend to have more visceral WAT and women more subcutaneous WAT [279].

Estrogen has been suggested to be a major factor contributing to this protective effect in WAT, and one proposed mechanism is that estrogens are involved in the favored fat distribution in the subcutaneous WAT through modifying the SNS tone [280, 281]. There is site-specific regulation by estrogen in WAT and evidence for that the ratio of adipose estrogen receptors (ERα to ERβ) affect insulin sensitivity [281, 282]. ERα null mice exhibit obesity accompanied with higher visceral adiposity [283]. Estrogen is also involved in multiple signaling pathways in the hypothalamus where it can modulate neuronal activity: it promotes anorexinergic signalling via POMC neurons and acts antagonistically for the NPY/AgRP neurons [281, 284, 285]. Studies in transgenic HD mice have shown that 17-β estradiol may modulate sex-specific differences in disease phenotype due to its correlation with striatal loss and motor impairment [286].

As is the case for estrogen, testosterone also has other effects outside the reproductive system. Testosterone can affect the growth of muscle mass, fat distribution and bone mineral density [287-289]. Testosterone and LH levels are significantly lower in males with HD than age-matched controls, and low plasma testosterone was associated with dementia [290]. Alterations in the HPG axis and reduced levels of testosterone has further been found in transgenic HD mouse models [178, 274]. In BACHD rats, lower testosterone levels were proposed to contribute to gender-dependent growth alterations [274]. Reduced musculoskeletal growth and reduction of brain mass was found in male but not female BACHD mice; this was despite that the expression levels of wtHTT and mHTT were comparable

[274]. Furthermore, there was transcriptional dysregulation of steroid biosynthesis in the testes [274], and according to our hypothalamus datasets from Paper 2 (female mice) [215], there is also significant downregulation of 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*Hmgcs1*) and Methylsterol monooxygenase 1 (*Msmo1*) in WT mice overexpressing mHTT in hypothalamus (mHTT vs. WT dataset) (Figure 15D). Hmgcs1 is also dysregulated in the striatum of R6/2 mice [291].

To try and conclude this section: the study of the role of mHTT in metabolic alterations in the body becomes increasingly complex considering the gender-specific differences in metabolic tissues and sensitivity to obesity. However, not only is this topic interesting in that there are interactions between the reproductive system and metabolic systems [285, 292, 293], HTT is highly expressed in both the brain and testis (Human Protein Atlas, proteinatlas.org, [18]). Therefore, continuing to explore gender differences in HD models and trying to understand how mHTT affects the levels of sex hormones in the body may help us to further delineate mechanisms underlying the various disease phenotypes.

# Conclusions

HTT plays a role in metabolic control via hypothalamic neurocircuits

Transcriptional dysregulation in hypothalamus contribute to metabolic alterations in HD

Adipose tissue changes are present during disease stages in R6/2 mice and could contribute to disturbed energy balance

Peripheral pathology contributes to metabolic alterations in HD

# Concluding remarks

*Are there different stages of metabolic dysfunction in HD mice?*

I want to conclude this thesis work with a few question marks that has been reoccurring during my PhD studies. The first one is whether metabolic dysfunction in HD, including both central- and peripheral forms of pathology, is non-linear/biphasic.

While many symptoms of HD progressively worsen over time, there are some with disease stage-specific changes. One example are the motor symptoms, where chorea is usually pronounced during the early stages of disease, and akinesia (rigidity/absence of movement) towards the late stages of disease [294-296].

By selective mHTT silencing in the hypothalamus of BACHD mice, the metabolic phenotype can be prevented [111]. The effect could be that certain components of the hypothalamic circuitry are rescued from mHTT and can recover before the deleterious effects (e.g. hyperphagia, transcriptional dysregulation) manifest. As shown in the *Key Results* section, mHTT-induced transcriptional dysregulation in hypothalamus of BACHD may be selective to just a few neuronal populations, at least during the early stages. Trying to silence mHTT in hypothalamus when the metabolic features have already established causes no effect [111]. Interestingly, our transcriptomics analysis of the BACHD hypothalamus found no common pathways and certain genes appear to change the direction of expression (downregulated vs. upregulated) between the early and late stages. The question is whether the targets and mechanisms inducing the metabolic phenotype are different from the ones driving the progression.

*How significant is peripheral pathology for metabolic dysfunction?*

Another missing component here is the effect on the periphery. Considering the BACHD mice with mHTT silenced in hypothalamus: what happens when you challenge this modified crosstalk between hypothalamus (mHTT is silenced) and periphery (mHTT is expressed) with, for example, a high fat diet?

There is a possibility that over the course of disease, the peripheral mechanisms could become more dominant and prevent any compensatory effects from the brain. There is severe progressive peripheral pathology in the R6/2 mice that may at least in part account for their end-stage weight loss. As for the BACHD mice, there could be disease-stage specific changes in the R6/2 mice. To illustrate: during the early

disease stages the R6/2 mice are able to gain body weight and body fat in response to diet [113], overexpression of mHTT in hypothalamus (Paper 1, [199]), ghrelin administration [297] and genetic leptin-deficiency (Paper 3 and [118]). Furthermore, the gene profile of the R6/2 adipose tissue changes over time [114]. However, the effects we see are still from the pathological system modified by mHTT. During this early stage the adipose tissue function in R6/2 is compromised. R6/2s fed a regular chow diet or a high fat diets accumulate significantly higher body fat than WT mice, changes that are disproportional to their body size [113]. In paper 3 we further showed that despite no change in body weight or body fat, R6/2 mice exhibit changes in adipose population dynamics. While it may be subtle changes, if we go back and look at metabolic syndrome, changes in multiple markers can be present more than 20 years before diagnosis of T2D [298]. We will need more elaborate studies on the role of mHTT in peripheral pathology, in particular the mechanisms behind the functional changes we see in HD models, such as the higher adiposity in early stage R6/2 mice. Can we define a 'pre-manifest' and 'manifest' stage of metabolic dysfunction in HD models?

*Then what about the clinic*?

The complications surrounding the HTT silencing trials show that we have yet not fully grasped how to work with bodies that have been pathologically modified by mHTT throughout life. What we have learned from the field of metabolic diseases is that there is significant heterogeneity between individuals. All obese individuals do not have the same disease course, not all develop insulin resistance and T2D, and T2D in itself is heterogenous in its pathophysiology [299-302]. On this basis, it could be possible to identify and define subgroups of individuals in HD. This is not only important to promote our knowledge of HD but the design of more effective and safe clinical trials. In the HD field we have the privilege of a strong community with rich ideas, close collaborations, and strong engagement in clinical trials that makes it hard to believe that it is anything else than a possibility for the future.

# Key methodology

## Animals

### Ethical considerations

Mice were housed in groups (2-5/cage) and maintained at a 12 h light/dark cycle in environmentally enriched Innocage mouse cages (InnoVive, San Diego, CA, US) with free access to a standard chow diet and water. All the experimental procedures were carried out in accordance with the approved guidelines approved by the Lund University Animal Welfare and Ethics committee in the Lund-Malmö region. Ethical permit numbers can be found in each respective Paper.

### BACHD mice

The BACHD mouse line is a transgenic HD mouse model expressing a full-length mHTT fragment. BACHD are named after the bacterial-artificial chromosome (BAC) technology that was used to generate the model. The BAC transgene contains the entire 170 kb human HTT locus with the exon 1 containing 97 mixed CAA-CAG repeats (mHTT) under the control by the endogenous HTT regulatory system The mHTT exon 1 segment is flanked by two loxP sites that allow for conditional inactivation using the Cre system. BACHD mice are bred on the FVB/N genetic background. [110]

BACHD mice exhibit progressive motor- and behavioral deficits including anxiety-like and depressive-like phenotypes, as well as late-onset neurodegenerative pathology in the striatum and cortex [110, 182]. The metabolic phenotype in BACHD is characterized by hyperphagic obesity, leptin- and insulin resistance [111].

### R6/2 mice

The R6/2 mice are another line of transgenic HD mouse models. The transgene was derived from a phage genomic clone from a clinical HD case. R6/2 mice express HTT exon 1 with an expanded CAG repeat and with the endogenous HTT promoter. [112]

The number of CAG repeats can vary between R6/2 mouse lines. In our R6/2 cohort, the CAG repeat lengths are higher and therefore results in a slower disease progression than that of R6/2 mouse lines with 150 CAG repeats [194]. However, the longer CAG repeat is not neuroprotective; it causes a delay in onset of disease phenotypes in the R6/2 mice.

The R6/2 mice develop a HD-like phenotype where many aspects are reminiscent of HD clinical cases. R6/2 mice develop neuronal intranuclear inclusions, striatal atrophy, motor- and cognitive impairments as well as weight loss [112, 195, 303, 304]. Furthermore, they develop severe and progressive peripheral pathology including adipose tissue dysfunction, skeletal muscle atrophy and development of hyperglycaemia and hyperinsulinemia [53, 113, 115, 117, 119, 150, 173, 178, 196, 198, 305-307].

### R6/2 Ob/Ob mice

R6/2 Ob/Ob mice are characterized by rapid early weight gain, high fat mass and low energy expenditure [118]. The R6/2 Ob/Ob mice are a crossbreed between the R6/2 mouse model of HD and Ob/Ob mice.

The Ob/Ob mice are genetically deficient in leptin, a hormone produced by adipocytes that suppresses hunger [308]. The Ob/Ob mice are characterized by a hyperphagic obesity, lower energy expenditure and they develop severe insulin resistance, hyperlipidaemia and hyperglycaemia [246]. The Ob/Ob mice are used as a model for obesity and T2D [309].

Leptin-deficient R6/2 mice (R6/2 Ob/Ob) are generated through a two-step breeding strategy [118]. Females in the P0 were Ob-/+ mice (heterozygous for the leptin-deficiency gene) with the C57BL/6J genetic background (Jackson Laboratory, Bar Harbor, ME, US). P0 males were R6/2 mice with the C57BL/6xCBA genetic background (Jackson Laboratory, Bar Harbor, ME, US). Female Ob-/+ and male R6/2 mice were crossed to generate the F1 generation. Males heterozygous for the R6/2 gene and the leptin-deficiency gene, R6/2 Ob-/+, were crossed with Ob-/+ females to generate the F2 generation. [118]

## Viral vector overexpression of HTT

To evaluate effects of increasing HTT expression in specific tissues we have utilized viral vectors that are injected into the hypothalamus and specifically deliver fragments corresponding to wtHTT or mHTT to neuronal cells.

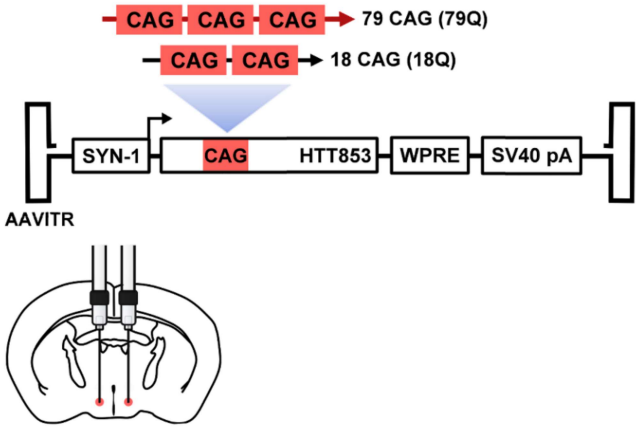


**Figure 16. Vector constructs used for the overexpression of HTT in hypothalamus.** The HTT transgene has a CAG repeat length of either 18 CAG (wtHTT) or 79 CAG (mHTT). Syn-1 = human Synapsin-1 promoter. AAVITR = inverted terminal repeats (ITRs) of the AAV2 flanking the expression cassette. WPRE = woodchuck hepatitis virus post-transcriptional regulatory element is used to enhance expression. Downstream of the WPRE is a poly-adenylation sequence from Simian vacuolating virus 40 (SV40 pA) that protects from endogenous endonuclease activity. An AAV5 capsid is used to package the vector construct. [310]

### Vector constructs

The vector construct used was a recombinant adeno-associated viral vector (AAV) of serotype rAAV2/5. The HTT transgene consisted of the first 853 amino acids of N-terminal HTT including the *HTT* exon 1 with an expanded CAG repeat [111]. The CAG repeat length corresponded to either wtHTT (18 CAG repeats; HTT853-18Q) or mHTT (79 CAG repeats; HTT853-79Q) (Figure 16). The promoter used to drive the expression was the human Synapsin-1 (Syn-1) to restrict expression to neurons [310, 311]. In addition, for Paper 1 a GFP vector was used as a control group to assess transgene efficiency in BACHD.

### Stereotactic surgery

At 8 weeks of age and under isoflurane anaesthesia mice were injected bilaterally in the hypothalamus with a vector volume of 0.5 µl/hemisphere using stereotaxic surgery. The stereotaxic coordinates for the hypothalamus were determined according to bregma (anterior-posterior, AP; medial-lateral, ML) and the dura mater (dorsal-ventral, DV), and were the following: AP = 0.6 mm, ML = 0.6 mm and DV = - 5.3 mm. The full surgery protocol is described in [312].

## Behavioral analyses

In paper 1 we performed a range of behavioral analyses to follow the development of disease phenotypes in the R6/2 mice. Behavioral testing was performed at two

timepoints: 4 weeks post-injection and 8 weeks post-injection. Prior to each behavioral tests, mice were habituated 1 hour to the behavioral room. Each station was cleaned with 30% ethanol between each test.

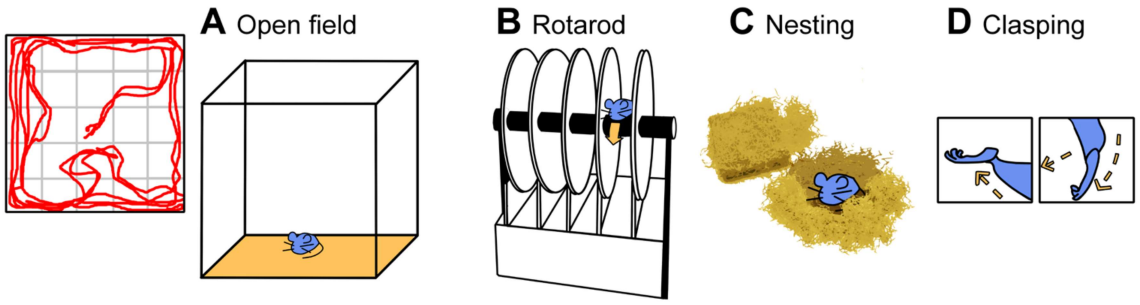


**Figure 17. Overview of behavioral analyses in Paper 1.** A) For the open field test, mice were placed in a 40 x 40 cm arena enclosed by transparent Plexiglas walls. Activity was monitored using the ANY-maze video tracking system (Stoelting Co, US). B) The rotarod test is based on that the mice have to try and stay on a rotating rod with set speeds or accelerating speeds over time. C) The nesting test is performed in a regular mouse home cage with nesting material provided. The mice are tested individually for their nest-building ability. D) The clasping test is performed by carefully lifting the mouse by the base of the tail from a horizontal surface. Healthy mice will splay the hindlimbs outwards.

## Open field

The open field test is used to monitor the general locomotor activity. The mice were placed in the center of an open arena for 60 min (Figure 17A) and their activity was tracked using the ANY-Maze video tracking system (Stoelting Co, Ireland). As a measure of activity we used the total distance moved over 60 mins.

## Rotarod

The rotarod test can be used to assess motor coordination and balance (Figure 17B). We used the Rotamex 4/8 system (Rota Rod Columbus Instruments, US). Prior to the rotarod tests, the mice were trained to run on the rotarod at a fixed speed of 4 rpm for 5 min. The rotarod tests (4-40 rpm over 5 mins) were performed in three rounds with 15 min rest in between. The mean latency to fall from the three tests were used as the representative for rotarod performance in each mouse.

## Nesting

Nest-building is a naturally occurring behavior in mice and can therefore be used to monitor well-being and to keep track of behavioral changes. However, nesting ability can also be affected by brain lesions, genetic mutations among other factors that cause cognitive and motoric impairment. To perform the nesting test, the cage is filled with bedding and provided with free access to food and water. One hour prior to the dark phase mice are individually placed in the cage and provided with a square of nesting material (Figure 17C). Mice are then left overnight to build nests.

In the morning, the nesting material is collected, weighed and scored according to the protocol by Deacon et al. [260-262]

### Hindlimb clasping

In the hindlimb clasping test, mice are carefully lifted by the base of the tail from a horizontal surface. When lifted, healthy adult mice instinctively splay the hindlimbs outwards and away from the abdomen [313] while mouse models with neurodegeneration display clasping behaviors in which the hindlimbs instead retract towards the abdomen [178] (Figure 17D). We used a 15 second duration with one clasping trial performed for each timepoint. The scoring criteria provided by Guyenet et al. was used [314].

## Metabolic analyses

### Body composition analysis using DEXA

The percentage body fat was measured by DEXA scanning in the Lunar PIXImus2 (Lunar Corporation, Madison, WI, US). The image analysis and data processing was performed using PIXImus2 2.10 software (Lunar Corporation, Madison, WI, US).

### Indirect gas calorimetry

The PhenoMaster Automatic Home Cage Phenotyping system (TSE Systems, Germany) was used for indirect gas calorimetry. We performed calorimetric measurements using a grouped setup. Female mice were grouped (n = 2-3/cage) based on vector injection (uninjected, 18Q or 79Q) and genotype (WT or R6/2). Mice were firstly habituated for 24 hours to the PhenoMaster water bottles inside their respective home cages (group-housed). The mice were then transferred to fully equipped PhenoMaster cages for a 24-hour acclimatization step. We provided bedding material from the home cage as an enrichment to reduce stress. Recordings were made during 24 hours. The sample interval was 3 min/cage. Metabolic parameters were calculated based on a correction for total body weight in the cage.

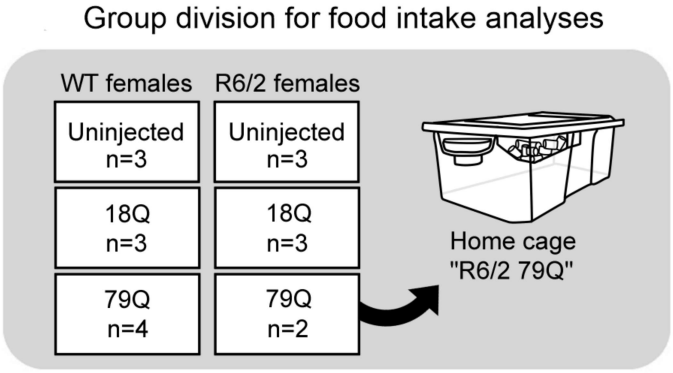


**Figure 18. Strategy for group division for the food intake analyses in home cages from Paper 1.**

## Food intake measurements

In Paper 1 we monitored how the food intake progressed from the surgery week until the end of the study. To minimize stress that could affect feeding behavior and metabolism, we performed the experiments in the mice's natural home cage environment with the environmentally enriched Innocages (InnoVive, San Diego, CA, US) during standard conditions (12 hour light/dark cycle) and with free access to a normal chow diet and water. After surgery we divided R6/2 females and their WT littermates into groups of 2-4 for each cage (Figure 18). Using this set-up, each experimental group was represented by one cage. To estimate the total weekly food intake for each cage (g/mouse/day) the amount of food consumed was measured using an electronic scale on day 0, 3 and 7 of each week.

## Adipose tissue analyses (Paper 3)

Primary gonadal adipocytes used for cellular analyses in Paper 3 were isolated using an established protocol [315]. Dissected gonadal tissue was suspended in Krebs-Ringer Bicarbonate HEPES (KRBH) buffer (pH 7.4, supplied with 200 nM adenosine and 3% bovine serum albumin (BSA). Addition of collagenase (1 mg/ml) followed by shaking at 37 ℃ for 1 hour was used to digest the tissue, followed by filtration through a 400 mikron mesh. Floating adipocytes were then washed 3 times in KRBH buffer before use in lipolysis and glucose uptake analyses.

Adipose cell size distribution was performed on fixed gonadal tissue (see *Cell size distribution* further below).

*Lipolysis*
Adipocytes, 10% v/v in KRBH buffer were incubated shaking at 37 °C for 30 min. We assessed three conditions: 1) non-treated (basal), 2) treatment with 10 nM isoprenaline and 3) combined treatment with 10 nM isoprenaline and 10 nM

insulin. This was followed by taking an aliquot of the media to measure glycerol release using the Free Glycerol Agent kit (F6428) (Sigma-Aldrich, US).

*Glucose uptake*
Adipocytes, 7.5% v/v in KRBH buffer were incubated for 30 min with or without 10 nM insulin. Thereafter, D-14C(U)-glucose (2.5 µl/ml, NEC042, Perkin Elmer, Akron, US) was added for an additional 30 min incubation. After completion, to separate the cell fraction from the media 300 µl of each cell suspension was centrifugated in microtubes containing 85 µl dinonylphtalate oil. For scintillation counting of the amount of glucose (fmol) taken up by the cells, the cell fractions were dissolved in scintillation fluid (Ultima Gold, Perkin Elmer, US). [316]

*Adipose cell size distribution*
Dissected tissue (~4 x 4 mg pieces) from the gonadal adipose depot was fixed in osmium tetroxide. The Beckman Coulter Multisizer 4e (Beckman Coulter Inc., US) was used for cell size distribution analysis, counting 6000 particles and taking the average from two runs. Data were analyzed in Multisizer 4e v. 4.03 using linear bins (20-240 µm, 400 bins, bin size 0.55 µm

# RNA analyses

## Tissue processing and RNA isolation

After a terminal dose of sodium pentobarbital (Apoteksbolaget, Lund, Sweden) via intraperitoneal injection, tissue was dissected and snap-frozen in liquid nitrogen for storage at − 80 °C until further use. Total RNA was extracted using the RNeasy Lipid Tissue Mini kit (Qiagen, US) for hypothalamus and the E.Z.N.A Total RNA Kit II (Omega Bio-Tek, US) for adipose tissue. RNA yield and quality was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, US).

## Quantitative real-time PCR (qRT-PCR)

Reverse transcription was performed on 1 µg of sample RNA using the Superscript IV Reverse Transcriptase kit (Invitrogen, US) (Paper 1) or the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, US) (Paper 3) according to the respective manufacturer's protocols. In Paper 1, qRT-PCR was performed using the LightCycler 480 (Roche, Switzerland) and SYBR Green I Master (Roche, Switzerland). In Paper 3, qRT-PCR was performed using the CFX96 touch real-time PCR detection system (Bio-Rad Laboratories, US) and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, US)

The 2-ΔΔCT method [317] was used to calculate gene expression relative to housekeeping genes for each respective tissue. Primer sequences can be found in each Paper.

## Histological analysis

In Paper 1, we performed immunohistochemical staining for HTT in the hypothalamus. At 11 weeks post-injection, under terminal sodium pentobarbital anesthesia (Apoteksbolaget, Lund, Sweden) mice were perfused transcardially with room-tempered saline (0.9%) followed by cold 4% paraformaldehyde (PFA) for 10 min. Brains were collected and submerged in 4% PFA at 4 °C for 24 hours followed by a switch to 25% sucrose at 4 °C for 24–48 hours. Coronal brain sections were cut in series of 30 μm and then stored at −20 °C in an antifreeze solution (30% glycerol, 30% ethylene glycol solution in Phosphate-buffered saline, PBS). For immunohistochemical staining, free-floating brain sections were washed 3 times with 0.05 M Tris-buffered saline (TBS) and then quenched in a 10% MeOH, 3% H2O2 solution in TBS for 30 min at room temperature (RT). After quenching, sections were washed 2 times in TBS and 1 time in TBS-T. Blocking was performed for 1 hour at RT with 5% normal horse serum in 0.25% Triton-X in TBS). The sections were incubated overnight with the goat anti-HTT (N18) antibody (sc-8767; Santa-Cruz Biotechnology, US) in a 1% bovine serum albumin (BSA)/TBS-T solution. After primary antibody treatment, sections were washed 3 times in TBS-T followed by 1 hour incubation at RT with a horse anti-goat antibody (BA9500; Vector Laboratories, US) prepared in 1% BSA/TBS-T. This was followed by washing 3 times in TBS-T before treatment with an avidin-biotin-peroxidase complex solution (PK-6100, Vector Laboratories, US) in TBS for 1 hour. Before developing with 3, 3′-diaminobenzidine (DAB), the sections were washed 2 times in TBS-T and 1 time in TBS. After the DAB reaction, stained sections were mounted on gelatin-coated glass slides. After drying overnight at RT, the slides were subjected to dehydration by increasing concentrations of ethanol and then cleared in xylene before cover-slipping using DPX mounting medium (Sigma–Aldrich, US).

# Transcriptomics and bioinformatics

## Microarray

Microarray analyses were performed on total hypothalamic RNA. RNA yield and quality of hypothalamic samples was measured in terms of RNA integrity number (RIN) using the Agilent 2,100 Bioanalyzer (Agilent Technologies, US). Samples with RIN < 7 were omitted. Microarray analysis was performed using the Affymetrix platform (Affymetrix, Thermo Fisher Scientific, US) using the Mouse Gene ST 1.0 array.

R software (R v.4.1.1.) [318] was used for data analysis. Microarray data from the raw .CEL files was processed and analyzed using Robust Multi-Array Averaging (RMA) followed by limma (linear models for microarray data). The full details of the microarray data processing can be found in Paper 2 [215].

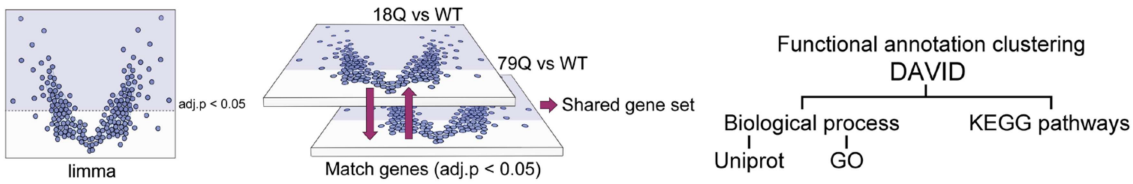


**Figure 19. Preparing gene lists for DAVID Functional Annotation Clustering in Paper 2.** The datasets used were wtHTT vs. WT and mHTT vs. WT. wtHTT and mHTT refer to the wild-type HTT overexpression vector (18 CAG repeats) and mutant HTT overexpression vector (79 CAG repeats).

## DAVID Functional Annotation Clustering

DAVID is an abbreviation for "Database for Annotation, Visualization and Integrated Discovery" and it is a free-to-use, web-based high-throughput functional annotation Bioinformatics resource (URL: https://david.ncifcrf.gov/, [319, 320]). Compared to other singular resources of clustering and pathway enrichment analyses, DAVID is built to analyse gene lists based on multiple sources of annotations. For example, pathway databases such as KEGG and Reactome can be combined with interaction/post-translational modification/protein domain databases to explore the biological meaning of a gene list.

In Paper 2, to generate gene lists for DAVID Functional Annotation Clustering we chose to look at genes that were significant. Only the wtHTT vs WT (18Q vs WT) and mHTT vs WT (79Q vs WT) datasets had genes that passed significance (adj.p-value < 0.05 criteria). To prepare gene lists for DAVID, genes filtered by significance were compared between the two datasets to create three separate gene lists: 1) shared genes, 2) unique genes for wtHTT vs WT and 3) unique genes for

mHTT vs WT (Figure 19). Functional annotation clustering was performed with default settings and using three categories: UP_KW_BP, GOTERM_BP_DIRECT, and KEGG_PATHWAY.

### Gene set enrichment analysis (GSEA)

The ClusterProfiler v.4.0.5. package in R was used to perform GSEA. To perform GSEA of GO terms and KEGG, the respective ClusterProfiler functions gseGO and gseKEGG were used. The full details of the procedure can be found in Paper 2 [215]

Instead of only performing enrichments and biological interpretations based on significant genes, GSEA takes into account the whole transcriptomics dataset. A disadvantage with using only significant genes for analysis is that there may not be any functional pattern and/or association within the list, making it difficult to interpret the biological significance of the findings [238]. We may miss genes with a lower magnitude of expression (non-significant) but that still show notable cross-correlation in gene expression and may therefore provide more biological context. The full description and algorithm behind GSEA can be found in [238]. In short, GSEA uses a template of predefined gene sets that have been grouped together based on their association with the same biological pathway and/or chromosomal location [321]. For a given transcriptomics dataset comparing two conditions (e.g. healthy vs. diseased) GSEA performs statistical scoring to estimate whether there are classes of genes that are over-represented based on the predefined gene sets and would therefore point towards an association with disease phenotypes. The GSEA output also provides a list of core genes that contributed the most in score for each respective pathway. This 'leading-edge' gene set may help in identifying candidate genes. [238]

## Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc., US). $p < 0.05$ was considered statistically significant. For parametric data with 3 or more groups, 2-way ANOVAs and 1-way ANOVAs were used to assess main effects and interaction effects followed by the Tukey's multiple comparisons test. For parametric data with 2 groups, we used the unpaired t-test. Non-parametric data with 3 or more groups were analyzed with the Kruskal-Wallis test followed by Dunn's post hoc. Non-parametric data with 2 groups were analyzed using the Mann-Whitney test.

In Paper 1, time-course body weight datasets that passed the normality test for parametric data were assessed for effects from vector, genotype and time. A linear mixed model was developed using Proc Mixed in SAS (SAS Enterprise Guide 6.1

for Windows, SAS Institute Inc., US), where body weight (or body weight change) were set as the dependent variable. The fixed effects in the model were vector, genotype and time. The repeated covariance was unstructured.

In Paper 1 we further had datasets with low sample number, e.g. in the case of weighed gWAT and iWAT fat depots (2-4/group), this data were presented using descriptive statistics.

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