Beyond the basal ganglia

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2008

Beyond the Basal Ganglia

Widespread pathology in Huntington's disease

Akademisk avhandling

Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet
för avläggande av doktorexamen i medicinsk vetenskap kommer att offentligen försvaras i
Belfrage Salen, Biomediciniskt Centrum, Lund,

Fredagen den 17 oktober 2008, kl 9:15

av

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Document name
DOCTORAL DISSERTATION

Date of issue
2008-10-17

Sponsoring organization
Marie Curie actions fellowship:
European Union,
RTN MRTN-CT-2003-504636

Title and subtitle
Beyond the Basal Ganglia, *widespread pathology in Huntington's disease*

Abstract
Huntington's disease (HD) is a fatal, hereditary disorder caused by a mutation in the gene encoding the protein huntingtin. Although mutant huntingtin is ubiquitously expressed throughout the body, HD research has mainly focused on the role of the basal ganglia. Dysfunction of these brain nuclei likely underlies motor disturbances in HD, including the conspicuous, uncontrollable, dance-like movements (chorea). However, HD is frequently complicated by other important signs and symptoms that cannot, or not solely, be attributed to basal ganglia dysfunction. Among these is unintended weight loss, which occurs in nearly all HD patients and can affect both quality of life and disease progression. Other symptoms that might occur irrespective of basal ganglia dysfunction are cognitive deterioration, psychiatric problems, sleep disturbances, cardiac failure and atrophy of skeletal muscle.

The past decade has seen an increasing interest in the role of mutant huntingtin in other areas of the brain and body. Although these effects are still poorly understood, their study could lead to a better understanding of the pathological mechanisms underlying HD, as well as to the identification of novel markers of disease progression and therapeutic options. The aim of this thesis was, therefore, to investigate the effects of mutant huntingtin outside the basal ganglia, especially those structures that might underlie weight loss in HD, including the hypothalamus, adipose tissue, and the gastro-intestinal tract. We found that weight loss in both HD patients and R6/2 mice (a transgenic model of HD) is not caused by changes in caloric intake or locomotor activity. Metabolism was, however, increased in R6/2 mice and this may be the cause of weight loss. Interestingly, weight loss increases with higher CAG repeat number in the mutant gene in both HD patients and R6/2 mice. This suggests that mutant huntingtin affects metabolic rate in a CAG repeat length dependant manner. The mechanism underlying this is unclear, but several regulators of metabolism, including the hypothalamus, were affected in R6/2 mice. In addition, the gastro-intestinal tract is affected in both HD patients and R6/2 mice and malabsorption of nutrients was observed in end-stage R6/2 mice. Although gastro-intestinal dysfunction is unlikely to be the cause of weight loss in HD, it may play an important role in the acceleration of weight loss in the final stages of the disease.

The findings in this thesis demonstrate that mutant huntingtin does not only affect the areas that have traditionally received the most attention in HD research, i.e. the basal ganglia. Other areas in the brain and body, such as the hypothalamus and gastro-intestinal tract, are also affected. Dysfunction of these structures could account for weight loss as well as several other, yet poorly understood, signs and symptoms of HD. Elucidation of the role of mutant huntingtin throughout the body could provide a better understanding of HD pathogenesis, lead to the development of novel markers of disease progression, and open new avenues for treatment.

Key words:
Huntington's disease, weight loss, metabolism, hypothalamus, gastro-intestinal tract

Classification system and/or index termes (if any):

Supplementary bibliographical information:

Language
English

ISSN and key title:
1652-8220

ISBN
978-91-86059-43-9

Recipient's notes

Number of pages

Price

Security classification

Beyond the Basal Ganglia

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Academic dissertation

by

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Cover art:
Research on Huntington's disease has primarily focused on the neuropathology of the basal ganglia. The disease-causing gene is, however, ubiquitously expressed throughout the body. The cover illustrates structures outside the brain that may also be affected in Huntington's disease.

Cover and thesis artwork:
Jorien van der Burg

Page lay-out:
Bengt Mattsson

ISBN 978-91-86059-43-9
Lund University, Faculty of Medicine Doctoral Dissertation Series, 2008:90

Printed by Grahns Tryckeri AB, Lund, Sweden

To my parents

Aan mijn ouders

Do not go where the path may lead,
go instead where there is no path and leave a trail.

Ralph Waldo Emerson
1803-1882

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Neurobiol Dis. 29 (1), 41-51.
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II. Wood N.I.*, Goodman A.O.G.*, **van der Burg J.M.M.***, Gazeau V., Brundin, P., Björkqvist M., Petersén Å., Tabrizi S.J., Barker R.A., Morton A.J., 2008. Increased thirst and drinking in Huntington's disease and the R6/2 mouse.
Brain Res Bull. 76(1-2), 70-79.
* The first three authors have equally contributed to the study

III. Aziz N.A., **van der Burg J.M.M.,** Landwehrmeyer G.B., Brundin P., Stijnen T., Roos R.A.C., 2008. Weight loss in Huntington's disease increases with higher CAG repeat number.
Neurology, in press.

IV. Van der Burg J.M.M., Maat-Schieman M.L.C., Winqvist A., Aziz N.A., Roos R.A.C., Bates G.P., Sundler F., Brundin P., Wierup N., Björkqvist M. Gastro-intestinal dysfunction in Huntington's disease.
Manuscript

PUBLISHED ARTICLES NOT INCLUDED IN THIS THESIS

Terpstra N.J., Bolhuis J.J., Riebel K., **van der Burg J.M.M.,** den Boer-Visser A.M., 2006. Localized brain activation specific to auditory memory in a female songbird.
J Comp Neurol. 494 (5), 784-791.

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Brain Res Mol Brain Res. 140 (1-2), 127-132.

SUMMARY

Huntington's disease (HD) is a fatal, hereditary disorder caused by a mutation in the gene encoding the protein huntingtin. Although mutant huntingtin is ubiquitously expressed throughout the body, HD research has mainly focused on the role of the basal ganglia. Dysfunction of these brain nuclei likely underlies motor disturbances in HD, including the conspicuous, uncontrollable, dance-like movements (chorea). However, HD is frequently complicated by other important signs and symptoms that cannot, or not solely, be attributed to basal ganglia dysfunction. Among these is unintended weight loss, which occurs in nearly all HD patients and can affect both quality of life and disease progression. Other symptoms that might occur irrespective of basal ganglia dysfunction are cognitive deterioration, psychiatric problems, sleep disturbances, cardiac failure and atrophy of skeletal muscle.

The past decade has seen an increasing interest in the role of mutant huntingtin in other areas of the brain and body. Although these effects are still poorly understood, their study could lead to a better understanding of the pathological mechanisms underlying HD, as well as to the identification of novel markers of disease progression and therapeutic options. The aim of this thesis was, therefore, to investigate the effects of mutant huntingtin outside the basal ganglia, especially those structures that might underlie weight loss in HD, including the hypothalamus, adipose tissue, and the gastro-intestinal tract. We found that weight loss in both HD patients and R6/2 mice (a transgenic model of HD) is not caused by changes in caloric intake or locomotor activity. Metabolism was, however, increased in R6/2 mice and this may be the cause of weight loss. Interestingly, weight loss increases with higher CAG repeat number in the mutant gene in both HD patients and R6/2 mice. This suggests that mutant huntingtin affects metabolic rate in a CAG repeat length dependant manner. The mechanism underlying this is unclear, but several regulators of metabolism, including the hypothalamus, were affected in R6/2 mice. In addition, the gastro-intestinal tract is affected in both HD patients and R6/2 mice and malabsorption of nutrients was observed in end-stage R6/2 mice. Although gastro-intestinal dysfunction is unlikely to be the cause of weight loss in HD, it may play an important role in the acceleration of weight loss in the final stages of the disease.

The findings in this thesis demonstrate that mutant huntingtin does not only affect the areas that have traditionally received the most attention in HD research, i.e. the basal ganglia. Other areas in the brain and body, such as the hypothalamus and gastro-intestinal tract, are also affected. Dysfunction of these structures could account for weight loss as well as several other, yet poorly understood, signs and symptoms of HD. Elucidation of the role of mutant huntingtin throughout the body could provide a better understanding of HD pathogenesis, lead to the development of novel markers of disease progression, and open new avenues for treatment.

SAMMANFATTNING

Huntingtons sjukdom (HS) är en ärftlig och dödlig sjukdom som orsakas av en mutation i genen som kodar för proteinet huntingtin. Trots att muterat huntingtin är uttryckt i hela kroppen genom proteinet ubikvitin, så har forskningen på HS till största del fokuserats på effekterna av sjukdomen på de basala ganglierna. En rubbad funktion i basala ganglierna ligger troligtvis bakom de motoriska störningar vid HS, som innefattar de iögonfallande, okontrollerbara och dansande rörelserna (därav den tidigare beteckningen danssjuka eller chorea). HS kompliceras oftast ytterligare av andra viktiga tecken och symptom som inte endast kan härledas till dysfunktion i de basala ganglierna. Ett av dessa symptom är ofrivillig viktminskning, som drabbar nästan alla HS-patienter och detta kan påverka både livskvaliteten samt hur sjukdomen framskrider. Andra symptom som uppkommer som inte enbart kan härledas till dysfunktion i de basala ganglierna, är en försämring av den kognitiva förmågan, psykiatriska problem, sömnsvårigheter, hjärtsvikt och förtvining av skelettmusklerna.

Det senaste årtiondet har intresset ökat att studera det muterade huntingtinproteinets roll i även andra områden av hjärnan och kroppen. Trots att kunskaperna fortfarande är bristfälliga, så kan studier av dessa effekter leda till en bättre förståelse av de patologiska mekanismerna som ligger bakom HS, samt till att identifiera nya markörer av sjukdomsförloppet och behandlingsalternativ. Målet med denna avhandling var därför att undersöka effekterna av muterat huntingtin utanför de basala ganglierna, framförallt i de strukturer som skulle kunna ligga bakom viktminskningen vid HS, som hypotalamus, fettvävnad och mag-tarmkanalen. Vi upptäckte att viktminskning hos HS-patienter och R6/2-möss (en transgen modell av HS) inte orsakas av förändringar i kaloriintag eller rörelseaktivitet. Ämnesomsättningen hade istället ökat hos R6/2-mössen och detta kan vara orsaken till viktminskningen. Intressant nog så ökade viktminskningen med ökat antal CAG-sekvenser i den muterade genen, bland både HS-patienter och R6/2-möss. Detta antyder att muterat huntingtin påverkar ämnesomsättningen och att den ökar med högre antal CAG-sekvenser. Mekanismen bakom detta är oklar, men ett flertal av de strukturer som reglerar ämnesomsättningen, hypotalamus medräknad, påverkades bland R6/2-mössen. Dessutom påverkades mag-tarmkanalen bland både HS-patienter och R6/2-möss och vi kunde observera att näringsämnena absorberades dåligt i R6/2-mössen. Trots att dysfunktion i mag-tarmkanalen inte är en trolig orsak till viktminskning vid HS, så kan det spela en viktig roll i slutskedet av sjukdomen när förloppet av viktminskningen påskyndas.

Fynden i denna avhandling visar att muterat huntingtin inte bara påverkar de områden som traditionellt uppmärksammats mest inom HS-forskningen, dvs. de basala ganglierna. Andra områden i hjärnan och kroppen, som hypotalamus och mag-tarmkanalen, påverkas också. Dysfunktion i dessa strukturer kan förklara viktminskningen samt flera andra tecken och symptom av HS som man hittills har dålig kunskap om. Ett klargörande över det muterade huntingtinproteinets roll i hela kroppen skulle kunna ge en bättre förståelse av HS-patogenes, samt leda till utvecklingen av tidigare okända markörer för sjukdomsutvecklingen och öppna upp vägar för nya behandlingar.

SAMENVATTING

De ziekte van Huntington is een fatale, erfelijke aandoening die wordt veroorzaakt door een mutatie in het gen dat codeert voor het eiwit huntingtine. Hoewel mutant huntingtine overal in het lichaam tot expressie komt, heeft het onderzoek naar de ziekte zich vooral geconcentreerd op de rol van de basale ganglia. Aantasting van deze hersenkernen ligt waarschijnlijk ten grondslag aan de bewegingsstoornissen, waaronder de in het oog springende, ongewilde, dans-achtige bewegingen (chorea), die bij de ziekte voorkomen, De ziekte van Huntington wordt echter ook gecompliceerd door andere, belangrijke symptomen die niet, of niet alleen, aan aantasting van de basale ganglia toegeschreven kunnen worden. Een van deze symptomen is ongewild gewichtsverlies. Dit komt bij bijna alle patiënten met de ziekte van Huntington voor en kan zowel de kwaliteit van leven als de progressie van de ziekte ongunstig beïnvloeden. Andere symptomen die wellicht niet alleen door aantasting van de basale ganglia veroorzaakt worden, zijn onder andere cognitieve achteruitgang, psychiatrische problemen, verstoring van de slaap, hartfalen en atrofie van skeletspierweefsel.

De afgelopen tien jaar is de interesse in de rol van mutant huntingtine in andere gebieden van de hersenen en het lichaam toegenomen. Hoewel deze effecten nog grotendeels onbekend zijn, kan het bestuderen ervan leiden tot een beter begrip van de pathologische mechanismen die aan de ziekte ten grondslag liggen. Ook draagt het mogelijk bij aan de ontdekking van nieuwe markers voor ziekteprogressie, alsmede de ontwikkeling van nieuwe medicatie. Het doel van dit proefschrift was daarom om de effecten van mutant huntingtin in andere gebieden dan de basale ganglia, waaronder de hypothalamus, vetweefsel en het maag-darmkanaal, te onderzoeken. We vonden dat gewichtsverlies in zowel patiënten met de ziekte van Huntington als in R6/2 muizen (dit is een transgeen dier-model van de ziekte van Huntington) niet wordt veroorzaakt door veranderingen in calorische inname of fysieke activiteit. Het metabolisme bij R6/2 muizen was echter verhoogd en dit zou mogelijk de oorzaak van het gewichtsverlies kunnen zijn. Interessant is dat het gewichtsverlies gecorreleerd was met het aantal CAG repeats in het *huntingtine* gen bij zowel patiënten met de ziekte van Huntington als de R6/2 muizen. Dit suggereert dat de sterkte waarmee mutant huntintine het metabolisme verstoort, afhangt van het aantal CAG repeats in het mutante gen. Het onderliggende mechanisme is onbekend, maar diverse structuren in het lichaam die betrokken zijn bij het regelen van de metabole snelheid, waaronder de hypothalamus, waren aangetast. Verder was ook het maag-darm kanaal bij zowel patiënten met de ziekte van Huntington als de R6/2 muizen aangetast. De opname van nutriënten door het maag-darm kanaal was verminderd bij muizen die in het eindstadium van de ziekte verkeerden. Hoewel aantasting van het maag-darm kanaal waarschijnlijk niet de oorzaak is van gewichtsverlies bij de ziekte van Huntington, speelt het vermoedelijk een belangrijke rol bij het excessieve gewichtsverlies in de eindstadia van de ziekte.

De bevindingen in dit proefschrift tonen aan dat mutant huntingtine behalve de basale ganglia ook andere delen van de hersenen en het lichaam aantast. De aantasting van deze gebieden zou ten grondslag kunnen liggen aan het gewichtsverlies, maar zou daarnaast ook kunnen bijdragen aan andere symptomen van de ziekte van Huntington waarvan de oorzaken nog steeds niet goed bekend zijn. Het ontrafelen van de rol van mutant huntingtine in het gehele lichaam, zou ons meer inzicht kunnen geven in het ziektemechanisme en zou kunnen bijdragen aan de ontwikkeling van nieuwe markers voor ziekteprogressie. Ook zou het mogelijk bij kunnen dragen aan het ontwikkelen van nieuwe behandelingen voor de ziekte.

ABBREVIATIONS

3V	Third ventricle
α-MSH	α-melanocyte stimulating hormone
ABC	Avidin-biotin complex system
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AGRP	Agouti-related protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
Arc	Arcuate nucleus
ATP	Adenosine triphosphate
AUC	Area under the curve
BAT	Brown adipose tissue
BDI	Beck depression inventory
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BSA	Bovine serum albumine
C57BL/6	Commonly used mouse strain
CAG	Cytosine-adenine-guanine
Cal	Calories
CART	Cocaine- and amphetamine-regulated transcript peptide
CCK	Cholecystokinine
cDNA	Complementary DNA
C/EBP-α	CAAT-enhancer-binding protein-α
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CRF	Corticotropin-releasing factor
CSF	Cerebrospinal fluid
D1	Dopamine receptor 1
D2	Dopamine receptor 2
DA	Dopamine
DAB	3,3-diaminobenzidine
DARPP-32	Dopamine- and cyclic AMP-regulated phosphoprotein 32
DMH	Dorsomedial hypothalamus
DNA	Deoxyribonucleic acid
EHDI	European Huntington's Disease Initiative
ENS	Enteric nervous system
ER	Endoplasmic reticulum
FAS	Functional assessment
FITC	Fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GH	Growth hormone
GHRF	Growth hormone-releasing factor
GnRH	Gonadotropin-releasing hormone
GPe	External segment of the globus pallidus
GPi	Internal segement of the globus pallidus

HAP	Huntingtin-associated protein
HD	Huntington's disease
H/E	Hematoxylin and eosin
HIP	Huntingtin-interacting protein
IL-6	Interleukin-6
IL-8	Interleukin-8
IR	Immunoreactive
IT15	Interesting transcript 15
kCal	Kilo calories
LH	Lateral hypothalamus
MAO	Monoamine oxidase
MCH	Melanin-concentrating hormone
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NeuN	Neuronal nuclei
NF-κβ	Neuronal factor κβ
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
P53	Protein 53
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
PGP	Protein gene product
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
PYY	Peptide YY; also known as peptide tyrosine tyrosine
Q-PCR	Quantitative polymerase chain reaction
R6/2 mice	Transgenic mice expressing exon 1 of the human *HD* gene
REST-NRSE	Respressor Element-1 silencing transcription factor/neuron-restrictive silencer element
RIA	Radioimmunoassay
RNA	Ribonucleic acid
SCN	Suprachiasmatic nucleus
S.E.M.	Standard error of the mean
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TFC	Total functional capacity
TH	Tyrosine hydroxylase
TNF	Tumor necrosis factor
UCP1	Uncoupling protein 1
UCP2	Uncoupling protein 2
UHDRS	Unified Huntington's Disease Rating Scale
VACht	Vesicular acetylcholine transporter
Vas	Vasopressin
VMH	Ventromedial hypothalamus
VO_2	Rate of oxygen consumption
WT	Wild-type (*not expressing mutant huntingtin*)
YAC	Yeast artificial chromosome

INTRODUCTION

In 1872 a young man named George Huntington published an article in which he described a hereditary, fatal disorder mainly characterized by uncontrollable movements (chorea) and mental impairment (Huntington, 1872). His description of the illness was so accurate that it gave the disease its eponymous name and served as the foundation for all subsequent work.

George Huntington proposed that 'chorea is essentially a disease of the nervous system', a view previously suggested by Paracelsus (table 1) (Huntington, 1872). On autopsy, Meynert (1877) observed that brains of Huntington's disease patients exhibit extensive atrophy of the basal ganglia (Bates et al., 2002). Subsequent research on Huntington's disease (HD) has therefore focused on the neuropathology of the basal ganglia, with limited attention to pathology in other regions (table 2).

Over the past century we have come to appreciate that HD not only presents with movement disturbances, but with a wide spectrum of other signs and symptoms, including cognitive deterioration, psychiatric problems, sleep disturbances, wasting of skeletal muscle, heart failure, and weight loss. Basal ganglia dysfunction alone may not be sufficient to explain the occurrence of such signs and symptoms. Are other brain areas and peripheral organs also affected in HD, and could they be held responsible for causing these 'non-motor symptoms'?

Clinical and pathological aspects of Huntington's disease

Symptoms and prevalence

Huntington's disease is an autosomal-dominantly inherited disorder. It has a prevalence of 4 to 8 per 100,000 people in European and Northern American populations and affects men and women equally (Harper, 1992). The first symptoms typically start appearing between the ages of 35 and 45 years and include minor uncontrollable movements and personality changes, such as depression and irritability (for review see Walker, 2007). Initially, motor symptoms mainly involve the small distal muscles, but later on also the larger postural muscles are involved, resulting in the characteristic dance-like movements called 'chorea'. Over the years, cognitive functions gradually deteriorate leading to impairments in memory and attention. The end-stage of the disease is characterized by rigidity, dystonia and dementia. In most cases, HD results in death about 15 to 20 years after clinical onset.

In about 7% of HD cases, the first symptoms appear in childhood and lead to death within 7 to 10 years after disease onset (for review see Nance and Myers, 2001). This juvenile form of HD is neuropathologically comparable to the adult variant, although the rate of brain atrophy is increased and neuronal inclusions are more abundant (Squitieri et al., 2006). Compared to the adult form, disease progression is faster in juvenile onset HD patients. Choreatic symptoms are less pronounced and epileptic seizures, rigidity and dystonia are more common (Squitieri et al., 2006).

Year	Event
1374	Epidemic dancing mania described
1500	Paracelsus suggests CNS origin for chorea
1686	Thomas Sydenham describes post-infectious chorea
1832	John Elliotson identifies inherited form of chorea
1872	George Huntington characterizes Huntington's disease (HD)
1877	Meynert proposes that lesions in the striatum underlie chorea in HD
1976	Striatal lesioning used to create an animal model of HD
1993	*HD* gene identified
1993-97	Huntingtin found to be expressed in many tissues and organs in the body
1996	First transgenic mouse models of HD developed
1999	Huntingtin aggregates discovered in non-CNS tissues in R6/2 mice
2000-08	Increasing evidence that skeletal muscle, testis, blood cells, pancreas, cardiomyocytes, and liver tract are affected in HD

Table 1. History of Huntington's disease.
(Table modified from Walker, 2007)

The cause of Huntington's disease: mutant huntingtin

Huntington's disease is caused by an increased number of cytosine-adenine-guanine (CAG) repeats located near the 5'-end in exon 1 of the 'interesting transcript 15' (*IT15*) gene (HD study group, 1993). Healthy individuals typically have less than 36 CAG repeats, and repeats of 40 or above cause HD with complete penetrance (for review see Gusella et al., 1993; Myers, 2004). Individuals with 36-39 CAG repeats are at risk of developing HD, but penetrance is incomplete (McNeil et al., 1997). The length of the CAG expansion is inversely correlated with age of disease onset, with juvenile onset characterized by expansions of more than 60 repeats (Trottier et al., 1994; Brandt et al., 1996).

The protein encoded by the *IT15* gene, huntingtin, is primarily localized in the cytoplasm, but it is also present in the nucleus (for review see Landles and Bates, 2004). Huntingtin is a multi-domain protein with many functions and it is widely expressed throughout the body, in both neuronal and non-neuronal cells (Hoogeveen et al., 1993; Trottier et al., 1995). It may act as a molecular scaffold and regulates several cellular processes including protein trafficking and vesicle transport (Engelender et al., 1997), transcriptional events (Marcora et al., 2003; Zuccato

Search	Number of publications
"Huntington's disease"	5313
"Huntington's disease" AND "basal ganglia"	449
"Huntington's disease" AND striatum	1299
"Huntington's disease" AND cortex	643
"Huntington's disease" AND "cerebral cortex"	345
"Huntington's disease" AND cerebellum	133
"Huntington's disease" AND hypothalamus	53
"Huntington's disease" AND muscle	148
"Huntington's disease" AND heart	36
"Huntington's disease" AND kidney	48
"Huntington's disease" AND stomach	4

Table 2. PubMed search for Huntington's disease.
PubMed is a search engine for abstracts of biomedical research articles. Searching for "Huntington's disease" in this database, gives a rough indication of the number of publications that has appeared about this disorder. All searches included in this table were performed on the 1st of August 2008. At that date, according to the PubMed engine, 5313 publications on Huntington's disease had appeared, of which 449 concerned the basal ganglia and 1299 the striatum, a structure that is part of the basal ganglia. The cerebral cortex was the next most investigated brain region, but the number of publications concerning this area was not even half of the number of publications on the striatum. Remarkably few publications had appeared on organs outside the central nervous system.
Note: Searches in PubMed can be conducted in different ways, resulting in different numbers of publications. This table is, therefore, a rough indication of the number of scientific publications that had appeared on the different topics until the July 30, 2008.

et al., 2003), and brain-derived neurotrophic factor (BDNF) production (Zuccato et al., 2001; Zuccato et al., 2003; Zuccato et al., 2005). The fact that huntingtin is highly conserved throughout evolution (Cattaneo et al., 2005) suggests that it is indispensable.

Mutant huntingtin has an expanded polyglutamine domain that induces a conformational change causing the protein to form intracellular aggregates. Most of these aggregates manifest as intranuclear inclusions (Lunkes et al., 2002), but aggregates are also found in the cell body, in axons and in dendrites (Li et al., 2003a). Inclusions are not unique to the brain. A study in the R6/2 mouse model of HD demonstrated their presence in many organs, including the liver, kidney, pancreas and gastro-intestinal tract (Sathasivam et al., 1999). Their role remains contro-

versial: some researchers believe the aggregates are a neutral by-product or even neuroprotective (Arrasate et al., 2004; Slow et al., 2005), whereas others believe they are toxic.

Huntingtin interacts with various proteins. Today, more than 30 huntingtin-interacting proteins have been identified, and the list is continuously growing (for review see Li and Li, 2004). Some are involved in trafficking and endocytosis, such as huntingtin-associated protein 1 (HAP1) (Li et al., 1995; Li and Li, 2005), and huntingtin-interacting protein 1 (HIP1) (Kalchman et al., 1997; Wanker et al., 1997). Others, such as nuclear factor-κB (NF-κβ), protein 53 (P53), and repressor element-1 silencing transcription factor/neuron-restrictive silencer element (REST-NRSE) are important transcription factors. Several are involved in signaling and cell metabolism. It is unclear if and how these huntingtin-interacting proteins are involved in HD pathogenesis.

Cellular dysfunction

There are various ways by which mutant huntingtin could cause cellular dysfunction and death (for review see Landles and Bates, 2004). Misfolded huntingtin for instance saturates the proteasome system that normally degrades un-needed and damaged proteins. Consequently, mutant huntingtin accumulates and forms insoluble aggregates that recruit and trap other proteins. Among these trapped proteins are transcription factors, such as CREB-binding protein, and depletion of such factors results in transcriptional down-regulation of many other proteins.

Presence of mutant huntingtin also causes mitochondrial impairment and oxidative stress (for review see Browne and Beal, 2004, 2006; Lin and Beal, 2006). Activity of different mitochondrial complexes is decreased in HD cells (Gu et al., 1996; Arenas et al., 1998; Tabrizi et al., 2000) resulting in impaired metabolism, reduced ATP production, decreased resting membrane potential and a reduced threshold for calcium induced depolarization of mitochondria. This renders cells more vulnerable to excitotoxic insults. Excitotoxicity is a pathological process by which cells die due to overstimulation by excitatory amino acids, such as glutamate. Overactivation of the N-methyl-D-aspartate (NMDA)- and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-receptors by the excitatory amino acids leads to increased influx of calcium into the cells, which activates free radicals and enzymes such as phospholipases, endonucleases, and proteases (includes caspases and calpains). Free radicals are highly reactive oxygen derivates that attack DNA and oxidize proteins. Phospholipases, endonucleases, and proteases digest the cytoskeleton, membranes, DNA, proteins and various other cellular components.

Apart from these toxic gain of functions, loss of wild-type huntingtin might also contribute to cell dysfunction and death. Ablation of wild-type huntingtin in mice is lethal (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995), indicating that huntingtin is necessary for cell survival. Loss of huntingtin has many consequences, all related to the functions it has. It could for instance cause depletion of BDNF. This growth factor is present in both the central and peripheral nervous system where it promotes survival and growth of neurons. Wild-type huntingtin normally binds to the transcription factors REST/NRSF that halt the production of BDNF. Absence of wild-type huntingtin leads to a constant inhibition of transcription of BDNF, resulting in decreased levels of this neurotrophic factor.

The role of the basal ganglia in Huntington's disease

Anatomy and function of the basal ganglia

Research on pathological mechanisms of HD has mainly focused on the basal ganglia. These are a group of interconnected brain nuclei that consist of the striatum (which includes the caudate nucleus, putamen and nucleus accumbens), and the external (GPe) and internal (GPi) segments of the globus pallidus. Because of their close functional and anatomical association, the substantia nigra (SN) and subthalamic nucleus (STN) are also often regarded as part of the basal ganglia.

The basal ganglia are mainly involved in motor control, but have also been implicated in cognition and emotion. The classical model of basal ganglia function developed by Albin and co-authors (Albin et al., 1989) and later modified by Chesselet and Delfs (1996), distinguishes a direct and indirect pathway. The striatum is the major input structure of the basal ganglia receiving information from the cortex through excitatory (glutamatergic) neurons. Input arises from most areas of the cortex, most extensively from the motor and prefrontal cortices (Parent and Hazrati, 1995a, b). The information that enters the striatum continues by the direct and indirect pathways to the GPi and GPe respectively. The GABAergic striatal neurons of the direct pathway express dopaminergic-1 (D1) receptors. They directly inhibit the GPi and in turn remove the tonic inhibition of the thalamus. The GABAergic striatal neurons of the indirect pathway express dopaminergic-2 (D2) receptors. They inhibit the GPe, reducing its tonic inhibition of the subthalamic nucleus. As a result of the disinhibition, the glutamatergic neurons of the subthalamic nucleus excite GABAergic neurons in the GPi and thereby promote inhibition of the thalamus. Removal of the tonic inhibition of the thalamus by the direct pathway facilitates movement, whereas the indirect pathway inhibits movement. (For review of basal ganglia function see Kandel et al., 2000)

The basal ganglia are affected in Huntington's disease

Soon after the publication of George Huntington's article, it was discovered that the striatum is severely atrophic in HD (Bates et al., 2002). Striatal atrophy occurs gradually and usually starts in the caudate nucleus (Vonsattel et al., 1985; Vonsattel and DiFiglia, 1998). Not all neuronal populations in the striatum are equally affected. Medium-sized spiny neurons, corresponding to about 90% of the striatal neuronal population, are generally the first to degenerate, whereas a-spiny interneurons are relatively spared (Ferrante et al., 1985; Ferrante et al., 1987; Cicchetti et al., 1996). Medium-sized spiny neurons include substance P-containing cells that project to the GPi and enkephalin-containing cells that project to the GPe. The latter, enkephalin-containing medium-sized spiny neurons, are the first to degenerate in HD (Sapp et al., 1995; Reiner et al., 2003), causing impaired activity of the indirect pathway and leading to increased locomotor activity and uncontrolled movements.

Why is the striatum so vulnerable?

It is not clear why the striatum is particularly vulnerable in HD. Many of the factors that are involved in cell death, such as caspases, proteases, and calcium are present in all cells. Selective cell loss in the striatum might result from a pathological interaction of mutant huntingtin with

striatum-specific molecules. Despite an extensive search for such molecules, only brain-specific proteins that interact with mutant huntingtin have been found (for review see Li and Li, 2004).

In the 1970s it was discovered that the striatum is very sensitive to toxic effects of excitatory amino acids, such as glutamate, kainic acid, and NMDA. Intrastriatal injections with kainate acid or NMDA in animals, affects medium-sized spiny neurons, while interneurons are spared (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976; Ferrante et al., 1985; Beal et al., 1986). The sensitivity of a certain group of neurons to 'excitotoxic' insults largely depends on the number of NMDA receptors that are present on the cells, as well as on the uptake of glutamate by surrounding glial cells. Interestingly, brain regions that are most vulnerable in HD are generally not those containing the highest numbers of NMDA receptors (Wagster et al., 1994). Moreover, levels of glutamate are not increased in the basal ganglia (Reynolds et al., 1988; Schwarcz et al., 1988b; Schwarcz et al., 1988a; Beal et al., 1990; Hickey et al., 2005), despite reduced levels of glutamate transporters in HD glial cells (Arzberger et al., 1997; Lievens et al., 2001). Moreover, several mouse models of HD replicate many of the HD symptoms, such as locomotor dysfunction, while their striata are resistant to excitotoxic insults (Hansson et al., 1999; Hansson et al., 2001).

BDNF and dopamine (DA) might also play a role in striatal cell death. Striatal neurons are especially reliant on BDNF and might also be oversensitive to the effects of DA. The latter may be well tolerated under normal circumstances, but could kill neurons with impaired energy metabolism. Among all brain regions, the striatum receives the densest DAergic innervation, which could explain its increased vulnerability in HD. Moreover, DA levels within the striatum show the same dorso-ventral gradient as the neuropathology in HD, indicating that DA is involved in the increased striatal sensitivity (Augood et al., 1997; Cass, 1997; Jakel and Maragos, 2000; Johnson et al., 2006).

Huntington's disease is not only a movement disorder

Although motor disturbances are a prominent feature of HD, the clinical phenotype is far more complex and variable than depictions of it as a progressive movement disorder. HD often presents with a wide spectrum of signs and symptoms, including cognitive deterioration, psychiatric and behavioral problems, sleep disturbances, autonomic nervous system dysfunction, heart failure, osteoporosis, wasting of skeletal muscle and weight loss (fig. 1).

Cognitive deterioration

Cognitive deterioration is an inherent feature of HD and is present from early in the disease (Zakzanis, 1998). Impairment of attention, concentration, visuospatial processing, and mnemonic function occur in early stages of HD, while deficits in executive function such as planning, problem solving, cognitive flexibility, and general cognitive deterioration are more pronounced during later stages of the disorder (Butters et al., 1978; Wolf et al., 2008a). Memory deficits progressively worsen over time, and include impairments in working memory, as well as declarative and procedural memory (Zakzanis, 1998; Wolf et al., 2007; Wolf et al., 2008b). In a meta-analysis of cognitive dysfunction in HD patients, verbal- and visual-delayed recall were found to be most impaired in HD, followed by tests involving executive skills, attention, and concentration (Zakzanis, 1998).

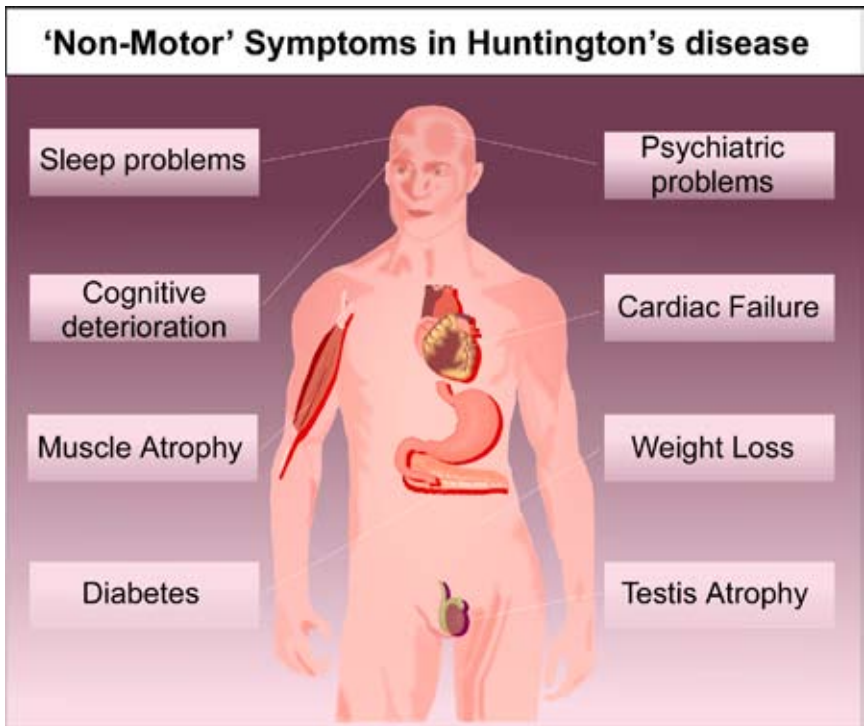

Figure 1. 'Non-motor symptoms' in Huntington's disease.
Huntington's disease not only presents with movement disturbances, but with a wide spectrum of other signs and symptoms, including cognitive deterioration, psychiatric problems, sleep disturbances, wasting of skeletal muscle, heart failure and weight loss.

Psychiatric and behavioral problems

The psychiatric symptoms in HD patients include depression, anxiety, irritability, apathy, schizophrenia-like states, behavioral and personality disorders, and aggression (Anderson and Marder, 2001; Berrios et al., 2001; Craufurd et al., 2001; Vassos et al., 2008). The prevalence of psychiatric disorders ranges from 35% to 76%, depending on the study design (van Duijn et al., 2007). The wide variation in the prevalence most likely results from definitional and ascertainment problems (Berrios et al., 2001; Vassos et al., 2008).

Sleep problems

Estimated from a questionnaire, almost 90% of HD patients suffer from sleep problems and nearly two-thirds of them report these as moderately or very much contributing to overall problems (Taylor and Bramble, 1997). Sleep problems include frequent nocturnal awakenings, increased non-rapid eye movement sleep and decreased slow wave sleep (Wiegand et al., 1991; Silvestri et al., 1995; Chokroverty, 1996; Petit et al., 2004). In addition, patients have disrupted circadian

rhythms (Morton et al., 2005). Interestingly, circadian sleep disruption is also evident in the R6/2 mouse model of HD (Morton et al., 2005) and treating the disrupted rhythm improved cognitive function in these mice (Pallier et al., 2007).

Autonomic nervous system dysfunction

Vegetative symptoms indicative of autonomic nervous system dysfunction have repeatedly been reported in patients with HD. They include defects in postural vasoregulatory mechanisms (Aminoff and Gross, 1973, 1974), hyperhydrosis of hands and feet, disturbances of micturition (Bruyn, 1968), and swallowing difficulties (Leopold and Kagel, 1985; Kagel and Leopold, 1992). Although vegetative symptoms are most prominent in the advanced stages of the disease (Nance and Sanders, 1996; Kirkwood et al., 2001), autonomic complaints such as dizziness following standing up, excessive perspiration and tachycardia can occur even in mildly disabled HD patients (i.e. Shoulson and Fahn stages I and II (Shoulson and Fahn, 1979)) and even in otherwise asymptomatic gene carriers (Kobal et al., 2004).

Heart failure

Multiple epidemiological studies have shown that cardiac failure is the second most common cause of death in HD patients (Lanska et al., 1988; Sorensen and Fenger, 1992). Around 25% of the patients die due to cardiac failure, compared to less than 2% of the age-matched non-HD patients in the general population. Huntington's disease patients have decreased heart rate variability, both at rest and during the Valsalva maneuver, which is inversely correlated to some aspects of the Unified Huntington's Disease Rating Scale (UHDRS) (Sharma et al., 1999; Andrich et al., 2002). Also changes in cerebral blood flow have been described (Deckel et al., 1998; Deckel et al., 2000) and even bradycardia and second-degree atrioventricular block have been reported in a case of juvenile HD (Stober et al., 1983).

Weight loss

Weight loss is an important feature of HD. It follows a progressive course that begins in presymptomatic *HD* gene carriers and ends with profound cachexia in advanced stage patients (Sanberg et al., 1981; Farrer and Meaney, 1985; Farrer and Yu, 1985; Stoy and McKay, 2000; Djousse et al., 2002; Trejo et al., 2004; Trejo et al., 2005; Robbins et al., 2006; Mochel et al., 2007). It is unclear what tissues are lost. Weight loss could result from reduced adipose tissue, but also osteoporosis (Bonelli et al., 2002; Otti et al., 2007) and wasting of skeletal muscle (Farrer and Meaney, 1985; Farrer and Yu, 1985; Ribchester et al., 2004; Trejo et al., 2004) could contribute to weight loss in HD. Loss of weight frequently leads to general weakening and affects the quality of life of HD patients (Nance and Sanders, 1996). In addition, a higher body mass index (BMI) has been associated with a slower rate of disease progression (Myers et al., 1991) and weight loss is therefore an important therapeutic target in HD.

Interestingly, HD patients lose weight despite adequate nutrition and have a normal, or even higher caloric intake compared to control subjects (Sanberg et al., 1981; Morales et al., 1989; Trejo et al., 2004; Mochel et al., 2007). Weight loss does not correlate with chorea scores (Djousse et al., 2002) and is most prominent in the final, hypokinetic stages of the disease (Sanberg et al., 1981), suggesting that it is not secondary to hyperactivity.

Despite these important observations, little is known about the pathogenesis of weight loss in HD. Maintaining body weight is a highly complex process that involves different organs and tissues (Flier, 2004; Badman and Flier, 2005). The hypothalamus is one of the key players in regulating appetite and energy metabolism. It continuously monitors the bodies energy levels and increases appetite or energy expenditure when levels are respectively too low or too high. Organs such as the stomach and small intestines, the pancreas, and adipose tissue play important roles in communicating energy levels to the hypothalamus, as well as regulating the uptake of nutrients. The role of these organs in weight loss in HD is, however, unknown.

What causes these 'non-motor signs and symptoms'?

Most of the above described signs and symptoms in HD are poorly understood. Some have been suggested to be secondary to basal ganglia dysfunction (Aylward et al., 1997; Aylward et al., 2000; Anderson and Marder, 2001; Rosas et al., 2001; Rosenblatt et al., 2003; Ruocco et al., 2006; Ruocco et al., 2008). Unwanted movements could for instance disturb sleep (Hurelbrink et al., 2005), or cause increased energy expenditure and weight loss. However, symptoms such as weight loss are most prominent in the final, hypokinetic stages of the disease, arguing against a role for the basal ganglia. As will be reviewed in the next chapters, it is possible that pathology outside the basal ganglia contributes to these non-motor features of HD.

Beyond the basal ganglia: multiple brain regions are affected

It has long been recognized that neurodegeneration in HD is not confined to the basal ganglia. Subjects with HD exhibit generalized brain atrophy and significant volume reduction of the cerebral cortex, hypothalamus, amygdala, hippocampus, brainstem, and cerebellum (Vonsattel et al., 1985; Aylward et al., 1997; Vonsattel and DiFiglia, 1998; Aylward et al., 2000; Rosas et al., 2001; Aylward et al., 2003; Ruocco et al., 2006; Ruocco et al., 2008).

Cerebral cortex

The cerebral cortex is, after the basal ganglia, the second most investigated brain region in HD (table 2). Many studies have demonstrated that the cerebral cortex is affected. Cortical volume (with the exception of the medial temporal lobe) is decreased by 25% (Halliday et al., 1998) and significant reductions in the number of neurons have been found (Cudkowicz and Kowall, 1990; Hedreen et al., 1991; Sotrel et al., 1991; Braak and Braak, 1992; Macdonald et al., 1997). Depending on the layer and area within the cortex, as many as 10 to 55% of the neurons are lost. Cortical pathology is already evident in pre-symptomatic HD gene carriers (Gomez-Anson et al., 2008) and progresses in middle (Rosas et al., 2003) and later stages (Heinsen et al., 1994; Wagster et al., 1994; Selemon et al., 2004) of the disease. Interestingly, a magnetic resonance imaging (MRI) study by Paulsen and co-authors (Paulsen et al., 2006) found increased volume of the cerebral cortex in preclinical HD, and these findings have, using a similar technique, been replicated in a mouse model of HD (Lerch et al., 2008). The volumes of cortical white matter, caudate and putamen were reduced.

Because of its extensive connectivity with the basal ganglia, most studies have focused on the frontal lobes. It has been shown, though, that the parietal cortex is also severely affected (Macdonald et al., 1997) and this could account for the slowing of saccadic eye movements, and deficits in performance of voluntary movements. Moreover, cortical neurodegeneration might also be involved in personality changes and dementia occurring in HD and it has been shown to contribute to some of the symptoms that previously had been primarily ascribed to the striatum (Rosas et al., 2008).

Cerebellum

The cerebellum plays an important role in the integration of sensory perception, coordination, and motor control. Neurodegeneration in the cerebellum has only been examined in a few cases of HD. Some studies reported cerebellar atrophy (Rosas et al., 2003; Ruocco et al., 2008), which manifests as thinning of the granule cell layer and loss of Purkinje cells (Jeste et al., 1984). Recently, loss of white matter in the cerebellum of HD patients was reported and it was suggested that the cerebellum and the integrity of cerebellar white matter might play a more important role in HD symptomatology (Fennema-Notestine et al., 2004).

Hippocampus

The hippocampus is located in the forebrain and plays a major role in short term memory and spatial navigation. It is one of a limited number of adult brain regions in which neurogenesis takes place. Late onset hippocampal degeneration in HD might contribute to memory deficits and general dementia. Several studies in HD patients reported a reduction of hippocampal area (de la Monte et al., 1988) and loss of neurons in the CA1 region of the hippocampus (Spargo et al., 1993). In the R6/1 (Lazic et al., 2004; Lazic et al., 2006) and R6/2 mouse models of HD (Gil et al., 2005) decreased cell proliferation was observed.

Thalamus

The thalamus is part of the circuitry connecting the basal ganglia and the neocortex. Neuronal loss in this area might therefore disrupt the striatocortical circuitry and contribute to motor dysfunction (Cepeda et al., 2007). As the thalamus also plays a role in the regulation of sleep and wakefulness, and emotions, thalamic degeneration might also contribute to the sleep problems and emotional disturbances frequently observed in HD patients.

Stereological quantification of the number of neurons and glial cells in the thalamus of terminal-stage HD patients, has demonstrated that in certain thalamic nuclei as many as 20 to 55% of the neurons are lost (Heinsen et al., 1996; Heinsen et al., 1999). Using MRI, Kassubek and co-authors also found the thalamus to be atrophic in HD patients and showed that this atrophy correlates with cognitive performance (Kassubek et al., 2005). A more recent study involving serial positron emission tomography (PET) found that thalamic metabolism is increased in presymptomatic HD gene carriers, but fell to subnormal levels as soon as they started developing symptoms (Feigin et al., 2007).

Brainstem

The brainstem is responsible for the regulation of many vital functions, such as breathing, digestion, heart rate and blood pressure. Little has been published on neuropathological alterations in the brainstem of HD patients, but neurodegeneration and atrophy in this area has been reported (Roos et al., 1986; Roos, 1986; Rosas et al., 2003). More recent findings indicated long-term potentiation plasticity to be impaired in the brainstem of HD patients (Crupi et al., 2008). In mice, HAP1 has recently been shown to be an important regulator of both cerebellar and brainstem development (Sheng et al., 2008), but it is unclear if depletion of HAP1 in HD could lead to cerebellar and brainstem pathology.

The hypothalamus

Anatomy and functions of the hypothalamus

The hypothalamus plays an important role in the regulation of sleep, reproduction, water homeostasis, body temperature, energy metabolism, appetite, and body weight (for review see Swaab, 2003). Several of these functions, such as body weight and sleep regulation, are disrupted in HD patients and hypothalamic dysfunction may contribute to this (Aziz et al., 2007).

The hypothalamus can be divided into three regions in a rostro-caudal direction: anterior (or preoptic), medial (or tuberal) and posterior region.

The anterior hypothalamus

The most anterior part of the hypothalamus is the preoptic area, which includes the suprachiasmatic nucleus (SCN) that acts as a circadian pacemaker or biological master-clock. The anterior hypothalamus is also involved in the control of blood pressure, body temperature, cycles of activity and reproductive activity.

The medial hypothalamus

The medial hypothalamus includes the dorsomedial, ventromedial, lateral, paraventricular, supraoptic, and arcuate nuclei. (See fig. 2 for an overview of the medial hypothalamus in the mouse brain and the neuronal populations that have been investigated in this thesis.)

The paraventricular nucleus (PVN) lies adjacent to the third ventricle, from which it derives its name. Based on cell size, two types of neurons are generally distinguished in the PVN: magnocellular and parvocellular neurons. The magnocellular cells possess long axons that terminate in the posterior pituitary gland. There they release either oxytocin or vasopressin, two peptide hormones that are produced in the cell bodies of the magnocellular cells and released into the blood stream in the posterior pituitary. Vasopressin, also known as arginine vasopressin or antidiuretic hormone, plays a major role in water homeostasis. It is released when the body is dehydrated and causes the kidneys to conserve water. It also raises blood pressure by inducing moderate vasoconstriction. Oxytocin has several roles, including the regulation of breastfeeding and uterus contractions during labor.

The parvocellular neurons of the PVN produce neuropeptides, such as corticotropin-releasing factor (CRF) and thyrotropin-releasing hormone (TRH) that are released into the hypothalamic-pituitary portal system that transports them to the anterior pituitary. There they stimulate the

production of, respectively, adrenocorticotropic hormone (ACTH) and thyroid-stimulating hormone (TSH). ACTH is a component of the hypothalamic-pituitary-adrenal axis and is produced in response to stress. Its principal effect is to increase cortisol production by the adrenal glands. TSH stimulates the thyroid gland to secrete the hormones thyroxine (T4) and triiodothyronine (T3) that increase the body's basal metabolic rate.

The arcuate nucleus contains neuroendocrine neurons, such as growth hormone-releasing hormone (GHRH) producing neurons, and projecting neurons, such as the neuropeptide Y (NPY), agouti-related protein (AGRP), pro-opiomelanocortin (POMC), cocaine-and-amphetamine-regulate transcript (CART) and somatostatin producing neurons. GHRH is transported to the anterior pituitary gland via the hypophysial portal blood system where it stimulates the secretion of growth hormone (GH). The other peptides are, among other things, involved in appetite regulation: NPY and AGRP stimulate appetite, whereas POMC and CART inhibit appetite and feeding.

The lateral hypothalamus contains neurons expressing the orexigenic neuropeptides melanin-concentrating hormone (MCH) and orexin. The latter, also known as hypocretin producing neurons, express the *orexin* gene, which codes for the precursor peptide prepro-orexin from which two orexin peptides are generated: orexin-A and orexin-B. Both MCH and orexins have several functions, including the regulation of appetite and energy metabolism.

The medial hypothalamus in humans and primates also contains the lateral tuberal nuclei. The function of these nuclei is not completely clear, but they are enriched with NMDA-receptors (Kremer 1993).

It has to be acknowledged that, apart from the above described functions, most peptides in the medial hypothalamus have various other roles. Orexins are for instance also known as regulators of wakefulness. Moreover, the expression of most peptides is not limited to the above described hypothalamic nuclei. CART, for instance, is also present in the dorsomedial and lateral nuclei (Elias et al., 2001).

The posterior hypothalamus

The posterior hypothalamus includes the mammillary bodies, which are involved in memory processing (Swaab, 2003; Vann and Aggleton, 2004), and the tuberomammillary nucleus. The latter contains the histaminergic system and is the only source of histamine in the brain. The histaminergic system is involved in a great number of different functions, such as modulation of the state of arousal, the control of vigilance, sleep and wakefulness, locomotor activity, food intake, neuronal plasticity, and learning and memory (Passani et al., 2000; Swaab, 2003).

Hypothalamic-pituitary axes

Hypothalamic output is either conveyed via neural projections, or through endocrine hormones. Neural projections include the medial forebrain bundle that connects the hypothalamus with the brain stem, basal forebrain, amygdala and cerebral cortex, and a periventricular fiber system that links the hypothalamus to the midbrain.

The endocrine projections of the hypothalamus are classically divided into different axes, including the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic-pituitary-thyroid (HPT) axis, the hypothalamic-pituitary-gonodal (HPG) axis, the somatotropic axis and the lactotropic axis. Via these axes, the hypothalamus regulates the production of many hormones in the periphery, including cortisol by the adrenal gland (regulated by the HPA axis), thyroid hormones

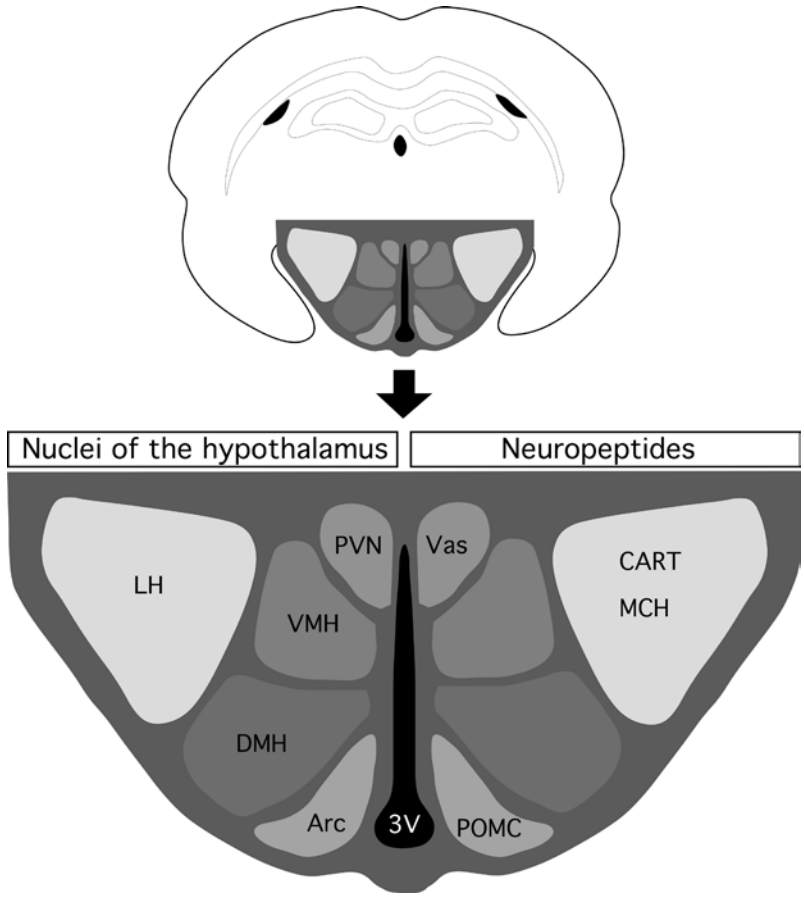

Figure 2. Overview of the medial hypothalamus in mice.
Schematic overview of the localization of the hypothalamus in a coronal section of the mouse brain (top figure). The figure below represents a rough indication of the location of the nuclei (on the left side) and neuropeptides (on the right side) that were investigated in the studies included in this thesis. Note that not all nuclei of the hypothalamus can be seen at the same rostro-caudal level. Moreover, the expression of most peptides is not limited to the nuclei that they have been depicted in.

by the thyroid (regulated by the HPT axis), and estrogen, progesterone and testosterone by the gonads (regulated by the HPG axis). As will be reviewed below, both the hypothalamus (Kremer et al., 1990; Kremer et al., 1991; Kassubek et al., 2004; Petersen et al., 2005; Aziz et al., 2008a) and the expression of several of these 'peripheral hormones', e.g. testosterone (Markianos et al., 2005) and corticosteroids (Heuser et al., 1991; Leblhuber et al., 1995; Bjorkqvist et al., 2006), are affected in HD. It is, however, unclear whether alterations in plasma levels of these peripheral hormones arise from hypothalamic-pituitary dysfunction (Petersen and Bjorkqvist, 2006; Aziz et al., 2007), or from pathology in the organs producing these hormones.

The hypothalamus is affected in Huntington's disease

The first indications for hypothalamic pathology in HD stem from a few papers published during the latter half of the last century (Schöpe, 1940; Vogt and Vogt, 1952; Wahren, 1959, 1964; Bruyn and von Wolferen, 1973). These studies were all qualitative in nature, describing single or a few cases without systematic quantitative morphological analysis (Aziz et al., 2007). Kremer and co-authors extended upon these early findings by demonstrating that up to 90% of the cells in the lateral tuberal nucleus of the hypothalamus are lost in end-stage patients (Kremer et al., 1990; Kremer et al., 1991). More recent studies also showed that the hypothalamus is atrophied (Kassubek et al., 2004) and that orexin-A, but not MCH, neurons are specifically lost (Aziz et al., 2008a). Loss of orexin was also found in the R6/2 mouse model and has been linked to narcoleptic episodes (Petersen et al., 2005). In addition, R6/2 mice have increased daytime activity and decreased nocturnal activity that is accompanied by disrupted expression of the circadian clock genes mPer2 and mBmal1 in the SCN (Morton et al., 2005).

Cells in the hypothalamus in a HD mouse model exhibit a high incidence of huntingtin aggregates and mRNA levels of vasopressin, oxytocin, and CART, are significantly reduced (Kotliarova et al., 2005). Genes that are predominantly expressed in the striatum, such as DARPP-32 and enkephalin, are also significantly reduced in the hypothalamus. Interestingly, the authors did not observe significant cell loss and therefore concluded that aggregate formation might result in down-regulation of specific genes.

In addition to transcriptional down-regulation of genes, HAP1 might also be involved in hypothalamic dysfunction in HD. This huntingtin-interacting protein is highly abundant in the hypothalamus (Li et al., 1996), where it might be involved in intracellular trafficking in certain types of endocrine cells (Liao et al., 2005). Mice lacking HAP1 die postnatal due to depressed feeding behavior (Chan et al., 2002) and display neuronal death resembling hypothalamic degeneration in HD (Li et al., 2003b). In addition, HAP1 is upregulated in response to fasting (Sheng et al., 2006). This all suggests a role for HAP1 in feeding and body weight regulation. Interestingly, HAP1 expression is reduced in brains of HD patients (Li et al., 1998) and the R6/2 mouse model (Li et al., 2003b) and HAP1 might therefore contribute to weight loss in HD.

Hypothalamic cell loss in HD patients has been suggested to result from excitotoxicity. Kremer and co-authors found the lateral tuberal nucleus to be enriched with NMDA-receptors (Kremer et al., 1993). In addition, orexin neurons in the lateral hypothalamus might be particularly susceptible to excitotoxic cell death (Katsuki and Akaike, 2004). These findings might explain the vulnerability of this neuronal population in HD.

Beyond the brain: peripheral pathology in Huntington's disease

As reviewed above, several areas in the HD brain are affected. This might contribute to several non-motor symptoms of the disease. Most of these symptoms are, however, complex and brain dysfunction might not be the only factor contributing to their occurrence. Mutant huntingtin is also expressed outside the brain (Hoogeveen et al., 1993; Trottier et al., 1995), and may affect, as will be reviewed below and in figures 3 and 4, many peripheral organs. Investigating peripheral pathology in HD might contribute to our understanding of non-motor symptoms. Moreover, it could lead to the discovery of new markers of disease progression, the so called 'biomarkers',

that are needed for monitoring disease progression in patients and can be used as output measurement during drug testing.

Cardiovascular system

Cardiac failure is the second leading cause of death in HD patients (Lanska et al., 1988; Sorensen and Fenger, 1992) and is also a common cause of death in the R6/2 mouse model of HD (Mihm et al., 2007). Studies of cardiac tissue from HD patients have never been reported. In R6/2 mice the myocardium is atrophied and mitochondria have abnormal shapes (Mihm et al., 2007). Consequently, systolic and diastolic performances are disrupted and cardiac output is reduced by 50% in these mice.

It could be assumed that altered autonomic function underlies cardiac abnormalities in HD. Interestingly, cardiomyocyte-specific expression of polyglutamine fragments with 83 CAG repeats in mice induces aggregate formation, autophagy, and necrotic death of cardiomyocytes and leads to heart failure (Pattison and Robbins, 2008; Pattison et al., 2008). This suggests that heart failure in HD could directly arise from cardiomyocyte dysfunction.

Digestive tract

The digestive tract plays a major role in ingestion, digestion and absorption of food. Especially the stomach and small intestines are important in digestion. They produce digestive enzymes and communicate energy levels to the hypothalamus and other organs by peptides such as ghrelin and cholecystokinin (for review see Coll et al., 2007).

Malfunctioning of the digestive tract could result in reduced nutrient uptake and might contribute to weight loss. This has, however, never been investigated in HD. Interestingly, despite a normal, or even higher caloric intake (Sanberg et al., 1981; Morales et al., 1989; Trejo et al., 2004; Mochel et al., 2007), nutritional deficiencies have been reported to occur in HD patients (Lanska et al., 1988; Mochel et al., 2007).

It is unclear which organs along the alimentary canal might be affected in HD. There are strong indications that the endocrine pancreas is impaired, as both HD patients and mouse models of the disease tend to develop impaired glucose tolerance or diabetes (Podolsky et al., 1972; Podolsky and Leopold, 1977; Farrer, 1985; Hurlbert et al., 1999; Duan et al., 2003; Bjorkqvist et al., 2005; Josefsen et al., 2008). In HD mice, pancreatic islets are reduced in size and the cells exhibit intranuclear inclusions (Bjorkqvist et al., 2005; Hunt and Morton, 2005), causing them to develop defects in insulin, somatostatin and glucagon production (Andreassen et al., 2002). Mass of the islets is, however, not decreased in HD patients (Bacos et al., 2008), suggesting that impaired glucose tolerance might be due to transcriptional defects, rather than cell loss. The exocrine pancreas has, to my knowledge, never been studied in HD.

In addition to the pancreas, the liver might be affected in HD. A recent study in mice has shown that mutant huntingtin suppresses CAAT-enhancer-binding protein-α (C/EBP-α), a crucial transcription factor for the transcription of urea cycle enzymes, in the liver (Chiang et al., 2007). This leads to high circulating levels of ammonia and subsequent behavioral deficits, such as locomotor dysfunction. Interestingly, a low protein diet reduces blood plasma levels of ammonia, and thereby ammeliorates brain damage and behavioral deficits in these HD mice, suggesting that classical neurological symptoms could even arise from peripheral deficits.

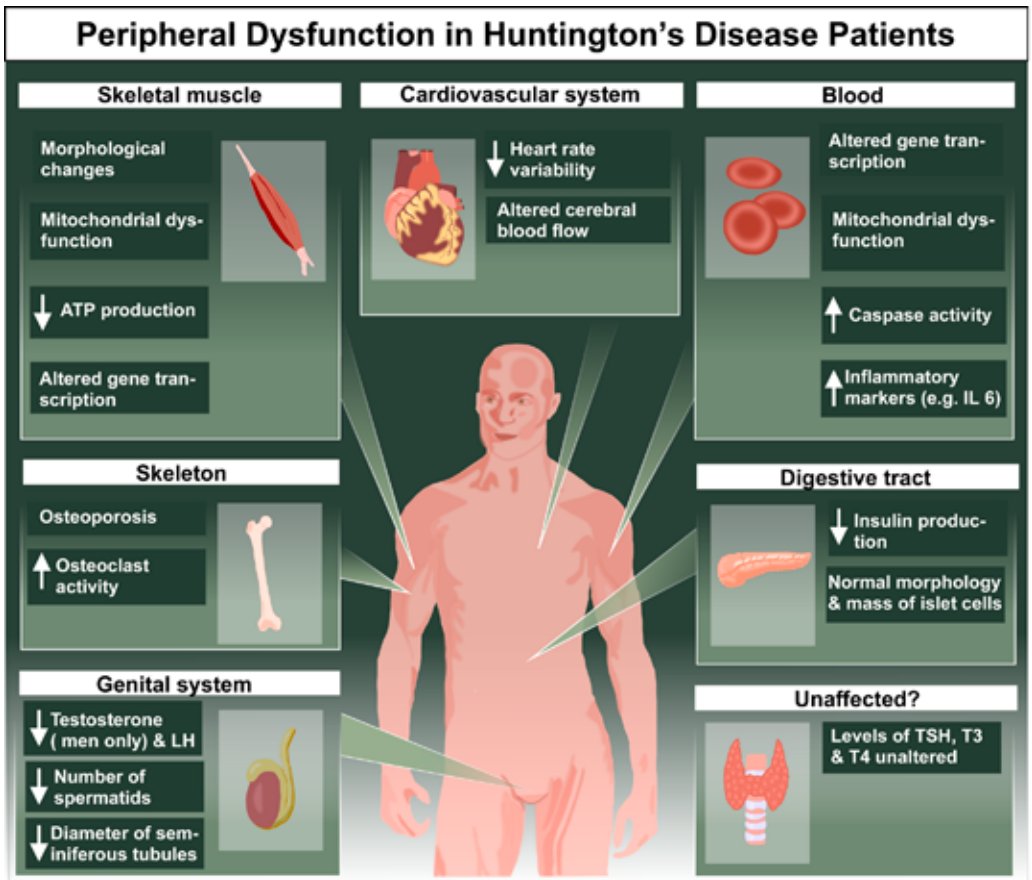

Figure 3. Peripheral dysfunction in HD patients.
Several peripheral organs and tissues have been found to be affected in HD patients

Loss of function of wild-type huntingtin might also play a role in weight loss in HD. Interestingly, levels of normal huntingtin modulate body weight in a dose-dependent way (Van Raamsdonk, 2006) and fasting alters the expression of both huntingtin and huntingtin-interacting proteins, such as HIP1 and NF-kβ, in the intestines of wild-type mice (Milka Sokolovic and Wouter Lamers, personal communication). These findings suggest a direct role of huntingtin in the regulation of nutrient uptake.

Skeletal muscle

Wasting of skeletal muscle commonly occurs in HD patients (Farrer and Meaney, 1985; Farrer and Yu, 1985; Ribchester et al., 2004; Trejo et al., 2004). *In vitro* cultures of skeletal muscle from pre-symptomatic and symptomatic HD subjects show several abnormalities, including mitochondrial depolarization, respiratory chain dysfunction, cytochrome c release, increased caspase activity, defective cell differentiation and apoptosis (Arenas et al., 1998; Ciammola et al.,

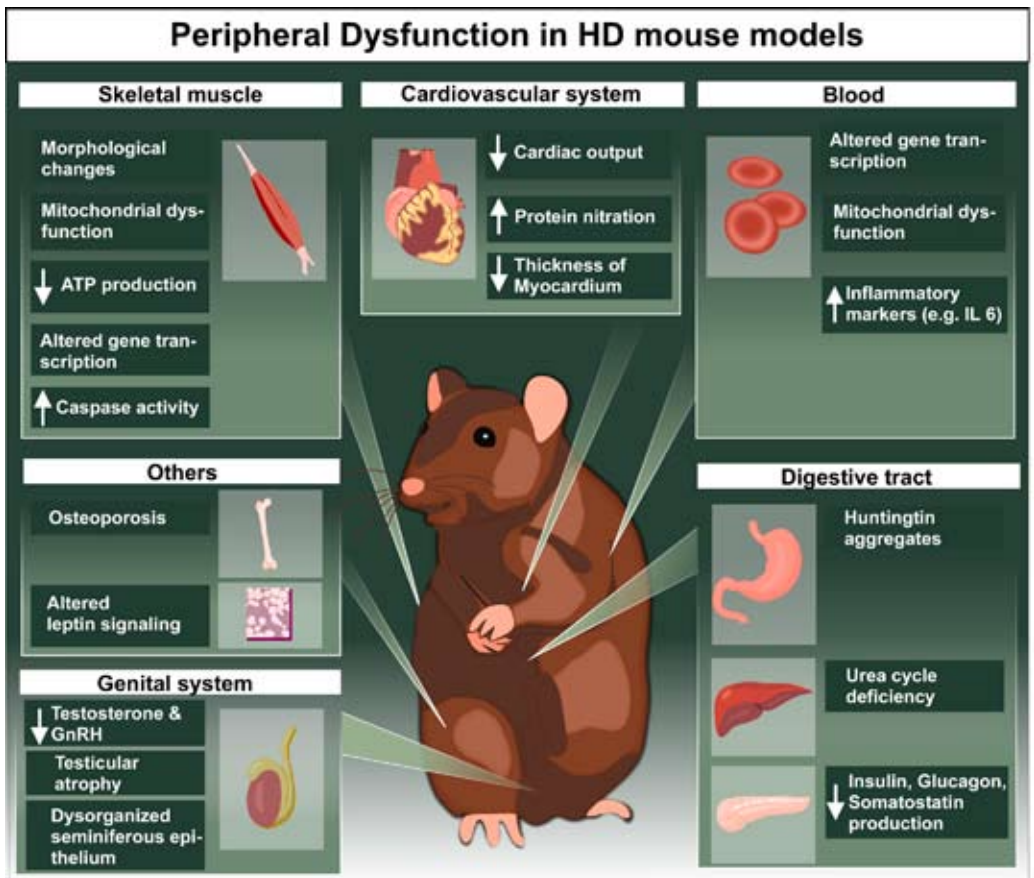

Figure 4. Peripheral dysfunction in HD mouse models.
Several peripheral organs and tissues have been found to be affected in mouse models of HD

2006; Turner et al., 2007). Moreover, the cells exhibit abnormal fiber morphology and enlarged mitochondria with abnormal cristae (Arenas et al., 1998). These findings point towards mitochondrial dysfunction underlying skeletal muscle pathology in HD.

In vivo, studies in HD patients also indicate mitochondrial dysfunction. During recovery from exercise, the maximum rate of mitochondrial adenosine triphosphate (ATP) production is largely reduced in both symptomatic HD patients and pre-symptomatic HD carriers (Lodi et al., 2000; Saft et al., 2005). In addition, progressive myopathy along with mitochondrial pathology has been reported in a marathon runner at risk for HD (Kosinski et al., 2007).

Apart from mitochondrial dysfunction, transcriptional dysregulation also contributes to skeletal muscle pathology. HD muscle cells show increased expression of genes encoding chaperones, heat shock proteins and proteasomal-subunits (Luthi-Carter et al., 2002; Strand et al., 2005). Transcriptional dysregulation in skeletal muscle of HD mice is highly comparable to changes seen in the brain of the same mice, although also muscle-specific mRNAs are altered

(Luthi-Carter et al., 2002). In addition, changes in gene expression in muscle in both HD patients and mice reflect disease progression and are therefore a valuable biomarker of the disease (Strand et al., 2005).

Genital system

The testes are together with the brain the sites with the highest huntingtin expression in the body. Although the number of offspring is not decreased in HD patients (Shokeir, 1975; Mastromauro et al., 1989; Pridmore and Adams, 1991), recent studies show that male patients have decreased numbers of germ cells along with thickened walls and narrower seminiferous tubules in the testes (Van Raamsdonk et al., 2007). Mature spermatids were often absent near the time of death (Van Raamsdonk et al., 2007).

The R6/2 and yeast artificial chromosome (YAC) 128 mouse models of HD also exhibit testicular pathology. Their testes are atrophied and the mice display decreased fertility (Leavitt et al., 2001; Papalexi et al., 2005; Van Raamsdonk et al., 2005; Van Raamsdonk et al., 2007). The seminiferous epithelium in the testis of YAC128 mice is disorganized and germ cell numbers are reduced (Van Raamsdonk et al., 2005; Van Raamsdonk et al., 2007). Interestingly, the onset of testicular atrophy coincides with the onset of neuronal atrophy in the striatum and was more severe (Mangiarini et al., 1996; Davies et al., 1997; Sathasivam et al., 1999), suggesting that testicular degeneration is a major event in the disease.

Male HD patients and mouse models of HD also have reduced plasma levels of testosterone (Markianos et al., 2005). Decreased levels of testosterone are generally associated with decreased libido and fertility, osteoporosis, and wasting of skeletal muscle and these are all signs and symptoms that have been attributed to either HD patients or mouse models of HD (Farrer and Meaney, 1985; Leavitt et al., 2001; Bonelli et al., 2002; Trejo et al., 2004; Papalexi et al., 2005; Otti et al., 2007). Moreover, low levels of testosterone have been associated with neuronal loss (Veiga et al., 2004) and could therefore even contribute to neurodegeneration in HD.

It is unclear what the cause is of reduced plasma testosterone levels in HD. Levels of luteinizing hormone in the patients (Markianos et al., 2005) and GnRH in R6/2 mice (Papalexi et al., 2005) are decreased, which suggests involvement of the HPG axis. However, GnRH and testosterone levels are normal at early time points when testicular degeneration is already present (Papalexi et al., 2005; Van Raamsdonk et al., 2007), suggesting that testicular abnormalities are not secondary to alterations in the HPG axis, but primarily arise from mutant huntingtin expression in the testis.

Blood

Blood cells in HD are affected in numerous ways. Gene transcription (Borovecki et al., 2005; Anderson et al., 2008), caspase activity (Maglione et al., 2006), and mitochondrial function (Parker et al., 1990; Sawa et al., 1999; Panov et al., 2002; Mormone et al., 2006) are affected in hematocytes. In addition, adenosine A2A receptor functioning (Varani et al., 2003; Varani et al., 2007), which might also be involved in striatal pathology, and monoamine oxidase (MAO) activity (Norman et al., 1987), that produces free radicals, have been reported to be altered.

It is unclear if and how dysfunction of blood cells contributes to HD symptoms. Wild-type huntingtin is suggested to be required for normal hematopoiesis (Metzler et al., 2000), and for iron utilization and hemoglobin production (Lumsden et al., 2007). Levels of hemoglobin and iron utilization are decreased in HD patients (Bonilla et al., 1991).

Interestingly, immune activation has also been suggested to play a role in HD. Blood plasma of HD patients shows signs of inflammation, such as increased levels of interleukins 8 and 6 (IL-8 and IL-6) (Dalrymple et al., 2007; Bjorkqvist et al., 2008). The immune system in HD might be activated in reaction to pathological changes, such as aggregate formation or necrotic cell death. However, levels of IL-6 are already increased as early as 16 years before the predicted onset of symptoms (Bjorkqvist et al., 2008). Moreover, monocytes from HD patients and asymptomatic *HD* gene carriers express mutant huntingtin and are, when isolated, pathologically hyperactive in response to stimulation (Bjorkqvist et al., 2008). This all suggests that inflammatory changes in HD result from cell autonomous dysfunction.

Other organs and tissues

In addition to the above described organs and tissues, other peripheral sites might be affected in HD. A study in R6/2 mice demonstrated that the mice suffer from Cushing's syndrome with enlargement of the intermediate pituitary lobe, increased cortisol levels, insuline resistance, wasting of skeletal muscle and decreased bone density. Several of these symptoms are also present in HD patients and, although rarely investigated, even osteoporosis might be part of the HD phenotype (Bonelli et al., 2002; Otti et al., 2007). Connective tissue is also affected, as fibroblast lines from HD patients and R6/2 mice exhibit reduced mitotic indices and a high frequency of aneuploidy (Sathasivam et al., 2001).

Studies in HD mouse models show that white adipocytes respond inadequately to starvation signals (Fain et al., 2001), and that brown adipocytes respond inadequately to cold exposure due to reduced peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and blunted uncoupling protein 1 (UCP1) expression (Weydt et al., 2006). Such alterations in adipose tissue could have major effects on energy metabolism and weight loss in HD.

Apart from all these non-neuronal tissues, studies in mice suggest that mutant huntingtin might also affect peripheral nerve cells, such as those forming the sciatic nerve (Wade et al., 2008), and those found in the retina (Jackson et al., 1998; Helmlinger et al., 2002; Petrasch-Parwez et al., 2004). Moreover, huntingtin has been shown to form aggregates in neurons of the enteric nervous system in the stomach and duodenum of R6/2 mice (Sathasivam et al., 1999), but possible malfunction of these neurons and organs has never been studied in HD.

Widespread pathology in Huntington's disease: still poorly understood

The evidence reviewed above indicates the occurrence of widespread pathology in HD. In spite of this evidence, pathology outside the basal ganglia and its relation to non-motor signs and symptoms in HD are still poorly understood. Several brain areas and other organs, including the lung, thymus, kidney and gastro-intestinal tract, have seldom or never been investigated in HD. Pathology outside the basal ganglia could be related to several of the disease characteristics, such as cognitive deterioration, psychiatric problems, sleep disturbances, wasting of skeletal muscle, heart failure and weight loss, and it is therefore tremendously important to investigate this. Moreover, studying structures outside the basal ganglia could contribute to a better understanding of the disease and its pathological mechanism, the identification of new biomarkers, and it could lead to the finding of new therapeutic targets.

This thesis aims to investigate pathology outside the basal ganglia and its relation to non-motor symptoms of HD. I have focused on weight loss, as this affects the quality of life of HD patients (Nance and Sanders, 1996) and is associated with the rate of disease progression (Myers et al., 1991). In doing so, I have addressed the research aims that are listed below.

Aims

- What is the cause of weight loss in HD?
(Papers I and III, and manuscript IV)

- Are the hypothalamus and gastro-intestinal tract affected in HD?
(Papers I and II, and manuscript IV)

- Are disturbances in water intake and water balance part of the HD phenotype?
(Paper II)

- Are nutrients properly absorbed in HD?
(Manuscript IV)

MATERIALS AND METHODS

Tools to study Huntington's disease

Several genetic models of HD have been generated. They include *in vitro* cellular models and non-mammalian animal models, such as those generated in *Drosophila melanogaster* and *Caenorhabditis elegans* (for review see Marsh et al., 2003; Brignull et al., 2006)). These models have been particularly useful tools to investigate intracellular disease processes. Moreover, they have been valuable for rapidly screening potential beneficial pharmacological compounds.

Although they provided important insights into the molecular mechanisms of HD, other models are needed to investigate the relationship between neuronal dysfunction and the development of abnormal behavior (Bates and Hockly, 2003). The recent announcement of a primate transgenic model of HD might represent a great advance, although technical challenges have to be overcome before further progress on these primate models can be made (Palfi and Jarraya, 2008; Yang et al., 2008).

Mouse models are less complicated to generate and have been proven to be very valuable tools to study HD. During the past 12 years various mouse models of HD have been generated (Menalled and Chesselet, 2002; Hickey and Chesselet, 2003). They primarily differ in the size of the expressed huntingtin fragment, the number of CAG repeats, the background strain, the promotor driving the transgene and consequently the expression of the mutant protein. Each model, therefore, exhibits a unique phenotype and replicates different aspects of the disease.

The R6/2 mouse model

The R6/2 mouse is the most extensively studied animal model of HD (Li et al., 2005). A partial explanation for its relative popularity might be its rapid disease progression. Symptoms often start within a few weeks after birth and generally result in death between 12 and 16 weeks of age. The cause of death is unknown, but cardiac failure (Mihm et al., 2007) and epileptic seizures (Mangiarini et al., 1996) have been suggested to play a role.

R6/2 mice express a part of the human *HD* gene with around 150 CAG repeats. They were created in 1996 by Mangiarini and co-authors by injection of a segment of the N-terminal part of the *IT15* gene, obtained from a single HD patient, into a CBAxC57BL/6 mouse embryo (Mangiarini et al., 1996). This transgene includes the promotor, exon 1 and a part of intron 1 and constitutes about 3% of the entire human *HD* gene. Transgene expression has been detected in many tissues in the R6/2 mouse and levels are approximately 75% of the endogenous huntingtin levels.

R6/2 mice replicate several aspects of HD, including progressive motor dysfunction, cognitive deficits, wasting of skeletal muscle and weight loss. They exhibit neuronal atrophy and dysfunction, but interestingly, no extensive cell loss is observed in their brains until the final stages of their lives, long after the onset of symptoms (Stack et al., 2005). The lack of extensive cell death in the brain of this and other mouse models of HD has led to them being widely criticized and debated. However, the R6/2 mouse remains a model that reproduces many aspects of HD, including neuronal dysfunction and phenotypic changes, and is therefore a useful tool to study certain aspects of HD.

Among the pathological alterations that occur in the brains of R6/2 mice are decreases in cell soma of various neuronal populations (Klapstein et al., 2001; Petersen et al., 2005), reduced brain weight and striatal volume (Davies et al., 1997; Stack et al., 2005), decreases in synaptic proteins (Morton et al., 2001; Freeman and Morton, 2004) and altered gene transcription (Luthi-Carter et al., 2000). Moreover, neuronal nuclear aggregates are formed in many neuronal populations, e.g. in the hippocampus, hypothalamus, cortex and striatum. They can be detected as early as postnatal day 1 (Stack et al., 2005) and are present in more than 80% of all neurons in the brain by 10 weeks of age (Morton et al., 2000; Meade et al., 2002). Also other organs and tissues, such as the liver, kidney, gastro-intestinal tract, pancreas and skeletal muscle, form huntingtin aggregates (Sathasivam et al., 1999). Some of these organs are atrophic in R6/2 mice (Papalexi et al., 2005).

At birth, R6/2 mice are phenotypically indistinguishable from their wild-type littermates, but around 4 weeks of age they start displaying their first motor symptoms. When placed in an open field, R6/2 mice appear hyperactive compared to wild-type mice. Around 6 to 8 weeks of age, R6/2 mice become hypoactive instead (Carter et al., 1999; Luesse et al., 2001; Bolivar et al., 2003, 2004). They also show a decline in motor coordination and by 8 weeks of age they start exhibiting tremor, stereotypical hindlimb grooming, irregular gait and paw clasping when suspended by the tail.

Apart from displaying locomotor dysfunction, R6/2 mice also replicate the progressive weight loss that characterizes HD. From around 9 weeks of age, mice lose body weight. The cause of weight loss in mice and patients is unclear, but it is suggested to be related to increased locomotor activity, metabolic alterations and reduced caloric intake. Neuronal dysfunction in the hypothalamus could result in metabolic and appetite changes and might therefore be related to the cachexia. Hypothalamic damage has been observed in both HD patients and R6/2 mice and might also underlie other signs and symptoms, such as sleep problems and testicular atrophy. R6/2 mice mirror the disrupted sleep-wake cycle that was observed in HD patients and have a marked disruption of circadian clock gene expression in the suprachiasmatic nuclei of the hypothalamus (Morton et al., 2005; Fahrenkrug et al., 2007).

Also fertility problems that occur in R6/2 mice might be related to hypothalamic dysfunction. Male R6/2 mice display reduced fertility and are generally infertile after 8 weeks of age. Females are often completely sterile (Mangiarini et al., 1996) and males are used to breed the colony.

Methods used in this thesis

Below follows a brief description of, and comments on, the main methods used in the studies comprising this thesis. For additional information about the applied methods, see papers I-III and manuscript IV.

Mice

The studies that are included in this thesis involve different colonies of R6/2 mice: a colony at Lund University (papers I, II and III and manuscript IV), a colony at King's College London (manuscript IV), and a colony at Cambridge University (papers I and II). Some of these colonies have developed different CAG repeat lengths. R6/2 mice at King's College London have the

original number of 150 CAG repeats, whereas R6/2 mice at Lund University and Cambridge University have developed higher numbers of approximately 160-180 and 240-280 repeats respectively. R6/2 mice in these colonies show similar behavioral phenotypes, but different rates of progression, with the mice in Cambridge having the longest life span. It is unclear why progression rates differ. It could result from the differences in repeat length, although a higher number of CAG repeats usually leads to a more rapid rate of disease progression (Illarioshkin et al., 1994; Penney et al., 1997; Rosenblatt et al., 2006; Ravina et al., 2008). Differences are more likely to result from genetic variations due to inbreeding, or from differences in animal welfare conditions. Variations in the number of mice per cage, enriched versus impoverished environment, and type of food may all play a role in the survival of the mice. At Cambridge University, chow food is soaked (100 g food in 230 ml water) until the pellets are soft and fully expanded. Feeding mice daily with this type of mashed food has been found to be beneficial for long-term health of the animals (Carter, Hunt and Morton, 2000).

R6/2 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and maintained on a C57BL/6 background by crossbreeding wild-type females with heterozygous males. All mice were housed under standard conditions. Unless stated otherwise, mice were housed with littermates of the same gender and had *ad libitum* access to chow food (which was mashed in case of the mice at Cambridge University) and water. The temperature in the room was 21-23°C, the humidity 55 ± 10% and the light-dark cycle was kept at 12 hours light and 12 hours dark. The use of laboratory animals in all experiments was approved by the ethical committees at Lund University, King's College London and Cambridge University.

Huntington's disease patients

Three articles in this thesis (papers II and III and manuscript IV) include data obtained from HD patients. In paper II, HD patients and their relatives or caretakers were subjected to a xerostomia questionnaire. All HD patients were recruited at the Cambridge Centre for Brain Repair HD clinic and patients that were taking medications that could have drying of the mouth as a side effect were excluded from the analysis. Urine and blood samples for measuring urine osmolality and serum vasopressin (paper II) were collected at the HD clinic at the National Hospital for Neurology and Surgery in London. All patients had a positive genetic diagnosis for HD and were in different stages of the disease. Healthy controls were recruited from relatives and friends of the patients attending the clinic.

The subjects from paper III participated in a randomized placebo-controlled trial to study the effects of riluzole on disease progression. Participants were all in an early stage of the disease (defined on the UHDRS as a motor score ≥ 5 and Total Functional Capacity (TFC)-score ≥ 8) and were free of neuroleptic treatment. Weight and UHDRS scores were measured 9 times over a period of 3 years. The UHDRS questionnaire form is divided into four components assessing cognition, motor performance, behavior and functional capacity (Group, 1996; Siesling et al., 1998; Djousse et al., 2002). Briefly, the motor component consists of 31 questions rated on a scale between 0 to 4, with 4 indicating the most severe impairment. Of the 31 questions, five assess maximal dystonia and seven evaluate maximal chorea. For dystonia, the following scores were assigned: 0 if the subject exhibited no sign of dystonia, 1 for slight/intermittent, 2 for mild/common or moderate/intermittent, 3 for moderate/common, and 4 for marked/prolonged dystonia. A comparable scoring was used for maximal chorea, rigidity and bradykinesia. The rating

of the functional capacity used information on occupation (score range: 0 to 3), finances (score range: 0 to 3), domestic chores (score range: 0 to 2), activities of daily living (score range: 0 to 3), and care level (score range: 0 to 2). Under this rating, a higher score indicates a higher function. The total sum of the scores on the five items is the TFC, which ranges from 0 to 13. The independence scale (Myers et al., 1991) ranges from 10 to 100 units (increments of 5), with 100 units indicating the highest functional level. The cognitive component of the UHDRS scale includes the Stroop Interference, Verbal Fluency, and Symbol Digit Modalities tests.

Manuscript IV involves post-mortem collected gastro-intestinal tissues of 9 HD patients. Control material consisted of biopsies of 5 age and gender matched subjects that had not been diagnosed with HD. Furthermore, manuscript IV involves 9 medication-free, early stage HD patients, and 11 healthy control subjects. These patients kept a three day food diary and were asked about their daily stool frequency and feces consistency. The groups were well matched for age and gender.

Body weight and food intake

Measurements of body weight and food consumption (papers I and III and manuscript IV) were carried out in male R6/2 mice and wild-type littermates from the Lund colony. Mice were singly housed and had *ad libitum* access to pre-weighed portions of food (15% fat on a caloric basis) and water. Experiments were performed from 6 to 12 weeks of age. As R6/2 mice have been reported to develop locomotor problems during disease progression, food was placed in a Petri dish on the bottom of the cage to facilitate feeding.

Caloric intake was measured by weighing pre-weighed portions of food one or several days later. The amount of sawdust present in the cage was minimized, so pieces of food were easily detectable. All food particles were collected by systematic screening of the entire bottom of the cage (including the sawdust) and included in the measurement. Large particles were lifted with a spoon, small particles with a forceps. Feces, sawdust or other materials that occasionally stick to the food, were carefully removed and excluded from the analysis. The weight of the food that the mice had consumed was dived by the number of study days in order to express the values as 'consumed grams per 24 h'. In papers I and III, caloric intake was measured four times per week over 24 h in male R6/2 and wild-type mice from 6 to 12 weeks of age. Body weight was measured twice per week in the same cohort of mice. In manuscript IV, food intake was measured only occasionally, several days prior to measuring whole gut transit time. This was performed in order to determine how much food each mouse had to receive during 24 h of 70% food deprivation, which was part of the whole gut transit time experiment.

In order to monitor energy intake in HD patients (manuscript IV), we asked 9 medication-free, early stage HD patients and 11 healthy control subjects to keep a standardized food diary for three days (two days during the week and one day in the weekend). These records were analyzed by dieticians using computerized programs based on the Dutch 'NEVO tables 2006' containing information about the composition of food consumed in the Netherlands.

Water consumption and xerostomia

Water consumption (paper II) was measured in male R6/2 and wild-type mice from the Lund colony from 6 to 12 weeks of age. Mice were singly housed and had *ad libitum* access to pre-weighed portions of food (15% fat on a caloric basis) and water. Water consumption was mea-

sured by weighing the water bottle and comparing this to the weight of the full bottle that had been put in the cage 3 or 4 days earlier. Long, non-leaking drinking sprouts were used to improve access to the water bottles. The weight of the water that the mice had consumed was divided by its density (0.99) and by the number of days (3 or 4), to express the values as milliliters water consumed per mouse per day.

In addition, drinking behavior (paper II) was measured in female R6/2 and wild-type mice from the Cambridge colony. Mice were singly housed with *ad libitum* access to food and water and their behavior was monitored for 24 h in the LABORAS apparatus (Metris b.v., Hoofdorp, The Netherlands). This apparatus is able to measure total locomotor activity, as well as grooming, eating and drinking by using the vibrations induced by movements of the animal. Each mouse was tested at 7, 10 and 18 weeks of age. Immediately before being put in the LABORAS cages, all mice were tested for glycosuria using Diastix (Bayer plc., Newbury, Berks, UK).

Xerostomia was investigated in HD patients and control subjects by means of a questionnaire. This questionnaire was taken from Eisbruch et al. (Eisbruch et al., 2001) and consisted of the following eight questions:

1. Rate your difficulty in talking due to dryness.
2. Rate your difficulty in chewing due to dryness.
3. Rate your difficulty in swallowing solid food due to dryness.
4. Rate the frequency of your sleeping problems due to dryness.
5. Rate your mouth or throat dryness when eating food.
6. Rate your mouth or throat dryness while not eating.
7. Rate the frequency of sipping liquids to aid swallowing food.
8. Rate the frequency of sipping liquids for oral comfort when not eating

Subjects rated each symptom on an ordinal Likert scale from 0 to 10, with higher scores indicating greater dryness and discomfort due to dryness.

Locomotor activity

Locomotor activity (paper I) of male and female R6/2 and wild-type mice from the Lund colony was measured at 4, 8 and 12 weeks of age, using a photobeam activity cage. This cage consists of an open field with 16 x 16 photobeams in a horizontal plane that are approximately 2.5 cm apart from each other (Flex Field: San Diego Instruments, San Diego, CA, USA). Mice were placed individually in a cage and the number of times they interrupted a photobeam was used to measure their activity. As R6/2 mice display decreased exploratory behavior when placed in a novel environment (Bolivar et al., 2003), we decided to record locomotor activity for a relatively long period (4 h). Recordings were performed during the first four hours of the dark phase and the results were expressed as total number of crossed beams during this period.

Additionally, in order to investigate locomotor analysis in a more detailed fashion and over a longer time period (24 h), we also used the LABORAS apparatus. Female R6/2 and wild-type mice from the Cambridge colony were tested at 7, 10 and 18 weeks of age. The mice were singly housed with *ad libitum* access to water and food. The cage was then placed on the LABORAS apparatus for 24 h and the vibrations produced by different movements of the mouse were measured. Apart from enabling us to study locomotor behavior over 24 h, this apparatus also enabled

us to study the mice while housed under relatively normal circumstances, in a cage similar to their home environment.

Analyses of hypothalamic peptides

Papers I and II include post-mortem analysis of the hypothalamus of 4-, 8- and 12-week-old R6/2 and wild-type mice from the colony at Lund University. Mice were anaesthetized with pentobarbital (200 mg/kg, intra-peritoneal) and perfused with saline, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS). Brains were post-fixed overnight in 4% PFA, and dehydrated in 20% sucrose/0.1 M PBS. They were cut in 30 µm thick sections using a freezing microtome.

Free-floating sections were processed for immunohistochemistry (IHC), using primary antibodies against MCH (diluted 1:500; Phoenix Pharmaceuticals Inc., Belmont, CA, USA), CART (diluted 1:1600; generated by Prof. M. Kuhar, Emory University, Atlanta, GA, USA), and POMC (diluted 1:400; Phoenix Pharmaceutical Inc. Belmont, CA, USA)(Paper I). In paper II sections were stained for vasopressin (1:5000; Chemicon International Inc., CA, USA). Tissues from R6/2 and wild-type mice were processed in parallel at all times. Staining procedures were started with rinsing the free-floating sections three times for 10 min in 0.1 M PBS. All sections that would be stained with a biotinylated secondary antibody during the following day, were placed in 3% hydrogen peroxide in 0.1 M PBS for 15 min to quench endogenous peroxidase, followed by three times rinsing for 10 min in PBS. In order to reduce background staining, all sections were incubated for 1 h in 5% normal serum and 0.3% Triton X-100 in PBS. The type of serum depended on the secondary antibody that would be used the following day. For instance, if the secondary antibody would be raised in goat, normal goat serum would be used. One series of sections was then overnight incubated at room temperature with either one of the primary antibodies in PBS.

The following day, sections were rinsed three times for 10 min in PBS. Sections were then incubated for 1 h at room temperature with the corresponding secondary antibodies in PBS. Both biotinylated (Dakopatts, Copenhagen, Denmark) and fluorescent (Alexa 488; Invitrogen, CA, USA) secondary antibodies were diluted 1:200 in PBS. Incubation was followed by rinsing three times in PBS. Bound biotinylated antibodies were then visualized using an avidin-biotin complex (ABC) system (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) using 3,3-diaminobenzidine (DAB) as the chromogen. All sections were mounted on glass slides and covered with either DPX-mouting solution (used for the DAB-stained sections) or PVA-dabco solution (used for the fluorescent sections), and subsequently overlaid with cover slips.

In addition to the IHC stainings, one series of brain sections was stained with cresyl violet. These sections were analyzed with the optical dissector method of stereology to assess the total number of cells in the hypothalamus. All cells in a random sample of 2% of total volume were counted using an Olympus CAST-grid system (Olympus Danmark, A/S, Albertslund, Denmark). In IHC stained sections all stained profiles were counted in all hypothalamic sections, as the total number of these cells was low. Also cell soma diameters were analyzed from 20 randomly selected immunopositive cells per animal. The total numbers of cells was then calculated using the formula by Abercrombie and Johnson's: $N = C \times T / (T+ D) \times F$, in which N = total cell number, C = number of counted cells in the sample, T = section thickness, D = average cell diameter, F = number of section series (Abercrombie and Johnson, 1946).

In addition to IHC, levels of the peptides CART, MCH, CRH, CHRF and POMC were measured in the hypothalami of 12-week-old R6/2 and wild-type mice from the Lund colony using commercially available radioimmunoassay (RIA) kits (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) (paper I).

Analysis of metabolism

In paper I, several factors related to metabolic rate were measured in R6/2 and wild-type mice from the Lund colony. Oxygen consumption was measured in male mice at 6, 8, 10 and 12 weeks of age using an Oxymax system (Columbus Instruments, OH, USA). Measurements were performed for 1 hour during the light phase at 28°C. Body temperature was measured rectally with a CTD-85 thermometer (Ellab, Roedovre, Denmark) once per week, from 6 to 12 weeks of age, in a separate group of male R6/2 and wild-type mice from the Lund colony. mRNA levels of uncoupling proteins (UCP) 1 and 2 were measured in brown adipose tissue from 12-week-old R6/2 and wild-type mice by means of quantitative polymerase chain reaction (Q-PCR) and Northern blot analysis.

Analysis of the gastro-intestinal tract

Morphological analysis

In paper IV we studied the enteric nervous system (ENS) in stomachs of 12-week-old R6/2 and wild-type mice from the colony at King's College London. Mice were sacrificed by cervical dislocation and their stomachs were isolated, sagitally opened with a knife and rinsed in saline to remove food. Tissues were fixed in 4% PFA, dehydrated in 20% sucrose/0.1 M PBS and embedded in O.C.T.™-compound (Sakura Finetek Europe, Zoeterwoude, the Netherlands) and frozen. The fundus of the stomach was cut along the short axis in 10 µm thick sections using a cryostat. In order to investigate which neurons of the ENS might be affected, we stained mounted stomach sections for several markers of both neurons that inhibit and neurons that stimulate gut motility: vasoactive intestinal peptide (anti-VIP; 1:1200 dilution; Eurodiagnostica, Malmö, Sweden), tyrosine hydroxylase (TH; 1:200 dilution; Pel, Freeze Biologicals, Rogers, Arkansas, USA), calcitonin gene-related peptide (CGRP; 1:1200 dilution; Eurodiagnostica, Malmö, Sweden), protein gene product (PGP; 1:1600 dilution; UltraClone, Isle of Wight, UK), vesicular acetylcholine transporter (VACht; 1:4000 dilution; Chemicon, Temecula, CA, USA), CART (1:800 dilution; Santa Cruz Biotech Inc., Santa Cruz, CA, USA), and somatostatin (1:800 dilution; Immunostar Inc., Hudson, Wisconsin, USA). Antibodies were diluted in PBS containing 0.25% Triton X-100 and 0.25% bovine serum albumin. Stainings were performed overnight (at 4 °C) in a moisturized chamber. The following day, sections were incubated with secondary antibodies with specificity for either rabbit, pig or goat primary antibodies that were coupled to fluorescein isothiocyanate (FITC; 1:100, Dako, West Grove, PA, USA) for 1 h at room temperature. Staining intensity was measured in the muscular layer or mucosa of the stomach wall by capturing images on a digital camera (Nikon DS 2-MV, Tokyo, Japan) using a fluorescence microscope (Olympus, Tokyo, Japan) and analyzing these with BioPix iQ2.0 software. Counting of VIP stained varicosities and cells was performed in randomly selected areas with NIS-Elements AR 2.30 software.

To investigate mucosal thickness and villus length, we stained sections of the stomach, duodenum, and colon of 12-week-old R6/2 and wild-type mice with hematoxylin/eosin (H/E). Animals were killed by cervical dislocation and organs were removed and fixed in 4% PFA and dehydrated in 20% sucrose in PBS. Stomach, duodenum and colon were sectioned along the short axis in 20 µm thick sections using a cryostat. Duodenal samples from HD patients and control subjects were also stained with H/E. Mounted sections were rinsed 3 times for 10 min in PBS and placed in deionized water for 5 min. Sections were then placed in hematoxylen (Mayers) for 5 min and briefly rinsed in deionized water and, for 20 min, in tap water. Sections were then transferred to eosin for 1 min, dehydrated in alcohol baths and xylene, and covered with DPX mounting solution and coverslips. Thickness of the mucosa in the stomach and colon, as well as villus length in the duodenum were measured using an Olympus CAST-grid system (Olympus Danmark, A/S, Albertslund, Denmark). Fourty (mice) or 30 (human) villi, or 40 mucosa parts were measured in a blinded fashion.

In addition, sections from different parts of the gut of R6/2 mice and HD patients were stained for EM48 (anti-huntingtin, 1:500 dilution; Chemicon, Temecula, CA, USA) and PGP to investigate if huntingtin aggregates occur in the gastro-intestinal tract. For the human sections, antigen retrieval was performed before the start of the staining by placing the sections in a 0.01 M citrate buffer and boiling them in a microwave (650 W) for 14 min. Further staining was performed as described above.

Gastro-intestinal function

To study gut motility, we measured whole gut transit time in 8- and 12-week-old male R6/2 and wild-type mice from the Lund colony according to a protocol that has been described by Nagakura et al. (Nagakura et al., 1996). Mice were food deprived (70% of normal food consumption) 24 h prior to experimentation. Mice received a single oral dose of 0.3 ml of a marker solution (3 g carmine in 50 ml of 1.5% methylcellulose) and were singly housed in a cage with a white sheet on the bottom. Time taken for excretion of the marker was measured.

In order to investigate whether malabsorption of nutrients occurs, male R6/2 and wild-type mice were singly housed with *ad libitum* access to water and food. Fecal output was determined at 8, 10 and 12 weeks of age from the dry mass of fecal pellets collected over 72 h. During the same period, food intake was measured as described above. Percentage food excreted as feces was calculated by dividing the fecal excretion (in grams) by food intake (in grams) and multiplying by 100. Caloric content of fecal samples was measured in a high-pressure oxygen calorimeter (performed by AnalyCen, Lidköping, Sweden). Stool water content was determined in spontaneously defecated stools in 8- and 12-week-old male R6/2 and wild-type mice from the colony at Lund University. In addition, we asked 9 medication-free HD patients and 11 healthy control subjects about their daily stool frequency and fecal consistence.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (papers I and II, and manuscript IV; GraphPad software Inc. San Diego, CA, USA), or SPSS version 14.0 and SAS version 9.1 (paper III; SPSS Inc, Chicago, Ill, USA, and SAS Institute Inc., Cary, NC, USA, respectively). In general, all statistical tests were two-tailed and values of $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Weight loss is not caused by decreased caloric intake or increased motor activity

Papers I, II, and III and manuscript IV

Body weight decreased significantly during a three year follow-up study in a large group of HD patients (paper III). All patients were at an early stage of the disease and free of neuroleptic treatment. Our findings are in line with previous studies indicating that weight loss occurs in HD (Sanberg et al., 1981; Morales et al., 1989; Nance and Sanders, 1996; Djousse et al., 2002; Trejo et al., 2004; Trejo et al., 2005; Mochel et al., 2007) and that it is an early disease phenomenon (Mochel et al., 2007).

Weight loss in early-stage HD patients did not correlate with indices of hyperactivity, such as UHDRS scores on total motor performance, chorea or dystonia (paper III). Weight loss is therefore unlikely to result from increased motor activity. Previous studies also found that total daily energy expenditure is unaltered in HD patients (Pratley et al., 2000) and that chorea scores do not correlate with weight loss (Djousse et al., 2002). Unaltered total daily energy expenditure might be explained by the fact that HD patients, despite having higher sedentary energy expenditure due to unwanted movements, engage less in voluntary activity (Pratley et al., 2000).

Caloric intake was unaltered in medication-free HD patients at early stages of the disease (manuscript IV). Other studies also found that caloric intake in HD patients is similar or higher compared to control subjects (Trejo et al., 2004; Mochel et al., 2007). Moreover, cognitive and behavioral scores, that could affect eating behavior and the motivation to eat, were not correlated with weight loss (paper III). This all suggests that weight loss in HD is also not due to decreased energy intake.

We confirmed all these findings in the R6/2 mouse model of HD (paper I). R6/2 mice lost weight from 9 weeks of age and onwards, but caloric intake only decreased from 11 weeks of age. Total locomotor activity, which we measured in two different R6/2 colonies with two different methods before and after onset of weight loss, was unaltered in R6/2 mice compared to wild-type littermates. This indicates that, similar to HD patients, R6/2 mice do not lose weight due to hyperactivity.

Although total motor activity was unaltered, R6/2 mice spent more time eating and drinking (paper I and II). This might be due to increased appetite or thirst, but could also indicate difficulties in eating and drinking due to impaired motor function. Previous studies have reported motor function to be impaired in late stage R6/2 mice. They display an irregular gait, resting tremor, reduced grip strength, stereotypical grooming, have impaired coordination and balance capacities, and are hypoactive (Carter et al., 1999; Hickey et al., 2002; Hickey et al., 2005; Stack et al., 2005; Johnson et al., 2006). Findings of hypoactivity conflict with our findings of unaltered total locomotor activity (paper I), but this likely reflects differences in experimental set-up. Previous studies have investigated spontaneous locomotor activity in R6/2 mice for relatively short periods (<30 min) in an activity cage (Hickey et al., 2002; Hickey et al., 2005), which is a novel environment to the mice. Bolivar et al. demonstrated that R6/2 mice exhibit decreased explor-

atory behavior compared to wild-type mice when placed in a novel environment (Bolivar et al., 2003). Previous findings of hypokinesia might therefore reflect decreased exploratory behavior, rather than decreased activity. Our studies were performed for 4 or 24 h periods and changes in exploratory behavior are expected to play a smaller role during such relatively long periods. Moreover, as was demonstrated in paper II, it is important to study behavior during both day and night. Compared to wild-type mice, R6/2 mice were slightly hypokinetic during the light period, but exhibited similar activity during the dark period. Importantly, overall total motor activity during day and night combined was not significantly altered in R6/2 mice.

Increased metabolism

Paper I

As neither hyperactivity nor hypophagia can explain weight loss in HD, we hypothesized that weight loss might be caused by increased metabolism. This hypothesis is in line with previous findings of a negative energy balance in HD (Underwood et al., 2006; Goodman et al., 2008). We found increased overall oxygen consumption in R6/2 mice compared to wild-type littermates (paper I), which was recently confirmed by Goodman and co-authors (Goodman et al., 2008). Increased oxygen consumption, which is indicative of increased metabolic rate, was already present at the earliest time point tested, i.e. 3 weeks prior to the start of weight loss (paper I). At this time point, R6/2 mice probably compensate for the increased metabolism by consuming more calories, effectively preventing weight loss. However, when caloric intake drops to similar levels as those in wild-type mice, R6/2 mice might no longer be able to compensate for the increased metabolic rate and start losing weight.

The cause of increased oxygen consumption in R6/2 mice is unclear. Increased levels of thyroid hormones cause increased metabolism and oxygen consumption. Levels of these hormones have not been investigated in R6/2 mice, but several studies in HD patients have indicated that thyroid hormone levels are unaltered (Lavin et al., 1981; Goodman et al., 2008).

Hypothalamic peptides, such as agouti-related protein (AGRP), CART, orexin, and CRF, as well as pro-inflammatory cytokines, including IL-6, IL-1β and TNF-α, are also known to affect whole body oxygen consumption (Drescher et al., 1994; Asakawa et al., 2001; Asakawa et al., 2002). Interestingly, several of these peptides, such as IL-6 (Bjorkqvist et al., 2008) and orexin (Petersen et al., 2005; Aziz et al., 2008b), are altered in HD. The mechanisms by which these factors could affect oxygen consumption are, however, complex and in most cases not well understood. It is therefore difficult to speculate upon their role in increased oxygen consumption in HD.

Increased oxygen consumption could also arise from mitochondrial defects. Several studies have shown that mitochondria are affected in HD (Browne and Beal, 2004, 2006). Complexes I-IV have decreased activity, which leads to decreased ATP production and altered membrane potential. In contrast to our findings (paper I), such changes would lead to decreased rather than increased oxygen consumption (Milakovic and Johnson, 2005; Browne and Beal, 2006). Here we found increased mRNA levels of uncoupling protein (UCP) 2 in brown adipose tissue. As UCP2 is expressed in many tissues, its increase might well explain the increased whole body oxygen consumption. Mitochondrial UCPs are mainly known for their ability to ‘uncouple’ oxi-

dative phosphorylation from ATP synthesis, releasing the electrochemical energy as heat. The exact function of UCP2 is unknown, but it has been shown that high levels of UCP2 protect neurons against excitotoxicity (Mattiasson et al., 2003). Increased UCP2 levels in HD could therefore reflect an adaptive rather than a pathological mechanism. Furthermore, UCP2 is expressed in many tissues and is therefore an interesting candidate with regard to increased whole body oxygen consumption.

Increased metabolism and UCP activity might cause an increase in body temperature. However, in our study we did not detect a significant increase in body temperature. Instead we observed a trend towards a lower body temperature (paper I). Others have reported hypothermia instead of hyperthermia in another transgenic mouse model of HD (Weydt et al., 2006).

Rate of weight loss increases with higher CAG repeat number

Paper III

Interestingly, weight loss in both HD patients and R6/2 mice increased with higher CAG repeat number (paper III). This suggests that mutant huntingtin affects metabolic rate in a CAG repeat length-dependant manner. The mechanism underlying this is unclear. Mutant huntingtin could affect the hypothalamus, or other regulators of metabolism, in a CAG repeat dependant-fashion. It is also possible that mutant huntingtin directly interferes with energy homeostasis at a cellular level. We did not investigate if CAG repeat number correlates with indices of increased metabolic rate, such as oxygen consumption, but it would be interesting to address this question in future studies.

Our findings indicate that weight loss is an inherent feature of HD. As weight loss directly correlates with the number of CAG repeats, it may reflect the pathological mechanism underlying HD and serve as a biomarker of disease progression. It also indicates that patients with higher CAG repeat numbers are at increased risk of unintended weight loss and their body weight should therefore be monitored more closely.

The hypothalamus is affected

Papers I and II

The hypothalamus is an important regulator of energy metabolism and at least two of its nuclei are affected in HD patients (Kremer et al., 1990; Kremer et al., 1991; Petersen et al., 2005; Aziz et al., 2008a). We examined levels of different hypothalamic peptides in R6/2 mice using immunohistochemistry and/or radioimmunoassay (RIA) (paper I). Several hypothalamic peptides were altered (paper I). In the lateral hypothalamus we observed a progressive reduction in the number of MCH-producing neurons. Loss of MCH usually leads to increased metabolism and weight loss (Shimada et al., 1998) and could therefore contribute to weight loss in HD. However, the number of neurons immunopositive for CART, an anorectic peptide expressed in the medial and lateral hypothalamus, also progressively decreased in R6/2 mice. In the arcuate nucleus, the anorectic peptide POMC declined with disease progression. Levels of the orexigenic peptide ghrelin were reduced in the stomach of R6/2 mice. The losses of MCH, CART, POMC, and

ghrelin were confirmed by RIA. This demonstrates that hypothalamic signaling is disturbed, but it is unclear how this might affect body weight as both anorectic and orexigenic peptides were decreased.

After finding reductions of MCH, CART, and POMC in the hypothalamus of R6/2 mice, we were interested if other hypothalamic peptides are also lost. Vasopressin is of particular interest, as it its loss would result in dehydration, which could contribute to weight loss. Indeed we found a progressive loss of vasopressin in the PVN of the R6/2 hypothalamus (paper II). It is, however, unclear whether this affects water balance and body weight in the mice.

It is unknown why levels of certain hypothalamic peptides are reduced in R6/2 mice. Loss of these peptides is specific, as not all peptides that we investigated were reduced in the R6/2 hypothalamus (paper I). For example, the level of GHRF was normal. Decreased levels of hypothalamic peptides could be explained by degeneration of the neurons producing these peptides, or by transcriptional down-regulation. We performed stereological counting to investigate the total number of cells in the hypothalamus of R6/2 and wild-type mice (paper I). As we could not detect significant cell loss (paper I), we concluded that neuronal death is unlikely to underlie the reduction in cells expressing the peptides. However, the neuronal populations that were investigated in our studies, such as MCH, POMC, CART and vasopressin, are small and stereological analysis of the entire hypothalamus might not detect loss of such small populations. Transcriptional down-regulation, which has already been shown to occur in the hypothalamus of another HD mouse model (Kotliarova et al., 2005), might be a more likely cause of the loss of these peptides.

Some of the anorectic and orexigenic peptides that have been investigated in R6/2 mice, have also been studied in HD patients. The number of orexin neurons was previously found to be significantly reduced in the lateral hypothalamus of both HD patients and R6/2 mice (Petersen et al., 2005; Aziz et al., 2008a). The peptides that were investigated in this thesis have, with the exception of MCH, never been investigated in brains of HD patients. The number of MCH-positive neurons was, in contrast to our data, not significantly reduced in brains of HD patients (Aziz et al., 2008a). It has to be noted, though, that there was a trend towards a loss of these cells in patients. The R6/2 mouse model might represent a more juvenile form of HD and it is therefore possible that analysis of brains of juvenile HD patients will reveal a significant loss of MCH.

Levels of CART and ghrelin were found to be increased in respectively CSF and serum of HD patients (Popovic et al., 2004; Bjorkqvist et al., 2007), but this does not necessarily contradict our findings in R6/2 mice. Both CART and ghrelin are expressed in other areas than respectively the lateral hypotalamus and stomach, and loss of these peptides in one area could therefore be compensated for by overproduction in other regions, resulting in net increases of these peptides in CSF or serum. Moreover, it is possible that, in spite of dysfunction of some of the neurons producing these peptides, the remaining neurons might still be capable of producing enough of these peptides to increase their levels in CSF and serum.

Thirst: a novel biomarker?

Papers I and II

After finding reductions in MCH, CART, and POMC in the R6/2 hypothalamus (paper I), we were interested if other hypothalamic peptides might also be affected in HD. We were primarily interested in vasopressin, because loss of vasopressin might cause dehydration and therefore contribute to weight loss.

We found that R6/2 mice spent more time drinking and also consume more water than wild-type littermates (paper II). The alterations in drinking behavior were not due to increased total locomotor activity, as total activity levels were comparable between R6/2 and wild-type mice (paper I and II). Increased drinking was neither due to diabetes, as increased thirst already occurred before the onset of altered blood glucose (Bjorkqvist et al., 2005). Only in end-stage mice, when diabetes was present and urine osmolality increased (paper II), pancreatic dysfunction might have contributed to increased water consumption in R6/2 mice.

Having found altered drinking behavior in R6/2 mice we were interested whether HD patients suffer from a similar problem. By using a xerostomia questionnaire, we found that HD patients reported significantly more often problems related to thirst, such as difficulties in talking, chewing, and swallowing due to a dry mouth (paper II). This is in line with previous studies reporting a high incidence of choking and coughing in HD patients (Leopold and Kagel, 1985; Kagel and Leopold, 1992; Nance and Sanders, 1996). Although other symptoms of HD, such as neuromuscular dysfunction, may contribute to these problems, it is important to note that HD patients themselves feel that xerostomia is a contributing factor (paper II). Interestingly, xerostomia scores correlated positively with UHDRS scores, suggesting that xerostomia increases with disease progression and might be a biomarker of the disease.

It is unclear what the cause is of increased thirst and drinking in HD. Vasopressin stimulates thirst in response to dehydration. Serum levels of vasopressin were increased in HD patients compared to control subjects, which is in line with increased thirst. However, R6/2 mice had progressive loss of vasopressin in the PVN of the hypothalamus. It has to be emphasized though, that vasopressin was not investigated in the same tissues in both species. It is possible that vasopressin-producing neurons degenerate in brains of both HD patients and R6/2 mice, but that the unaffected neurons are still capable of producing sufficient vasopressin to increase serum levels of this peptide in response to dehydration. It would therefore be very interesting to investigate vasopressin-producing neurons in the brains of HD patients.

In addition to increased serum vasopressin, thirst could also be caused by reduced saliva production. Cholinergic neurons are important for saliva production. These neurons are affected in the HD brain (Smith et al., 2006), which might suggest that they are also affected in the periphery.

The digestive tract is affected

Paper I and manuscript IV

Various studies, including our own (paper I), suggest that weight loss in HD is not caused by decreased caloric intake (Morales et al., 1989; Trejo et al., 2004; Mochel et al., 2007). However, the absorption of nutrients across the gastro-intestinal wall has never been studied in HD, while reduced nutrient uptake might explain weight loss.

Interestingly, we found the gastro-intestinal tract to be affected in both HD patients and R6/2 mice (papers I and manuscript IV). Neurons in the ENS of both species exhibited huntingtin aggregates, which is in line with a previous study in R6/2 mice (Sathasivam et al., 1999). Although not yet addressed in a systematic way, we observed that most of these aggregates were present in the mucosa, submucosa and muscle layers of the gastro-intestinal tract and that they co-localize with neuronal markers (manuscript IV). Huntingtin aggregates were rarely present in the goblet cells lining the gastro-intestinal wall.

To further explore which neurons of the ENS are affected in HD, we performed immunohistochemical stainings for both neuropeptides that inhibit and neuropeptides that stimulate gut motility in the stomach of R6/2 mice. We found both markers of excitatory neurons, including TH, VACht, and CGRP, and markers of inhibitory neurons, such as VIP, CART, and SOM, to be decreased in R6/2 mice compared to wild-type littermates (manuscript IV). In addition, the number of PGP-positive cells, which is a general neuronal marker present in both excitatory and inhibitory neurons, was reduced, suggesting that the decrease in neuropeptides in the stomach might be due to cell loss, rather than down-regulation of specific peptides. This is in line with findings of reduced mucosal thickness in the wall of the stomach and colon in these mice. We also found the length of the villi in the duodenum of both HD patients and R6/2 mice to be decreased.

As both neuropeptides stimulating gut motility and peptides inhibiting gut motility were lost, we hypothesized that the net effect of such a general impairment of the ENS would be a reduction in bowel movements. This hypothesis was in line with what we observed. Whole gut transit time was increased in R6/2 compared to wild-type mice (manuscript IV), which suggests a reduction in gut motility.

Altered gastro-intestinal motility and decreased villus length could affect the absorption of nutrients and we therefore investigated nutrient uptake in R6/2 mice. We found that end-stage mice excreted a higher percentage of the food that they had consumed (manuscript IV), indicating that they suffer from malabsorption of nutrients. As caloric values of R6/2 and wild-type feces did not differ, malabsorption was suggested to be a general, rather than a specific impairment in nutrient uptake.

Moreover, we also found indications that gastro-intestinal function is affected in HD patients. Medication-free patients that were at an early stage of the disease reported having a significant higher number of stools than control subjects (manuscript IV). They also reported often having watery stools, which was in line with findings of increased fecal water content in R6/2 mice (manuscript IV).

Uptake of nutrients in R6/2 mice was only impaired in the end-stage, after the onset of weight loss, which suggests that malabsorption of nutrients is not the cause of weight loss in HD. Malabsorption could, however be an important contributor to the acceleration of weight loss in the end stage. It would therefore be of great interest to investigate nutrient absorption in HD patients.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The clinical phenotype of HD is more complex and variable than a mere progressive movement disorder dominated by neostriatal pathology. Mutant huntingtin is ubiquitously expressed throughout the body and affects other brain regions and organs.

Not only the basal ganglia are affected in Huntington's disease

This thesis demonstrates that the hypothalamus is a major site for cellular dysfunction in the R6/2 mouse model of HD. Peptides such as CART, POMC, MCH and vasopressin are decreased in the hypothalamus. In addition, peripheral organs, such as the gastro-intestinal tract and adipose tissue, are affected. Enteric neurons, including those producing TH, VACht, CGRP, VIP, CART and SOM, are lost in the stomach of R6/2 mice, resulting in decreased gastro-intestinal motility. Moreover, length of intestinal villi is decreased in the duodenum of both HD patients and R6/2 mice, which could contribute to malabsorption of nutrients across the gastro-intestinal wall.

Weight loss in Huntington's disease: a multi-factorial problem

This thesis shows that weight loss commonly occurs in medication-free HD patients in an early stage of the disease. The findings suggest that weight loss in both HD patients and R6/2 mice is not caused by hyperactivity or hypophagia. Increased metabolism, possibly due to mitochondrial dysfunction, is likely to be the cause of weight loss in HD. Interestingly, weight loss in both HD patients and R6/2 mice increased with higher CAG repeat number, which suggests that mutant huntingtin affects metabolic rate in a CAG repeat length-dependant manner. Furthermore, our studies in the R6/2 mouse indicate that malabsorption of nutrients by the gastro-intestinal tract might contribute to weight loss in the end stage.

Taken together, these findings indicate that weight loss in HD is a multi-factorial problem. Loss of weight is likely caused by altered metabolism, but malabsorption of nutrients across the gastro-intestinal wall and decreased caloric intake might contribute to it in later stages of the disease. Further studies in HD patients are, however, required to verify these findings in R6/2 mice.

Weight loss and thirst: novel biomarkers in Huntington's disease

The studies included in this thesis indicate that both weight loss and thirst might be valuable biomarkers of disease progression in HD patients that possibly could be used as a read-out in drug testing.

Future perspectives

Cellular pathology in HD is not limited to the basal ganglia. Other brain regions and other organs are also affected. It is, however, unclear if these changes stand on their own, or if they are

secondary to pathology elsewhere. Several peripheral alterations could indeed be due to cell-autonomous dysfunction in peripheral organs, but they could also result from hypothalamic dysfunction.

Animal models that selectively express mutant huntingtin in certain areas in the body could be helpful to address these issues. A mouse model with selective expression of mutant huntingtin only in the hypothalamus could e.g. contribute to unraveling the role of the hypothalamus in several non-motor symptoms of HD, including weight loss. A balanced hormone replacement therapy might for instance be beneficial in HD, but as long as the role of the hypothalamus in HD symptomatology is unclear, this remains highly speculative.

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to everyone who has assisted me during the past four years. In particular, I wish to convey my gratitude to the following people:

First and foremost I would like to thank my supervisor, Prof. Patrik Brundin. You gave me the opportunity to undertake this journey and you were always there to support me. Even on paternity leave or on holidays, you were a constant help with the preparations for my half time defense and my thesis. Your radiating enthusiasm and passion for science have been a great inspiration and I deeply admire your great knowledge of neuroscience. I cannot express too strongly how grateful I am for everything you have done for me during the past four years. I could not have had a better supervisor!

Secondly, I would like to thank my co-supervisor, Dr. Jia-Yi Li, who has been abundantly helpful, and who assisted me in numerous ways. I always enjoyed discussing science with you. I am grateful to Dr. Brigitte Pettmann, my other co-supervisor, for being, although far away, very supportive for my thesis work. It was always a great pleasure to work with you and I would like to thank you and Regine for all you have done for the NSR network!

The endless support and enthusiasm of Dr. Maria Björkqvist has been indispensable! Thank you for all your help with different projects and with this thesis, and for teaching me more about endocrinology. You were an inspiring mentor and I always enjoyed working with you! I will miss you a lot after leaving Lund!

I am also very grateful to Dr. Åsa Petersén. Thank you for the interesting discussions we had about HD, for introducing me to many HD researchers, for all your help with writing 'paper I', the discussion clubs you organize, and for many other things. It was a great pleasure to work with you!

I would like to thank Dr. Hindrik Mulder. You were always willing to help and discuss the weight loss project, although you were extremely busy. It was also fun to talk (a few) Dutch words with you every now and then!

A big 'thank you' to Britt Lindberg and Birgit Haraldsson for their very skilled technical assistance and many other things. Det var ni som lärde mig svenska och därför vill jag gärna tacka er på svenska. Ni har båda varit som en andra mor för mig, som en familj. Det är inte alltid så lätt att vara i ett annat land, långt borta från sina föräldrar och vänner. Ni har alltid varit där för mig: tack så jätte, jättemycket!

I also would like to thank Elsy Ling and Doris Persson. Your technical assistance was invaluable and it has always been a great pleasure to work with you!

I am very grateful to Bengt Mattsson for his help with the lay-out of this thesis.

Many thanks to our administrators and grant managers: Birgitta Larsson, Emma Idman, Dr. Martha Escobar and Dr. Jens Forsberg for all the paperwork you have been helping me with during the past years. Many thanks also to Dr. Emma Lane, Dr. Vanessa Hall, Dr. Phil Gaughwin, and Lt. Dave Marsh for helping me with various 'grammar issues', including those regarding my thesis.

Due to a viral outbreak in our animal house and failed breeding I was unable to perform experiments in R6/2 mice for more than one and a half years. This has been a major burden for my

thesis work. Thanks to Prof. Gillian Bates and Dr. Ben Woodman in London, I still managed to get the tissues necessary for completion of my projects. Dr. Nico Dantuma, Dr. Christa Maynard and Dr. Olga Rudenko have been extremely helpful with reestablishing our R6/2 colony. Thank you so much for your help!

I am also very grateful to all collaborators and co-authors that have not been mentioned elsewhere in this text: Prof. Roger Barker, Prof. Charlotte Erlanson-Albertsson, Dr. Veronique Gazeau, Dr. Anna Goodman, Prof. Michael Kuhar, Dr. Bernhard Landwehrmeyer, Dr. Andreas Lindqvist, Prof. Ruth Luthi-Carter, Dr. Marion Maat-Schieman, Dr. Jenny Morton, Prof. Raymund Roos, Prof. Theo Stijnen, Prof. Frank Sundler, Dr. Sarah Tabrizi, Jackie Wamsteeker, Dr. Nils Wierup, Annika Winqvist, and Dr. Nigel Wood. I especially thank Karl Bacos for making boring experiments so much fun!

I am greatly indebted to all the friends I had in Lund: Sergey Anisimov, Karin van der Borght, Sofia Correia, Tomas Deierborg, Marloes Dekker-Nitert, Valentina Fedele, Joana Gil, Nora Hagemeier, Helene Hall, Stephan Hermening, Emma Lane, Liselijn and Jeroen Langbroek-Wisman, Hannah Lewis, Anna Magnusson, Ingrid van Marion, Caterina Oliveira, Gesine Paul, Stine Rasmussen, Laurent Roybon, Jelena Pesic, Geraldine Petit, Diogo Ribeiro, Irene Kanter-Schlifke, Ruben Smith, Denis Soulet, Karin Staflin, Ayse Ulusoy, Hongyu Zhang, and Thole Züchner, You helped me with moving, with labwork, and we spent many great evenings and weekends together. You all have a special place in my heart. Without you, my time in Lund would have been much less fun!

I have been extraordinarily lucky to have spent the last four years in the Wallenberg Neuroscience Center (WNC) and in Patrik's Neuronal Survival Unit (NeSu). There are so many wonderful things that could be said about the WNC: the people, the open doors, the atmosphere, the many lectures, and interesting visitors. It is hard to imagine a more stimulating and encouraging academic environment. I would like to thank all of you that have contributed to this great working environment, especially Prof. Anders Björklund, Tomas Björklund, Dr. Manolo Carta, Dr. Angela Cenci-Nilsson, Dr. Eskil Elmer, Dr. Magnus Hansson, Ulla Jarl, Dr. Deniz Kirik, Dr. Cecilia Lundberg, Prof. Harold Robertson and Dr. Simon Stott who have directly contributed to my thesis by interesting scientific conversations or by helping with laboratory issues. I am also very grateful to all current and previous NeSu members (that have not been mentioned elsewhere in this text) for being the nicest, warmest and most helpful colleagues I could have ever imagined: Dr. Ines Araujo, Dr. Nicolaj Christopherson, Hinfan Chung, Darren Cleare, Dr. Irakli Darsalia, David Dias, Dr. Helena Frielingsdorf, Dr. Tord Hjalt, Dr. Shorena Janelidze, Caroline Kiaer, Pontus Klein, Ana Rita Lourenço Inacio, Dr. Yeliz Koc-Schmitz, Dr. Paul Mohapel, Dr. Asuka Morizane, Ingar Nilsson, Dr. Ilknur Özen, Anna Persson, Dr. Nataljia Popovic, and Sara Rundquist.

I would like to thank all students of the 'Nervous System Repair (NSR)'-network for being great friends and good company during the workshops we had at different European institutes: Liviu Aron, Jorge Aquino, Judith Bellemare, Christina Cantarella, Thijs Drenth, Julien Dusonchet, Max Elkouris, Ali Ertürk, Pablo Garcia Reitböck, Manuel Garrido, Özgun Gökce, Fiona O'Keeffe, Maurice Perrinjaquet, Veronique Planchamp, Frank Veenman, and Ian Williams. I would also like to express my gratitude to all principle investigators and other researchers that have been involved in the NSR-network. I am especially grateful to professor Christopher Hendersson, who laid the foundations of this network. Being an NSR-student was a great

experience and the training I received at the different European host institutes, the company visits and interactions with many scientists and even Nobel laureates, have contributed a lot to my scientific development.

The informal support and encouragement of many Dutch friends has been indispensable, and I would like particularly to acknowledge: Ruth Brinkmann, Nicoline Dijksma-Bakker, Hannah Groenendijk, Else Hellinga, Paul and Pauline Kraakman, Eliza Nauta, Susanne Scheer, Jessica Janssen and Stephan van der Sman, and Eveline van der Stoel. You have all come to visit me, some of you even more than once, and supplied me with an enormous amount of 'stroopwafels', and Gouda cheese! Your friendship is very important to me and I am looking forward to living closer to you soon!

I thank my family, especially my godparents Leo and Agaath, uncle Nol and aunt Wil, my sister Kirsten, and my grandmother, for being a constant source of support throughout life. Without you, I would not be who I am today.

I would like to thank Mr. and Mrs. Aziz, Ibrahim, Ali and Hosein for welcoming me so warmly into their family and making me feel like their daughter and sister. Mr. Aziz made me realize that it is never too late to make big [career] changes in life. You built up a new life in another country. I admire you for your courage. You set a great example for Ahmad and me. Mrs. Aziz: Thank you for all the delicious Persian dishes!

I am greatly indebted to my beloved Ahmad for helping with many issues regarding this thesis, but more importantly for his endless love, for sharing life with me, with its laughter and tears, and for always encouraging me in following my dreams. Science brought us together one-and-a-half years ago and since then you have always been on my side. You are the best that has ever happened in my life. *Man doestet daram.*

And finally, I would like to thank my parents, Rinus and Corry van der Burg, for making me who I am and for always being there for me. You are simply the best parents in the world! To you, I dedicate my thesis.

This thesis was supported by a Marie Curie actions fellowship: European Union, RTN MRTN-CT-2003-504636.

Curriculum Vitae

Jorien Maria Margaretha van der Burg was born on the 7th of December 1977 in Zwolle, The Netherlands. In 1990 she started high school at the Develstein College in Zwijndrecht. She graduated in 1996 and started her studies in Biology the same year at Leiden University. Her first master's research project was performed at the department of Behavioural Biology under supervision of Professor Johan J. Bolhuis and Dr. Nienke Terpstra. This project aimed to investigate the neural mechanisms involved in song recognition in zebra finches. After finishing this project, Jorien started a second research project at the Mental Health Research Institute in Melbourne, Australia. She worked in the laboratory of Dr. Maarten van den Buuse, under the supervision of Prof. Ron E. de Kloet and Dr. Melly Oitzl from the Leiden Amsterdam Center for Drug Research. She investigated the effects of long term estrogen deprivation on learning and memory in aromatase knock out mice. In February 2003 she graduated *cum laude*. In November the same year she was awarded the Unilever Research Prize for outstanding science students.

Since 2000 Jorien has combined her studies in Biology with a part time job as a lecturer at the Leiden University of Applied Sciences (Hoger Laboratorium Onderwijs, Hogeschool Leiden). After finishing her studies she obtained a full time position as a lecturer in this institute. In 2004, Jorien was elected, together with 15 other outstanding students, for the European research training program 'Nervous System Repair (NSR)'. Through this network she received education at different European institutes, such as the Karolinska Institute, Cambridge University, and the Max Planck Institute. At the same time (from September 2004 until August 2008), Jorien worked as a PhD student in the Wallenberg Neuroscience Center at Lund University, Sweden. There she performed the work that is described in this thesis under the supervision of Professor Patrik Brundin, Associate Professor Jia-Yi Li and Dr. Brigitte Pettman. Besides her studies Jorien has chaired the students steering committee of the NSR network and was on the board of the PhD student association (Samrådsgruppen för doktorander) at the department of Experimental Medical Science at Lund University. During her PhD training period, Jorien has been rewarded several prizes, among which the prize for the best scientific poster at the 2006 International Neuroscience Day in Lund, and the prize for the best mini-review, that was written together with two teammates during the NCoE/NSR workshop 2005.

In spring 2008 Jorien was elected for the Selective Utrecht Medical Master (SUMMA) at Utrecht University and enrolled the medical training program in September the same year.