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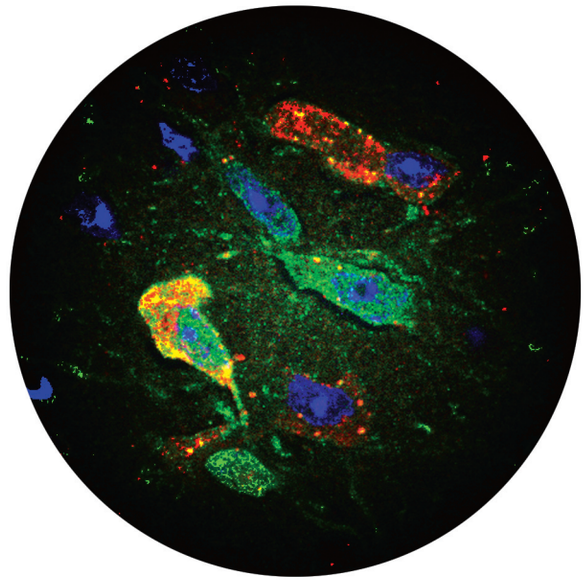

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Cover illustration: Confocal microscope image of neurons in the mouse hypothalamus, visualized with immunohistochemistry techique.

Green: interleukin-6 receptor α (IL-6Rα)
Red: melanin concentrating hormone (MCH)
Blue: cell nucleus stained with TO-PRO 3

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Abstract

Previous studies have shown that mice lacking interleukin-6 (IL-6), an important cytokine in the immune system, develop obesity, and that central, but not peripheral, administration of IL-6 induces energy expenditure. These findings suggest that IL-6 suppresses fat mass through the central nervous system. The mechanism behind this, however, is not understood.

The aim of this thesis was to investigate possible neurobiological mechanisms, by which IL-6, during health, could exert its fat suppressing effect. Using immunohistochemistry, we aimed to map the distribution of the IL-6 receptor α (IL-6Rα) in human and mouse hypothalamus. In IL-6 knockout mice, we measured the gene expression of key hypothalamic neuropeptides known to regulate energy homeostasis.

In mice, IL-6Rα was present mainly on neurons, and was widely distributed throughout the hypothalamus. IL-6Rα was found in a large number of neurons in the fat suppressing arcuate nucleus (ARC) and paraventricular nucleus (PVN), as well as in the fat promoting lateral hypothalamic area (LHA). We also found the IL-6Rα to be co-localized with several energy balance regulating neuropeptides in these hypothalamic sites, for instance with orexin and melanin concentrating hormone (MCH) in the LHA. In humans, IL-6Rα was only found in MCH neurons, but virtually all MCH neurons contained IL-6Rα.

Depletion of IL-6 reduced the expression of the fat suppressing neuropeptides corticotrophin-releasing hormone (CRH) and oxytocin, as well as of arginine-vasopressin (AVP). In addition, we found IL-6Rα on neurons that produce these neuropeptides. This indicates that IL-6 could directly act on these neurons to increase the expression of CRH, oxytocin and AVP.

Depletion of IL-6 induced the expression of the fat suppressing cytokine IL-1. In addition, IL-6 expression was reduced in mice with IL-1 receptor 1 knockout. This indicates that, in the hypothalamus, IL-1 receptor 1 signaling increase IL-6 expression, while IL-6 decreases IL-1 expression.

Based on our findings in this thesis we speculate that IL-6 could act on several hypothalamic neurons and sites involved in energy homeostasis to increase energy expenditure and eventually weight loss in mice, while a similar effect could by exerted via the pro-obesity neuropeptide MCH in humans.

Previous studies show that gut microbiota contributes to obesity, in part by facilitating nutritional uptake, but probably also through other mechanisms. We aimed to investigate possible effects of gut microbiota on central energy balance regulation. We

measured the gene expression of several important energy balance regulating neuropeptides in the hypothalamus and brainstem of germ free mice.

The fat suppressing neuropeptides glucagon-like peptide-1(GLP-1) and brain-derived neurotrophic factor (BDNF) was downregulated in the presence of gut microbiota, which could explain the elevated fat mass. In addition, we found that mice with gut microbiota were less sensitive to leptin, providing another mechanism by which gut microbiota could increase fat mass.

In conclution, our findings are in line the assumption that components of the immune system and the commensal gut microbiota can affect fat mass in part via energy balance-regulating circuits in the brain.

Populärvetenskaplig sammanfattning

Tidigare studier har visat att interleukin-6, en viktig signalmolekyl i vårt immunförsvar, kan ge viktminskning. Detaljerna kring hur detta går till är dock oklara. I denna avhandling har vi identifierat specifika celltyper i hjärnan som kan agera som målceller för interleukin-6. Många av dessa målceller i hjärnan visade sig tillverka ämnen, s.k. neuropeptider, som reglerar vår kroppsvikt genom att påverka hunger- och mättnadskänslor samt förbränning av fett. Vi har även funnit att interleukin-6 kan öka nivåerna av ett flertal neuropeptider i hjärnan som i sig verkar viktminskande, vilket skulle kunna förklara varför interleukin-6 ger viktminskning. Våra fynd klargör därmed en rad möjliga mekanismer för hur interleukin-6 kan ge viktminskning.

Det är vida känt att vid en infektion ökar interleukin-6 i blodet dramatiskt och det påverkar bl.a. hjärnan, där interleukin-6 tillsammans med ytterligare signalmolekyler ger feber och andra typiska sjukdomssymptom, såsom nedsatt hunger och orkeslöshet. Normalt är nivåerna av interleukin-6 i blodet relativt låga. På senare år har man funnit att interleukin-6 kan spela en viktig roll även hos friska individer vid reglering av ämnesomsättning. Ett viktigt bevis för detta var vårt tidiga fynd att möss som saknar interleukin-6 blir feta. Man har funnit att interleukin-6 frisätts i stora mängder från muskler vid träning, samt från fettvävnaden. Vi tror dock att det är interleukin-6 i hjärnan, vid normala nivåer, som ger viktminskning. Nivåerna av interleukin-6 hos en frisk person, skulle alltså kunna vara knutet till om personen är benägen att utveckla övervikt. En person med hög interleukin-6 produktion i hjärnan kan således skyddas mot övervikt.

Hur interleukin-6 kan påverka kroppsvikten är inte helt klarlagt, men mycket tyder på att delar av hjärnan som reglerar kroppsvikt kan vara involverade. Ett sådant område skulle kunna vara hypotalamus. Om man t.ex. injicerar råttor med interleukin-6 direkt till hjärnan nära hypotalamus, så ökar detta förbränningen, vilket på sikt kan leda till viktminskning. Tidigare har man inte vetat vilka områden och celltyper i hypotalamus som interleukin-6 kan utöva sin viktminskande effekt. Genom att i denna avhandling lokalisera receptorn för interleukin-6 kan vi få indikationer på vilka områden i hjärnan som interleukin-6 påverkar.

För att interleukin-6 ska kunna utöva sina effekter, krävs att interleukin-6 binder till en specifik receptor för interleukin-6, som sitter på målcellens yta, vilken sedan vidarebefordrar interleukin-6 signalen till cellens inre. Signalen kan sedan omsättas till att specifika ämnen börjar tillverkas och frisättas från cellen. Denna receptor har vi alltså funnit i specifika områden i hypotalamus och på särskilda nervceller som

tillverkar och frisätter neuropeptider som reglerar hunger- och mättnadskänslor samt kroppens förbränning av fett. Vi har även funnit att normala nivåer av interleukin-6 ökar nivåerna av er rad av dessa neuropeptider som minskar hunger och ökar förbränningen. Våra fynd i denna avhandling klargör därmed delvis detaljerna kring hur interleukin-6 kan verka för att ge viktminskning.

Tidigare studier visar att tarmflora kan bidra till övervikt, delvis genom ett ökat näringsupptag, men troligen också via specifika signaler från tarmflora till olika delar i kroppen. I denna avhandling har vi för första gången funnit att vår tarmflora kan påverka neuropeptider i hjärnan som reglerar vår kroppsvikt. Detta fynd att tarmbakterier kan påverka vår hjärna och kanske därmed specifikt ändra vårt beteende vad gäller födointag är förvånade och nytt.

Sammanfattningsvis kan vi i denna avhandling visa stöd för att både faktorer i immunförsvaret och den normala tarmfloran kan påverka fetma via effekter på hjärnan.

List of papers

This thesis is based on the following papers:

Paper 1. **Interrelation between interleukin-1 (IL-1), IL-6 and body fat regulating circuits of the hypothalamic arcuate nucleus**
Erik Schéle, Anna Benrick, Louise Grahnemo, Emil Egecioglu, John-Olov Jansson
Manuscript

Paper 2. **Interleukin-6 gene knockout influences energy balance regulating peptides in the hypothalamic paraventricular and supraoptic nuclei**
Anna Benrick, Erik Schéle, Scarlett Pinnock, Ingrid Wernstedt-Asterholm, Suzanne Dickson, Linda Karlsson-Lindahl, John-Olov Jansson
Journal of Neuroendocrinology. 2009 Jul;21(7):620-8. Epub 2009 Apr 13.

Paper 3. **Interleukin-6 receptor α is co-localised with melanin-concentrating hormone in human and mouse hypothalamus**
Erik Schéle, Csaba Fekete, Péter Egri, Tamás Füzesi, Miklós Palkovits, Éva Keller, Zsolt Liposits, Balázs Gereben, Linda Karlsson-Lindahl, Ruijin Shao, John-Olov Jansson
Journal of Neuroendocrinology. Epub 2012 Feb 1.

Paper 4. **The gut microbiota inhibits the expression of the obesity suppressing neuropeptides brain-derived neurotrophic factor (BDNF) and proglucagon in the hypothalamus and the brainstem**
Erik Schéle, Louise Grahnemo, Fredrik Anesten, Anna Hallén, Fredrik Bäckhed, John-Olov Jansson
Manuscript

Table of content

Abbreviations

ACTH	Adrenocorticotropic hormone
AgRP	Agouti-related peptide
Angptl 4	Angiopoietin-related protein 4
ARC	Arcuate nucleus
AVP	Arginine vasopressin
BBB	Blood–brain barrier
BDNF	Brain-derived neurotrophic factor
BSF-2	B-cell stimulatory factor-2
CART	Cocaine and amphetamine regulated transcript
CCK	Cholecystokinin
cDNA	Complementary DNA
CLC	Cardiotrophin-like cytokine
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
Cox-2	Cyclooxygenase-2
CRH	Corticotropin-releasing hormone
CT-1	Cardiotrophin-1
DMN	Dorsomedial nucleus
ER-stress	Endoplasmic reticulum stress
FIAF	Fasting induced adipose factor
GFAP	Glial fibrillary acidic protein
GLP-1	Glucagon-like peptide-1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gp130	Glycoprotein 130
HPA-axis	Hypothalamic-pituitary-adrenal axis
Iba-1	Ionized calcium-binding adapter molecule 1
IFN-β2	Interferon β2
IgG	Immunoglobulin G
IL (IL-1, IL-6 etc)	Interleukin
IL-6Rα	Interleukin-6 receptor α
IRE-1	Inositol-requiring protein 1

JAK	Janus kinase
LDA	Low density array
LHA	Lateral hypothalamic area
LIF	Leukemia inhibitory factor
Mc4R	Melanocortin receptor 4
MCH	Melanin-concentrating hormone
Myd88	Myeloid differentiation primary response gene 88
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
OSM	Oncostatin M
POMC	Pro-opiomelanocortin
PTP-1B	Protein-tyrosine phosphatase 1B
PVN	Paraventricular nucleus
PYY	Peptide YY
RT-PCR	Reverse transcription polymerase chain reaction
RVLM	Rostral Ventrolateral Medulla
SNS	Sympathetic nervous system
SOCS-3	Suppressor of cytokine signaling 3
SON	Supraoptic nucleus
STAT-3	Signal transducer and activator of transcription 3
Th17	T helper 17 cells
TLR-4	Toll-like receptor 4
TNF-α	Tumor necrosis factor α
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
VMN	Ventromedial nucleus
VTA	Ventral tegmental area
XBP-1	X-box binding protein
α-MSH	α-melanocyte-stimulating hormone

Background

Obesity

Obesity is a medical condition defined as a state of having excess fat mass to the extent of causing adverse effects on health, leading to increased health problems and decreased life expectancy. The development of excess fat mass in an individual will only occur if the energy balance equation is tilted, making energy intake, as food intake, exceed total body energy expenditure, which can be divided into physical activity, basal metabolism and adaptive thermogenesis [1].

Global epidemic

During the recent decades a dramatic increase in the incidence of obesity worldwide has brought attention to the question of what might be the cause of this current and future global health threat. Not too long ago obesity was seen upon as a result of bad character and lack of self control and will power [2]. Today, however, the picture of how obesity occurs is much more nuanced, and more often genetic factors in combination with environmental and phsycoscocial factors are suggested to contribute to the development of obesity. To have an impact on adiposity, these factors must influence the energy balance equation to favor the storage of access energy as triglycerides in adipose tissue. Much attention has been drawn towards environmental factors such as our western world lifestyle, with an endless availability of food in combination with reduced requirement of physical activity. This "energy-saturated" environment in combination with our genetically given ability to store energy has become a dreadful fusion [1].

Hunt for fat genes

The ability to store energy as fat is believed to be an evolutionary favorable trait that has been selected for over thousands of years, to cope with periods of food shortage. However, today in the western world we rarely or never experience any long time food shortage, making the ability to store fat a yoke for some people and a predisposition to develop obesity. This idea that has been given the name "the thrifty gene hypothesis" has been a consensus over the last 50 years [3]. Lately, this hypothesis has however been questioned, based on the fact that severe famine historically actu-

ally is rather rare, and that the mortality mostly is a result of infectious disease rather than starvation, as well as being restricted to very young and old individuals, giving little impact on the reproducible part of the population, and thus little evolutionary impact on a selection for the ability to store fat [4]. However, lack of energy reserves may impair the immune response to infections. Thus, thrifty genes could still be beneficial.

As the ability to store fat is an essential part of obesity development, one may conclude that the answer to the obesity epidemic may lie in genes involved in adipose tissue development, and that direct manipulation of such genes may limit obesity. However, genetically modulated experimental animals having reduced fat cell differentiation, growth and survival capacity, are not lean and healthy. As a matter of fact these animals show typical obesity related features, such as high blood lipids and fatty liver, as a result of lack of appropriate energy storage depot [1]. This highlights the importance of understanding energy balance and its components to be able to genetically understand obesity.

It is known that obesity is a highly heritable trait, and therefore possible genetic components have been extensively studied. In the last fifteen years, in the wake of the discovery of leptin, a lot of hope has been canalized towards genetic studies to find a cure for obesity. These studies have unfortunately not been successful in explaining the obesity epidemic and understanding the development of obesity in most people, thus giving no cure. However, several new so called monogenic disorders have been discovered, where one single gene in a limited number of people leads to severe obesity. Notably, all these genes affect components of the energy balance sensing pathways of the brain, including the hypothalamus and the central melanocortin system within the hypothalamus [5].

Leptin and gut hormones

Even though obesity now is very common, the majority of all people are able to maintain a steady body weight. This steady state is achieved through a process known as energy homeostasis, where food intake is adjusted over time to meet our needs, and maintain a stable body weight. Important components of energy homeostasis is the adipose tissue-derived hormone leptin, as well as gastrointestinal-derived hormones such as ghrelin, cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide-1 (GLP-1), which all communicate to the brain [6].

Leptin, is produced and released by adipocytes into the circulation in relation to adipose tissue mass, and it regulates food intake and energy expenditure by acting on energy balance regulating centers of the brain, leading to suppression of fat mass. In particular, leptin action is mediated by neurons in the arcuate nucleus (ARC) of the hypothalamus that produces the neuropeptides pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) or neuropeptide Y (NPY)

and agouti-related protein (AgRP). Leptin stimulate POMC and CART which reduce food intake, and at the same time inhibits NPY and AgRP which increase food intake, thus giving a fat mass suppressing effect [6].

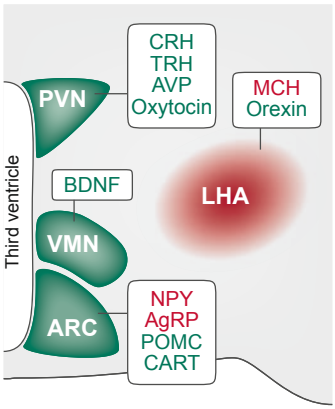
At discovery, leptin was believed to be the future cure for obesity because of its satiety mediating properties. Soon however, it was found that obese individuals already had elevated circulating levels of leptin as a result of their high fat mass, and that chronic elevated leptin levels were associated with decreased leptin sensitivity. This made leptin treatment useless to most obese patients [7-9]. One exception is a very small group of obese individuals that actually have dysfunctional leptin, and therefore respond positively to leptin therapy [10]. Interestingly, patients with lipodystrophy, which have impaired ability to store fat due to lack of adipocytes and low endogenous levels of leptin, also respond positively to leptin treatment [11].Today, the mechanisms behind leptin resistance are extensively studied and are believed to involve key elements in understanding obesity.

In contrast to the long-term effects of leptin on fat mass, the gastrointestinal-derived hormones ghrelin, CCK, PYY and GLP-1 all have short-term effects on hunger and satiety, where ghrelin stimulates hunger , and CCK, PYY and GLP-1 induce satiety (Murphy 2006). In addition to being produced by the intestines, GLP-1 is also produced by a limited number of neurons in the brainstem, which project to numerous part of the brain, including the hypothalamus [12].

Hypothalamus

The hypothalamus is a very important part of the brain involved in regulating energy balance [13]. It is composed of several nuclei having individual functions. The ARC (or infundibular nucleus) which is located at the base of the hypothalamus, serves as a link between the brain and the circulation, receiving information of fat mass status from, in particular, leptin [6]. The ARC is considered to overall have fat suppressing activities, as lesions in the region will cause a gluttonous appetite and obesity [14, 15]. However, individual neurons within the ARC comprise both fat suppressing and fat promoting properties (e.g. POMC- and NPY-neurons respectively) [6]. The neighboring ventromedial hypothalamic nucleus (VMN) is also considered a fat suppressing nucleus and is densely enriched with the fat suppressing neuropeptide brain-derived neurotrophic factor (BDNF) [16]. The lateral hypothalamic area (LHA), on the other hand, overall elicits fat promoting features [14, 15]. The neuropeptides melanin concentrating hormone (MCH) and orexin are highly abundant and predominantly restricted to the lateral hypothalamic area [17, 18]. MCH promotes obesity, while orexin suppress obesity by increasing energy expenditure more than food consumption [19-24]. The paraventricular nucleus (PVN), mostly known as an important regulator of hormones released from the pituitary, such as adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), oxytocin and

arginine vasopressin (AVP), has been proposed to be involved in energy balance regulation as a fat suppressing centre [25]. The paraventricular nucleus harbours multiple subpopulations of neurons that produce the neuropeptides corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) oxytocin and AVP which all has been showed to have fat suppressing properties (Figure 1) [26-29].



Figur 1. Hypothalamic nuclei and neuropeptides involved in energy balance regulation. The paraventricular nucleus (PVN), the ventromedial hypothalamic nucleus (VMN) and the arcuate nucleus (ARC) are all considered, overall, to be fat suppressing nuclei (green), while the lateral hypothalamic area (LHA) is considered fat promoting (red). Individual neurons within the ARC and LHA, however, comprise both fat suppressing and fat promoting properties. The fat suppressing neuropeptides indicated by green characters are; corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), arginine-vasopressin (AVP), oxytocin, orexin, brain derived-neurotrophic factor (BDNF), pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART). The fat promoting neuropeptides indicated by red characters are; melanin concentrating hormone (MCH), neuropeptide-Y (NPY) and agouti-related protein (AgRP).

Obesity and inflammation

During the past years an expanding body of evidence suggests that inflammation in peripheral tissue is a key feature of obesity. In a classical sense, inflammation in described as the primary response to injuries including local swelling, redness and pain as well as fever, caused by a serial of molecules and signaling pathways. The inflammation seen in relation to obesity do not involve any of the classical features of inflammation, but engages a similar set of molecules and signaling pathways, and is triggered by various nutrients or overindulgence [30].

A close relationship between metabolism and the immune system may not strike one as very surprising, as the ability to cope with nutritional shortage and infections are both very critical for the survival of an organism, and both crave energy. Indeed, in early evolution many of the functional units in metabolism and in the immune system was incorporated in the same biological structure, as for example the “fat body” in insects, which possess the functions equivalent to our adipose tissue, liver and immune cells together [30].

Immune factors in the hypothalamus in relation to obesity

In addition to peripheral tissue, inflammation may also involve neurons in the hypothalamus in response to nutrient excess. Unlike the periphery, hypothalamic inflammation has the potential of actually causing obesity, because of the crucial negative feedback from leptin. It has been suggested that leptin resistance can be provoked by neuronal inflammation in the hypothalamus, induced by high-fat rich diets. The mechanism is thought to involve the activation of the immune factors toll-like receptor 4 (TLR-4), myeloid differentiation primary response gene 88 (Myd88) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), which eventually could lead to the induction of suppressor of cytokine signaling 3 (SOCS-3), a factor known to inhibit leptin signaling. Endoplasmatic reticulum (ER) stress has also been implicated to induce leptin resistance (Table 1). When input from leptin is reduced, as in leptin resistance, the hypothalamus receives false information about fat mass status, which will lead to an inadequate feedback, and sustained weight gain [2].

The immune system does not only have deleterious effects on metabolism. Indeed, many immune factors have been showed to have beneficial effects on obesity, causing decreased fat mass, when acting on the central nervous system (CNS). The pro-inflammatory mediators interleukin (IL)-1, IL-6, IL-7, IL-18, granulocyte-macrophage colony-stimulating factor (GM-CSF) and prostaglandin E2 all show fat suppressing properties, as judged from various knockdown mouse models, and the site of action has been found to be within the CNS (Table 1) [31-36]. The mechanisms behind the fat suppressing effects of these immune factors remain, however, to be investigated. In this thesis, the possible sites and mechanisms of the anti-obesity effect by centrally acting IL-6, has been investigated.

Table 1. Immune factors in CNS implicated to affect energy homeostasis

Supress obesity	Promote obesity
IL-1	NFκB
IL-6	MyD88
IL-7	ER-stress
IL-18	
EP-3 rec	
GM-CSF	

Interleukin-6

Effects in immune function

IL-6, is a 26 kDa protein, that was first cloned in 1986 by two independent research groups and was initially designated B-cell stimulatory factor 2 (BSF-2) and interferon β2 (IFN-β2). This multifunctional cytokine has over the years been found to be important, in particular, in various immune functions, but also to play a role in a number of other biological activities. In the immune system, IL-6 is for example involved in the general immune response, inflammation, the formation of blood cells, including B-cells and the synthesis of acute-phase proteins by the liver [37]. The major source of IL-6 is endothelial cells, fibroblasts and monocytes/macrophages [38].

During an acute inflammation, IL-6 and various chemokines are released from endothelial cells in the vascular wall, initially leading to the recruitment of neutrophils and eventually to the recruitment of monocytes and T-cells, to the site of the infection. In addition to the recruitment of leukocytes, IL-6 is also important in the maturation of macrophages, and the differentiation of B-cells, and also T-cells. In fact, IL- 6 was first discovered as a factor that stimulates B-cells to produce antibodies, as the initial name BSF-2 implies [39]. In line with this, IL-6 seems to be of importance for the development of multiple myeloma [40]. Recent findings indicate that IL-6 has a very important role in the differentiation of a specific type of T-cell, known as T helper 17 cell (Th17), which is a key player in the pathogenesis of autoimmune disease [41].

IL-6, next after IL-1β, is considered the most important endogenous mediator of fever. During an infection, IL-6 accompanied by IL-1β and other cytokines, is released in high levels into the circulation, and possibly also into the cerebrospinal fluid. In the medial preoptic nucleus of the hypothalamus, these cytokines are known to induce fever, as well as anorexia (Figure 2). IL-6 has been shown to be an irreplaceable component of the fever response, even though IL-6 by itself, without IL-1β, cannot induce fever. The mechanism for IL-6 induced fever in the brain is not fully understood. However, as for IL-1β, IL-6 induced fever is believed to be dependent on hypothalamic cyclooxygenase-2 (cox-2) activity, indicating that IL-6 triggers fever through prostaglandin E2 in the hypothalamus [42].

In addition to being a pro-inflammatory cytokine, IL-6 also has anti-inflammatory activities through the stimulation of the anti-inflammatory cytokines IL-1 receptor antagonist and IL-10, and the inhibition of pro-inflammatory tumor necrosis factor-α (TNF-α) [43]. IL-6 also induces CRH-release which will result in elevated levels of glucocorticoids known to turn immune activity down [44, 45].

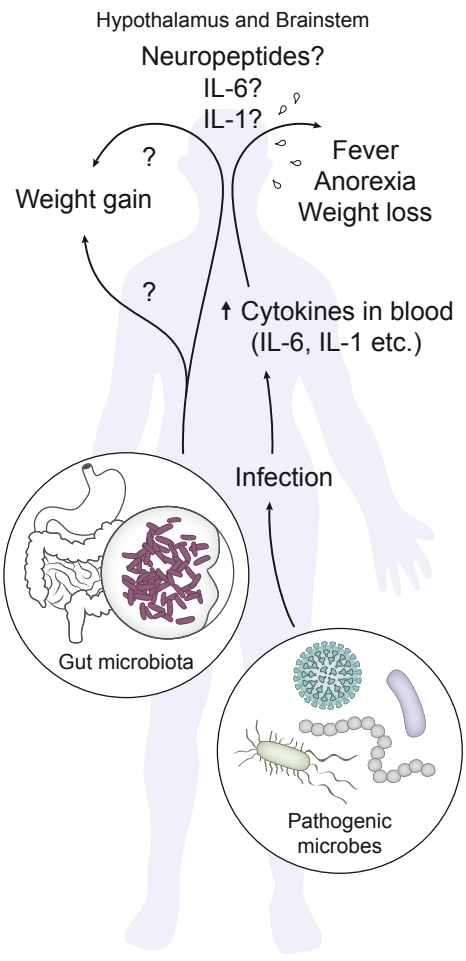


Figure 2. Central responses to infection and commensal gut microbiota.
During an infection, caused by pathogenic microbes, cytokines in blood are dramatically elevated, including interleukin-6 (IL-6) and interleukin-1 (IL-1). The cytokines reaches the hypothalamus and induce fever and anorexia which eventually lead to weight loss. Commensal gut microbiota facilitate nutritional uptake, and may increase body weight this way. In addition, gut microbiota may affect the hypothalamus and the brainstem, through components of the immune system and/or neuropeptides. Thus, both pathogens and commensal gut microbiota may exert central effects on body fat, via neuropeptides and cytokines, such as IL-6 and IL-1, to increase body weight.

Signaling pathway

IL-6 signals through a cell-surface receptor complex, composed of the ligand binding IL-6 receptor α (IL-6Rα) subunit and the IL-6 signal transducer (IL-6ST) subunit, also designated glycoprotein 130 (gp130). IL-6 selectively binds the non-signaling IL-6Rα subunit in the membrane of a target cell. Secondly, two molecules of the gp130 subunit are recruited and bind the IL-6/IL-6Rα complex, which enables full function of the receptor complex and the IL-6 signal is transmitted downstream the signaling pathway. In addition, IL-6 can also bind a soluble form of the ligand binding IL-6R subunit (sIL-6R), forming a soluble IL-6/IL-6R complex, which then binds gp130. Thus, any cell having gp130 can respond to IL-6, with or without the

membrane bound IL-6Rα, a process called trans-signaling. Whereas the gp130 subunit is commonly expressed among most tissues and cell types, the IL-6Rα is confined to only a few cell types, for instance, hepatocytes and immune related cells [39].

The biological significance of having two types of signaling procedures is not known. But it has been suggested that the pro-inflammatory effects of IL-6 is mediated mainly through the soluble form of the IL-6R, and thus via trans-signaling, whereas anti-inflammatory properties of IL-6 are mediated through classical-signaling, involving the membrane bound IL-6Rα [39].

The assembly of IL-6 and the IL-6 receptor (membrane bound or soluble), with two subunits of gp130 in the cell membrane, triggers gp130 associated Janus kinases (JAK) to phosphorylate signal transducer and activator of transcription (STAT3), which facilitates dimerization of STAT3. The STAT3-STAT3 homodimer then enters the cell nucleus, where it can bind DNA and acts as a transcription factor [37]. The signal pathway is negatively regulated by SOCS protein 1 and 3, which are both induced by STATs, and also act as a STAT inhibitor [46].

IL-6 family

IL-6 is not the only factor that signals through gp130. Indeed, gp130 is shared with several other cytokines including leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), Oncostatin-M (OSM), IL-11, IL-27, cardiotrophin-1 (CT-1) and cardiotrophin-like cytokine (CLC). Besides sharing the signal transducing receptor subunit gp130, these cytokines also comprise structural similarity and functional redundancy, which to a large extent can be explained by the fact that they share the gp130 subunit (Jones 2011). Gp 130 may also be important in elicit compensatory effects among the gp130 related cytokines. For most of these cytokines, complete lack of the cytokine, in mouse knockout models, do not cause as severe phenotypes as one would expect, even though all of these cytokines are being involved in several important physiological functions [47].

So far, IL-6 is the only one of the gp130 related cytokines that is known to perform trans-signaling, making IL-6 unique in that it can act on any cell expressing gp130 in the presence of soluble IL-6R, and consequently fulfill functions of the other gp130 related cytokines. The other cytokines solely act on cells expressing their ligand-binding receptor subunit [47].

Non-immune effect

IL-6 is often referred to as being a pleitrophic cytokine, meaning that it influences multiple phenotypic traits. Besides being an important factor in the immune system at a cellular level, IL-6 is also involved in several other physiological functions. Below follows some of the most important ones.

Hepatocytes are among the limited number of cell types that express IL-6Rα, indicating that IL-6 is of importance in the liver. In addition to induce acute phase reactants, IL-6 may also improve liver regeneration. Liver regeneration is accompanied by increased circulating IL-6, and liver growth after partial loss of tissue may be dependent on IL-6 [48, 49].

IL-6 has also been implicated to be involved in bone metabolism. In experimental studies, osteoblast-derived IL-6 promotes bone resorption, possibly by stimulating the differentiation, activation and survival of bone degrading osteoclasts. However, in vivo, under normal physiological conditions IL-6 is not required for osteoclast formation, as seen in IL-6 knockout mice. If IL-6 is important in the development of osteoporosis in humans is not clear, although IL-6 polymorphisms have been associated with bone mineral density [50, 51].

One very interesting feature of IL-6 is that during exercise the production and release of IL-6 from skeletal muscle is dramatically increased, leading to a up to 100-fold increase in IL-6 levels in the circulation. This exercise induced boost of IL-6, has both local effects in the muscles and, when released into the circulation, peripheral effects in several organs in an endocrine fashion. It has been suggested that muscular derived IL-6 may have anti-inflammatory effects, as the exercise induced increase in IL-6 is not accompanied by increased TNF-α, and is followed by an increase in the anti-inflammatory cytokines IL-10 and IL-1R [52].

IL-6 in relation to metabolism

Under normal physiological conditions, IL-6 levels are relatively low, but is considerably up-regulated is response to various stressors such as infection and exercise , where metabolism needs to be altered to fit the new challenge [52-54]. Nevertheless, endogenous IL-6, under normal conditions has also been shown to impact metabolism. Most importantly, IL-6 suppresses fat mass, as judged from developed obesity in IL-6 deficient mice, and the fact that body fat mass in humans is associated with variations in the IL-6 gene [36, 55, 56]. In experimental animals, IL-6 treatment increase energy expenditure, an effect that is solely found when administered to the ventricular system in the brain, indicating that IL-6 may suppress fat mass through increased energy expenditure, and that this effect is mediated by the CNS [57]. IL-6 has also been found to increase lipolysis and fat oxidation, which may also contribute to reduction of fat mass [58, 59]. While the affect of IL-6 on energy expenditure, both in health and disease, is rather clear, IL-6 affect on appetite is still uncertain and is believed to be moderate, if any [60].

IL-6 in the CNS

IL-6 has several reported functions involving the brain, including fever, sickness behavior, physiological stress and sleep [42, 61-63]. The origin of IL-6, in functions that involves the brain, is unclear. Basically, there are two possible sources, either from peripheral tissue via the circulation or from local production within the brain (Figure 3). Peripheral IL-6 may derive from either immune cells, adipose tissue or from muscles during exercise (Figur 3) [38, 52, 64, 65].

Blood borne molecules cannot unconditionally access the brain. Circulating blood is separated from the brain extracellular fluids by the discriminating blood-brain barrier (BBB). Small hydrophobic molecules like oxygen and carbon dioxide can easily diffuse across the BBB, while any passage of larger molecule (e.g. peptide) or hydrophilic molecules are either restricted or regulated. In addition to pure diffusion, molecules may however cross the BBB through various transport mechanisms [66]. Several components of the immune system have been found to interact with BBB to regulate its function, and in some cases even facilitate disruption of the BBB. As IL-6 is a large hydrophilic peptide, it has been questioned whether it can cross the BBB or not. It has been reported that IL-6 may cross the BBB by a saturable transport system, thus making it possible for peripherally derived IL-6 to act on brain function (Figure 3) [67]. The uptake rates of saturable transport systems are much higher than trans-membrane diffusion, and the rate is often regulated [68]. Many saturable transporters are selectively present in specific brain regions resulting in that any particular molecule may enter one brain area while at the same time restricted from entering another [69]. Even though IL-6 has been reported to cross the BBB at a fairly high rate, the majority of the molecules seem to be degraded once entered the brain [67].

IL-6 can also be locally produced within the brain. Under physiological conditions, low levels of IL-6 are produced by endothelial cells, glial cells and neurons, and IL-6 is commonly found in the cerebrospinal fluid (Figure 3). The role of locally produced IL-6 in the brain under normal healthy conditions is poorly understood, as IL-6 so far, mostly have been studied in relation to various neurological diseases, when the expression of IL-6 is dramatically elevated [70].

IL-6 has been found to have neurotrophic effects, and thus, this may suggest a neuroprotective role of IL-6 during health. The view of IL-6 as a neurotroph factor may not strike as surprising, as many of the other gp130 related cytokines are well known neurotrophic factors, as for instance CNTF. In fact, even gp130 by itself has been shown to be essential for survival of subgroups of neurons. The neurotrophic influence of IL-6 on the survival and differentiation on neurons, may be either direct, by IL-6 itself, or indirect, by subsequent stimulation of glial cells which support local supply of yet other neurothrophic factors [70].

A requirement for local effect of IL-6 on cells in the brain is the presence of either membrane bound IL-6Rα, or soluble IL-6Rα, in addition to gp130. To date, the whereabouts of IL-6Rα in the brain has been poorly studied, and again mostly

studied in the relation to various diseases. In this thesis, we have aimed at investigating the presence of IL-6Rα, during absence of inflammation, in certain parts of the hypothalamus that are of importance for energy balance.

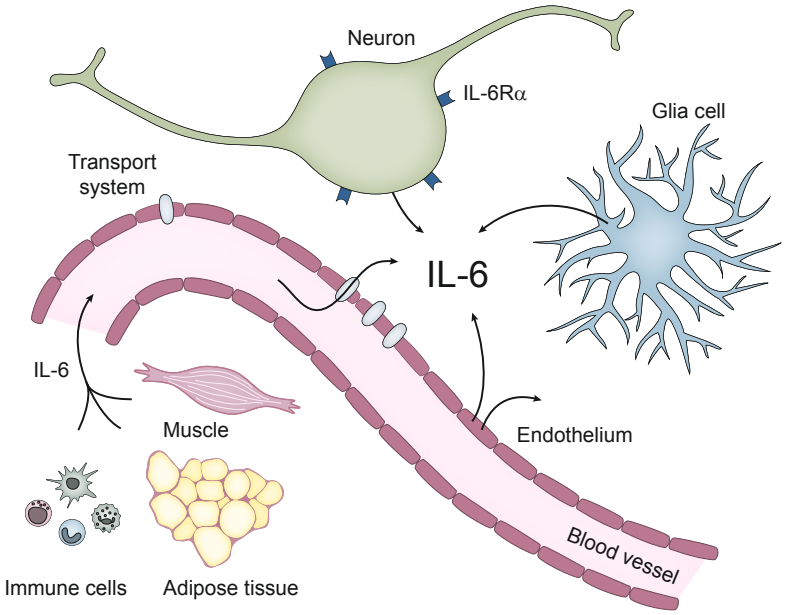


Figure 3. Possible origin of centrally acting IL-6.
Central IL-6 may derive either from the periphery or from local production within the central nervous system (CNS). The major peripheral sources of IL-6 are immune cells, adipose tissue and skeletal muscles during exercise. Circulating IL-6, from peripheral tissues, may then enter the brain parenchyma through the blood-brain barrier via, e.g. saturable transport systems. IL-6 is locally produced within the brain by the endothelium, glial cells and neurons.

Gut microbiota

Germ Free mice

In the 1940-ies, Bengt Gustafsson and colleges described a reliable system of rearing laboratory animals in a sterile environment [71]. This launched the use of germ free rats and mice in science, which at first was generally unpopular and many did not see the purpose of these animals [72]. Initial studies with germ free animals addressed the composition of the gut microbiota and the role of gut microbiota in nutrition uptake [72, 73], and as of today germ free mice have contributed to numerous new insights.

The overall morphology and physiology of germ free life was first described 1959, where germ free mice and rats were reported to develop impaired growth of the small intestine, dramatically enlarged cecum and reduced body weight [74]. Recently, a more thorough study of the germ free mouse metabolism reveals that, besides being lean, these mice surprisingly consume more food, and have decreased metabolic rate. As a result of reduced fat mass, germ free mice also have low circulating leptin levels [75]. The leanness, and increased food intake in germ free mice could be explained by their reduced capacity to harvest energy from the diet (Figure 2). Thus, germ free condition can be compared with calorie restriction [76].

Gut microbiota and obesity in humans

The gut microbiota, which constitutes approximately ten times more microbes than our own cells, is situated in the intersection between the diet and the host, and is important in food processing and making nutrients available to us. The gut microbiota, is first established at birth, and is constantly modulated throughout life, for instance by caloric intake. The human gut microbiota is dominated by two bacterial phyla, namely Bacteroidetes and Firmicutes. Obese individuals have been showed to have reduced microbial diversity, including reduced proportion of Bacteroidetes. Whether altered gut microbiota may cause obesity, or is rather its consequence is, however, not known. Interestingly, animal experiment have showed that transplanted gut microbiota, from an obese mouse to a healthy and lean germ free mouse, will cause more obesity in the recipient mouse compared to gut microbiota transplanted from a lean donor mouse. This indicates that the gut microbiota can modulate fat mass [76].

Gut-Brain axis in relation to the gut microbiota

While the role of the gut-brain axis in metabolism has been extensively studied in relation to food intake [77], so far, little is known about the role of gut microbiota in this communication (Figure 2). Some gut microbiota-brain interactions has been reported, even though not in relation to metabolism, suggesting that gut microbiota can influence the hypothalamic-pituitary-adrenal (HPA) axis, behavior and BDNF levels [78].

Gut microbiota in relation to the immune system and cytokines

Gut microbiota have profound effect on the immune function in the gastrointestinal tract, and is proposed to increase intestinal permeability [79, 80], which enables endotoxin penetration and subsequent immune system activation [76]. It has been hypothesized that a high fat rich diet, may change the gut microbiota composition so that to favor increased intestinal permeability, causing increased endotoxin levels and metabolically related inflammation [79]. Indeed, germ free mice have reduced adipose inflammation, and antibiotic treatment to obese mice reduces endotoxin levels and inflammation [76].

In general, there seems to be a close and complex relationship between the gut microbiota, the immune system and metabolism. Based on this, we used germ free mice to investigate whether the gut microbiota potentially could regulate various cytokines (e.g. IL-1 and IL-6), as well as fat regulating neuropeptides in the hypothalamus (Figure 2).

Aims

The overall aim of this thesis was to investigate potential routes in the hypothalamus and brainstem, by which immune modulating factors, such as interleukin-6 (IL-6), and the gut microbiota, can exert metabolic effects during health.

Specific aims

Paper 1 To investigate interaction between the energy balance regulating cytokines IL-6 and IL-1 in the arcuate nucleus (ARC) of the hypothalamus.

Paper 2 To investigate the effects of loss of endogenous IL-6 on the expression of energy balance regulating neuropeptides, mainly in neurons of the paraventricular nucleus (PVN) of the hypothalamus. To relate these findings to the distribution of IL-6Rα, which enables direct effects of IL-6 on these neurons.

Paper 3 To investigate the distribution of IL-6Rα in the human hypothalamus, and to relate these findings to the mouse hypothalamus, especially in the lateral hypothalamic area (LHA).

Paper 4 To investigate the effect of the gut microbiota on leptin sensitivity and expression of energy balance regulating neuropeptides in the hypothalamus and brainstem.

Methodological considerations

Immunohistochemistry

The basic concept of immunohistochemistry is to demonstrate the presence of antigens, in tissue, by specific antibodies directed against the specific target antigen. The antigen-antibody bond is then visualized by detection molecules, visible under a microscope.

General

In paper 1, 2 and 3, immunohistochemistry technique was used to investigate the localization of the IL-6Rα protein in mouse brain. The used protocol was initially optimized to detect IL-6Rα alone and later optimized to simultaneously detect IL-6Rα and additional peptides.

For tissue preparation, mice were fixed by transcardial perfusion with paraformaldehyde, to ensure a desirable homogenous fixation, and the brains were then dissected out and initially put in a solution with paraformaldehyde and sucrose, followed by a solution with solely sucrose, to protect the tissue from damage caused by freezing [81]. The fixation time was measured from the point where the muscles started to contract spontaneously (formalin dance), as a result from the fixative, rather than from the point of needle insertion. Free floating technique was used to enable optimal access of the antibody to the epitopes. The brains were sectioned with a cryostat or sliding freezing microtome, with a section thickness of 20-30 μm, and the sections were then stored in cryoprotectant solution.

The immunohistochemistry was performed by indirect methods, meaning that the detection was carried out by labeled secondary antibodies, which bind the antigen binding primary antibodies. In paper 1 and 2 different flourochrome conjugated secondary antibodies was used for the detection of all peptides except IL-6Rα. In paper 3, also IL-6Rα was detected with a flourochrome conjugated secondary antibody (Figure 4 A). In paper 1 and 2, on the other hand, a tyramine amplification method was performed to amplify the very weak signal of IL-6Rα (Figure 4 B, C). The amplification procedure is based on a horseradich peroxidase driven deposition of label-conjugated tyramine at the location of the antigen. Horseradich peroxidase is coupled to the antigen-antibody complex, and tyramine molecules, which has the ability to chemically adhere to the tissue section, is catalysed and droped in mul-

tiple numbers, in the immediate proximity of the antigen. The high numbers of conjugated tyramine molecules are then detected with appropriate method. This amplidication-method can increase the sensitivity of the antigen-antibody reaction at least 10-fold [82]. In paper 1, a horseradish peroxidase conjugated secondary antibody was used along with fluorescein conjugated tyramine, which will result in the deposition of fluorescent fluorescein molecules in the vicinity of the IL-6Rα protein (Figure 4 B). In paper 2, a biotinylated secondary antibody was used along with a streptavidin-horseradish peroxidase conjugate. Biotin and streptavidin form an irreversible bond with very high affinity, enabling the horseradish peroxidase to unite

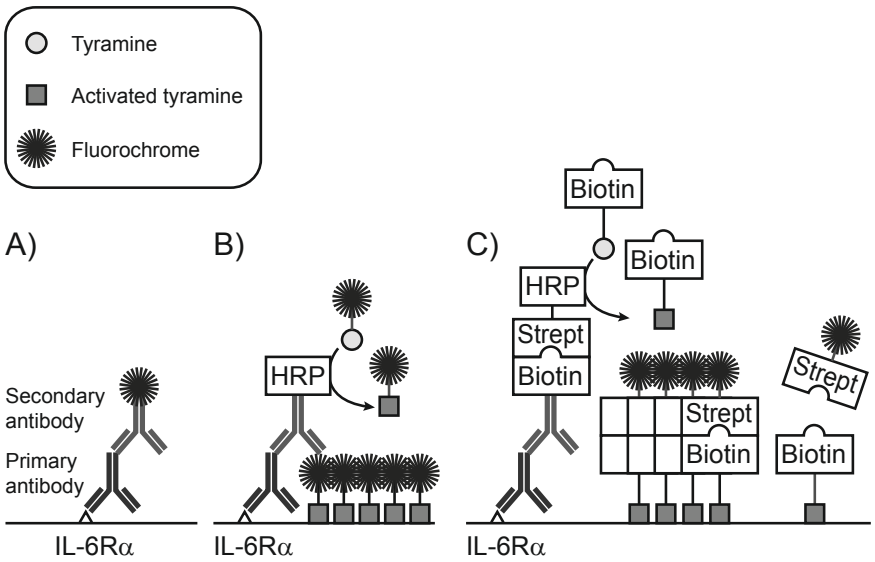


Figure 4. Immunohistochemical detection methods used to detect interleukin-6 receptor α (IL-6Rα).
In addition to the primary IL-6Rα antibody, different secondary antibodies and amplification methods were used in this thesis to detect IL-6Rα. In all studies a fluorochrome was used, in a final step, to visually detect IL-6Rα with a confocal microscope. **A)** A fluorochrome conjugated secondary antibody was used in Paper 3. **B)** In Paper 1, a horseradish peroxidase (HPR) conjugated secondary antibody was used to facilitate the deposition of fluorochrome conjugated activated tyramine. **C)** In Paper 2, a biotinylated secondary antibody was used together with a streptavidin-HRP conjugate. Streptavidin (Strept) binds irreversibly to biotin, and thereby HRP is united to the secondary antibody.The HRP then catalyzed the deposition of biotinylated activated tyramine in the vicinity of IL-6Rα, and subsequently a streptavidin-fluorochrome conjugate binds the deposited biotinylated thramine.

with the used secondary antibody and then catalyze the deposition of biotin from a tyramine-biotin conjugate. The deposited biotin was subsequently detected with a streptavidin-fluorochrome conjugate (Figure 4 C).

Cellular nuclei were stained with the DNA binding dye TO-PRO 3, which was choosen because of its absorption and fluorescent emission properties that was optimal for the used confocal microscope settings [83].

Specificity of antibodies

Antibodies (immunoglobulins) are large Y-shaped proteins composed of two identical light chains, and two identical heavy chains, and are produced by B-cells. Antibodies are grouped into several isotypes, and the most common isotype used in immunohistochemistry is immunoglobulin G (IgG). At the tip of the arms of the Y,called F(ab) fragments, IgGs have two specific binding sites which can bind antigens. The antigen binding site is a hypervariable region, and this portion differs depending on which B-cell line the antibody is produced from [84]. The tail of the Y, called Fc fragment, has one binding site, which permits binding of other antibodies, and is essential in multistep immunohistochemistry [82].

Antibodies used in immunohistochemistry are made by immunizing animals with an antigen of interest, which most often are either a syntethic peptide or a purified protein. The animal responds by producing antibodies that are directed against, and bind, that specific antigen. Two types of antibodies can be harvested from an immunized animal; polyclonal and monoclonal. Polyclonal antibodies derive from multiple B-cell lines, thus binding to, not only multiple sites of the antigen, but also to other epitopes depending on previous immune experience of the animal. As a matter of fact, a rather small portion of all antibodies produced bind the actual target antigen. This is however often overcome, even though not entirely, by affinity purification. Polyclonal antibodies are then often considered to have high sensitivity and affinity, but low specificity. Monoclonal antibodies are produced by harvesting B-cells from the spleen of an immunized animal, and then the B-cells are fused with myeloma cells, making the B-cell line immortal and dividing continuously. A single B-cell line is selected, producing identical antibodies, which are directed against one particular epitope on the antigen. Monoclonal antibodies are therefore more specific than polyclonal antibodies, but have low sensitivity because it only binds one single site of the antigen. However, some monoclonal antibodies derive from B-cells that are maintained in the animal peritoneum (to increase the concentration), thus allowing contamination with endogenous antibodies from the host. Consequently, no antibodies available are entirely specific against the antigen it is directed against. In addition to un-specificity, as a result from un-purity, a actual specific antibody, directed against the correct antigen, may also bind other epitopes in the fixed tissue. Such epitopes could, for instance, be proteins with similar amino acid sequence as the original anti-

gen, or similarity in the three-dimensional structure. The Fc-region of the antibodies can also be responsible for some un-specificity, as a result of non-immune adherence of the Fc-region to the tissue [82, 85].

Consequently, antibodies are very likely to bind to variable epitopes, other then, or in addition to, the actual antigen it is supposed to bind. Therefore it is very important to conduct control experiments to minimize the risk of false positive results.

In this thesis, several control experiments have been conducted, by us and others, to ensure specificity of the antibodies used. All the primary antibodies used, except antibodies against IL-6Rα, have been well characterized and controlled for specificity elsewhere, by other investigators. The IL-6Rα antibodies, have however not before been used in brain tissue, and were therefore characterized by several control experiments during the work of this thesis. First, two different antibodies directed against different parts of the IL-6Rα protein was used in a double immunohistochemistry experiment, which showed that the two antibodies stained the same cells. Secondly, western blot experiment showed one single band of the expected size, and thirdly, absorption experiment with the immunizing IL-6Rα agent, blocked the IL-6Rα immunoreactivity. The specificity of the secondary antibodies, and detection systems was controlled for by incubating them with brain sections without primary antibodies, resulting in no immunoreactivity. In addition, to ensure that the secondary antibodies did not cross-react in double-staining experiments, primary antibodies were incubated with mismatching secondary antibodies, which also resulted in negative staining.

With this broad repertoire of control experiment, one may conclude that the used IL-6Rα antibodies are specificly binding IL-6Rα in brain tissue.

Confocal microscopy

To determine co-localization of two or more molecular structures, a regular fluorescence microscope is not sufficient. What at a first may look like co-localization may not be, but rather a consequence of the two structures situated on top and below one another (Z-direction) in the tissue section. To overcome this problem, confocal microscopy was used throughout this thesis. The principle of confocal microscopy is based on the feature of eliminating out-of-focus signals, which is enabled by a pinhole in front of the detector [86]. The practical effect is that the obtained image only shows light from a very thin optical section, in the micro meter range, which permits correct determination of co-localization by optically separating structures, situated at various points in the Z-direction.

During the work of this thesis tree different flurochromes was used in co-localization studies namely; Alexa Fluor 488, Alexa Fluor 568 and TO-PRO 3. Lasers at the wave length of 488 nm, 543 nm, 637 nm was used to excite the fluorochromes, and the method (e.g. dichroic mirrors and emission filters) was optimized to ensure that no signal from one fluorochrome would spill light, and be falsely detected as another fluorochrome.

Colchicine treatment

Colchicine is a substance widely used in neurohistochemical studies to improve visualization of compounds, in the cell bodies of neurons. Colchicine arrests the axonal transport by interfering with microtubules, thus facilitating accumulation of synthesized compounds to the cell body [87-89]. In Paper 1 and 2, colchicine was injected intracerebroventricular for the purpose of detecting the peptides CRH, TRH oxytocin, AVP, NPY and α melanocyte-stimulating hormone (α-MSH) in the cell bodies, to investigate possible co-localization with IL-6Rα.

RT-PCR

To measure the level of expression of a gene, quantitative real-time polymarese chain reaction (RT-PCR) was used. RNA from mouse hypothalami was purified, and complementary DNA (cDNA) was synthesized. With cDNA as the template, the target gene was amplified using DNA-polymerase and flanking primers. A probe, that bind the gene, with a fluorescent dye and a quencher, was used to measure the amount of amplification. The fluorescent dye is suppressed by a quencher as long as they are situated in close proximity to each other. During the extension phase of the PCR reaction, the fluorescent dye and quencher are released from one another and the dye fluoresce. The intensity of the fluorescent signal, which correlates with the amount of amplification, was measured. Thus, a highly expressed gene will result in a highly intense signal [90]. To adjust for potential sample variation such as different starting material or different efficiency of cDNA synthesis, reference genes were measured and related to the target gene, in order to obtain a normalized value. An ideal reference gene should be constitutively expressed in the studied tissue both under normal and experimental conditions. In this thesis, the obtained results from the fluorescent signals were used to calculate the relative expression, of the target gene in the experimental groups, to the control group. Hence, no absolute quantities of any genes were measured.

In paper 4, multiple genes were simultaneously measured with custom made TaqMan low-density array card (LDA), using RT-PCR. Primers and probes of 48 genes, including reference genes, had been spotted onto the LDA card by the manufacturer. For each sample, cDNA was loaded onto the LDA-card and would reach every 48 genes. Thus, the same mix of cDNA and enzymes were used for both the target genes and the reference genes, giving a very accurate method. As reference gene, Gusb was evaluated as the most appropriate by using the NormFinder algorithm [91].

Key results and discussion

Paper 1, 2 and 3

IL-6Rα is present throughout the hypothalamus

To investigate possible routes in the hypothalamus of which IL-6 could act to influence metabolism, the distribution of IL-6Rα was determined by immunohistochemistry.

In the mouse hypothalamus, we found that IL-6Rα was widely distributed throughout the hypothalamus, with variable staining intensities in discrete regions and nuclei. IL-6Rα staining was most intense in the PVN and in the ARC, and moderately intense in the supraoptic nucleus (SON). In the LHA, the distribution of IL-6Rα positive cells was scattered, which would result in a less intense appearance. However, since the LHA is a rather large region, the total number of IL-6Rα positive cells may be comparable, or even higher, with the number of cells in the PVN and in the ARC. These findings indicate that IL-6 could act in these hypothalamic regions, known to be involved in energy homeostasis [6], to influence metabolism (Figure 5, 6)

The extensive presence of IL-6Rα in the ARC, maybe not surprisingly, also include two distinct populations of cells that either contain the fat mass promoting peptides NPY and AgRP, or the fat mass suppressing neuropeptides POMC and CART [6], as IL-6Rα was found to be localized on subsets of cells of these two populations. Similarly, in the PVN, IL-6Rα was found to be co-localized with CRH, TRH, AVP and oxytocin. In the LHA, IL-6Rα was found on most MCH- and orexin containing cells which are predominantly expressed in the LHA [17]. MCH and orexin, both affect body fat mass, even though at opposing directions. MCH is believed to promote fat mass [20, 23, 92], while the net effect of orexin is to suppress fat mass [19, 93]. Noticeably, orexin promotes food consumption, as the name implies, which may strike as contradictory. However, orexin also promotes energy expenditure [22, 94], and the effect on energy expenditure has been suggested to exceed the effect on food consumption, giving orexin a net fat mass suppressing effect.

Given the widespread distribution of IL-6Rα, as well as the broad presence of IL-6Rα in hypothalamic nuclei, and individual neuronal populations involved in energy homeostasis, suggest that IL-6 may act in parallel in multiple sites in the brain to regulate energy balance (Figure 5, 6). Indeed, many of the hypothalamic nuclei

involved in energy homeostasis are heavily interconnected. In particular, neurons in the ARC project to both the LHA and the PVN [95, 96], and neurons in the LHA project to the ARC (Figure 5) [97-99].

To our knowledge, no mapping of IL-6Rα in hypothalamus of healthy mice has previously been performed. However, the expression of IL-6Rα mRNA, in two separate studies, has been investigated in rat brain using in situ hybridization technique [100, 101]. These studies show variable results and detect limited expression of IL-6Rα in the hypothalamus. Schöbits et al only mention VMN and dorsomedial hypothalamic nucleus (DMN) as being IL-6Rα positive in the hypothalamus, while Vallieres et al in general did not find IL-6Rα expression in the hypothalamus, with the exception of the median eminence and in the PVN after endotoxin treatment. The reason for the discrepancy between these two studies, and our present results may be due to low expression of IL-6Rα in the hypothalamus during normal physiological conditions, as judged from own observation, making it difficult to detect. In addition, an effective translation may explain low mRNA levels, while at the same time as clearly detectable protein levels [102]. One may also consider the species difference.

IL-6Rα was mostly, if not solely, found in neuronal cells. Co-localization experiments with astrocyte (glial fibrillary acidic protein; GFAP) and microblia (ionized calcium-binding adapter molecule 1; Iba-1) markers showed that these cells, when located in the ARC, PVN and LHA, did not have IL-6Rα. This is in line with previous reports, showing that human astrocytes in vitro lack IL-6Rα mRNA [103]. In addition, it has been suggested that IL-6 only can trigger astrocytes in the presence of soluble IL-6 receptor [104]. However, IL-6Rα mRNA has been found on human microglia in vitro [105].

It should be emphasized that our results demonstrate the circumstances of healthy mice, under normal physiological condition, and that the situation may be different during a pathophysiological state.

In the human hypothalamus, IL-6Rα was found to be present predominantly in the LHA, with the most intense staining in the posterior parts, while it was absent from PVN and the infundibular nucleus (equivalent to the ARC in the mouse hypothalamus). As in the mouse hypothalamus, IL-6Rα was co-localized with the fat mass promoting neuropeptide MCH. Unlike in mice, the IL-6Rα- and MCH-stainings showed a complete overlap in humans. In contrast to in mice, IL-6Rα was not found on orexin neurons in the human hypothalamus. This clear cut finding, suggests that there is a biologically significant interaction between IL-6 and MCH in the human hypothalamus. However, it is unknown which of the IL-6 effect(s) in the CNS that is exerted by the modulation of MCH neurons. One possibility is that the anti-obesity effect of IL-6 could be mediated, at least in part, by MCH, as both IL-6 and MCH have been shown to influence body fat mass [20, 23, 56, 57, 106, 107].

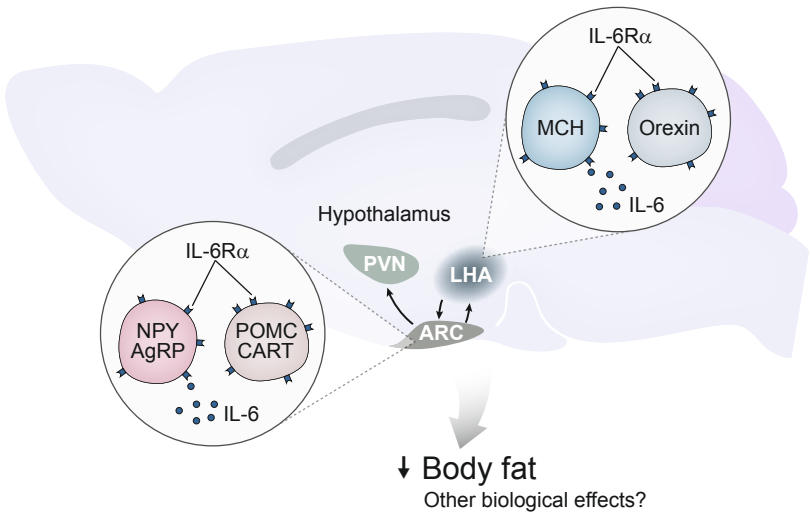


Figure 5. Interleukin-6 receptor α (IL-6Rα) is localized on neurons and hypothalamic sites known to influence energy balance.
We found IL-6Rα to be localized on NPY/AgRP neurons and POMC/CART neurons in the arcuate nucleus (ARC) (Paper 1), as well as on MCH neurons and Orexin neurons in the lateral hypothalamic area (LHA) (Paper 3). Neurons in the ARC project to both the paraventrivular nucleus (PVN) and the LHA, and neurons in the LHA project to the ARC. We hypothesize that IL-6 could act on these neurons and hypothalamic sites, not only to influence energy homeostasis to decrease body fat mass, but also other centrally regulated biological effects.

AgRP, agouti-related peptide; CART, cocaine and amphetamine regulated transcript; MCH, melanin-concentrating hormone; POMC, pro-opiomelanocortin.

IL-6 influence PVN neuropeptides

To investigate potential mechanisms by which IL-6 could influence energy homeostasis through the CNS, we investigated the effect of endogenous IL-6 on numerous key hypothalamic neuropeptides involved in energy balance regulation. We measured the mRNA levels of these neuropeptides with RT-PCR in IL-6 knockout mice.

The hypothalamic expression of CRH, oxytocin and AVP was clearly decreased in older IL-6 knockout mice. This is in line with the result of previous experiments that IL-6 stimulates the release of CRH, oxytocin and AVP from hypothalamic explants in vitro [108]. Taken together with our finding that IL-6Rα is located on CRH, oxytocin and AVP neurons in the PVN, this indicates that endogenous IL-6 directly stimulates the production of these neuropeptides.

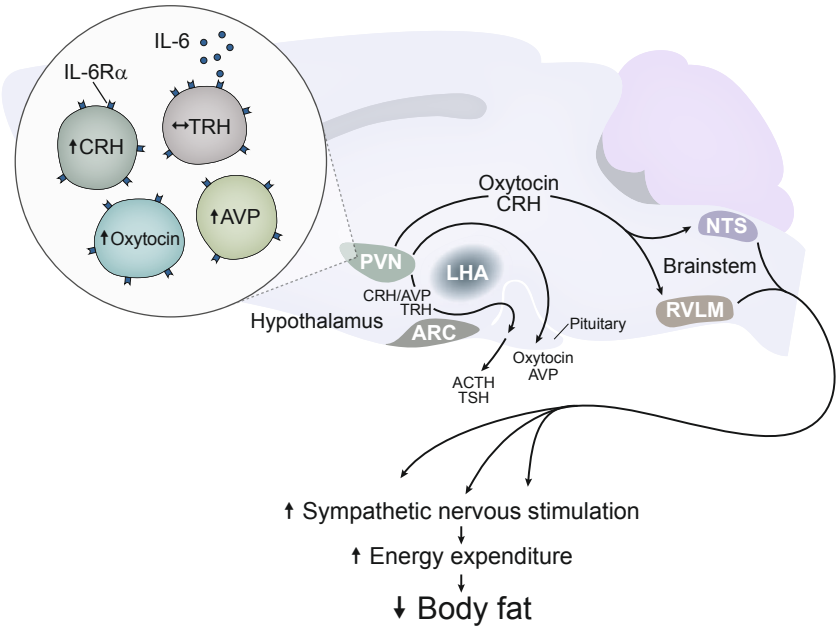


Figure 6. Interleukin-6 (IL-6) may act through CRH, Oxyocin and AVP neurons in the paraventricular nucleus (PVN) to exert fat suppressing effects.
Based on our findings that the expression of the anti-obesity neuropeptides CRH, Oxytocin and AVP in the hypothalamus was decreased in IL-6 knockout mice, and that IL-6Rα are located on neurons in the PVN that produce these neuropeptides, we speculate that IL-6 may directly act on these neurons in the PVN to increase the expression of CRH, oxytocin and AVP to exert its anti-obesity effects (Paper 2). Furthermore, we speculate that the route by which IL-6 exert its anti-obesity effects involves CRH and oxytocin neurons that project from the PVN to the nucleus of the solitary tract (NTS) and the rostral ventrolateral medulla (RVLM) in the brainstem. In the brainstem CRH and oxytocin may enhance the sympathetic outflow through these nuclei, which will increase energy expenditure and eventually decrease body fat mass. IL-6Rα was also found on TRH neurons, but the expression of TRH was not changed in IL-6 knockout mice.

CRH/AVP neurons, along with TRH neurons project from the PVN to the median eminence and the release of ACTH and TSH from the anterior pituitary is triggered. In addition, oxytocin and AVP neurons in the PVN project to the posterior pituitary, to release oxytocin and AVP into the circulation. We do not believe, however, that these routes through the pituitary are of importance in exerting the anti-obesity effects of IL-6. However, it cannot be excluded that dendritic release of oxytocin in the PVN and SON, from neurons projecting to the pituitary, is of importance in suppressing fat mass.

CRH, corticotropin-releasing hormone; AVP, arginine vasopressin; ACTH, adrenocorticotropic hormone; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone.

Although IL-6Rα was found in the ARC and LHA, and was co-localized with several important neuropeptides known to influence energy homeostasis, namely NPY, AgRP, POMC, MCH, orexin and BDNF, the expression of these were not affected in IL-6 knockout mice. This suggests that the PVN is more important than other nuclei in the hypothalamus, in mediating central metabolic effects of IL-6. The PVN is believed to influence energy homeostasis, as attenuation of PVN function result in hyperphagia and obesity [109-111]. In addition, several of the PVN neuropeptides has been reported to be involved in regulating energy balance.

The role of CRH in metabolism is well studied, mostly as being the initial step in the HPA-axis and subsequent stress response [112]. In addition, CRH has also been found to directly influence food intake and to increase energy expenditure, via activation of the sympathetic nervous system (SNS) [113]. There are indications that changes in sympathetic outflow, eventually could lead to alteration in total body fat mass [114]. Hence, we speculate that endogenous IL-6 may decrease obesity partly by stimulating CRH production and that subsequent activation of the SNS leads to increased energy expenditure (Figure 6). The fact that IL-6 has been found to increase energy expenditure [36, 57, 115], and also been suggested to activate SNS, support this hypothesis [116]. It should be mentioned that IL-6 also been found to stimulate the HPA-axis [44], which could be an additional pathway by which IL-6 can influence metabolism. Even though this has been proposed to be of biological importance during inflammation [117], it is most likely not of importance during normal physiological conditions, as the serum corticosterone levels were not effected in IL-6 knockout mice [36, 116].

Oxytocin is primarily produced by neurons in the PVN which mainly project to the posterior pituitary, where oxytocin is released from nerve terminals into the circulation [118]. In addition to its well established role in reproduction and well being, growing evidence indicate that oxytocin is an important regulator of energy balance effecting both food intake and energy expenditure [28, 110, 119]. This effect may be exerted within the CNS, as central administration of oxytocin is capable of reducing weight loss [120]. This is supported by the fact that oxytocin also could be released within the brain as a neuropetide [121]. Indeed, oxytocin has been shown to be released from dendrites of neuroendocrine neurons projecting to the posterior pituitary, and it cannot be excluded that this dendritic oxytocin exert a local anti-obesity effect in the PVN and SON [122]. Based on these, and our own findings, we speculate that IL-6, through IL-6Rα on oxytocin neurons, may influence oxytocin, and that oxytocin, in part, could be involved in mediating the anti-obesity effects of IL-6 (Figure 6).

There is little evidence that AVP is directly involved in regulating fat mass, but there are indications that AVP potentiates CRH actions [123]. Hence, it is less likely that AVP is of importance to mediate the anti-obesity effects of IL-6.

The route by which CRH and oxytocin may mediate the anti-obesity effect of IL-6

is likely to involve the projections from the PVN to the brain stem. Axonal projections from the PVN, to the brainstem appear to mediate effects on energy homeostasis [25]. A subpopulation of both CRH and oxytocin neurons has been suggested to project to the nucleus of the solitary tract (NTS) and rostral ventrolateral medulla (RVLM) in the brainstem (Figure 6) [124, 125]. RVLM is involved in regulating sympathetic outflow, by sending excitatory catecholaminergic fibers to the spinal cord [126, 127]. The NTS receives input from the gastrointestinal tract, primarily via the vagus nerve. In addition, it has been suggested that neurons in the hypothalamus project to the NTS, and interact with neurons receiving satiety signals from the gastrointestinal tract [6]. Leptin has also been suggested to act on the NTS directly to amplify the gastrointestinal signaling [128]. Thus, NTS is important in regulating energy homeostasis. Based on this we speculate that it is through these routes, via NTS and RVLM, that CRH and oxytocin mediate the anti-obesity effect of IL-6, mainly by enhancing the sympathetic outflow (Figure 6), although additional effects on food consumption cannot be excluded.

IL-6 and IL-1 interact in the hypothalamus

IL-6 and IL-1 has been suggested to interact, during illness, to elicit their actions in the central nervous system [129]. To investigate if IL-6 and IL-1 also interact during healthy conditions, we measured IL-6 and IL-1 mRNA levels in IL-6 knockout mice, as well as IL-1R1 knockout mice.

We have found that IL-6 mRNA levels were decreased in IL-1R1 knockout mice, indicating that downstream IL-1R1signaling have a stimulatory effect on IL-6 expression. In addition, IL-1β mRNA was increased in IL-6 knockout mice, indicating that endogenous IL-6 has possible inhibitory effects on IL-1. This may reflect a negative feedback mechanism, as has been proposed to be the case during pathophysiological conditions [117].

Based on these findings, we hypothesize that IL-6 and IL-1 may interact to regulate metabolism. Both IL-1R1 and IL-6 deficient mice independently develop obesity, but the obesity is more pronounced and of earlier onset in mice deficient in both IL-1 and IL-6 [130], which supports a connection between the IL-1/IL-6 interaction and energy homeostasis.

Paper 4

Gut microbiota influences BDNF and GLP-1

It is known that germ free mice have decreased fat mass, and are resistant to develop diet induced obesity. This indicates that gut microbiota influences energy homeostasis. It has been found that germ free mice have a reduced capacity to harvest energy from the food they consume, and this has been suggested to contribute to leanness [76]. However, our present results indicate, that other mechanisms may also contribute to leanness, caused by gut microbiota. We sought to investigate possible mechanism within the brain, by which the gut microbiota could influence fat mass. Interestingly, we found that the expression of pro-glucagon, precursor of the fat suppressing peptide GLP-1 was down regulated in the brain stem of mice with gut microbiota. Thus, low GLP-1 levels in the presence of gut microbiota may contribute to elevated fat mass (Figure 7). Central pro-glucagon is exclusively found in the brain stem nuclei NTS, which is known to be an important integrator of central and gut signals, and to receive input from the gut via vagal afferents[131, 132]. It remains to be investigated if enables the gut microbiota could inhibit GLP-1 producing neurons via the vagus nerve.

We found that the fat suppressing neuropeptide BDNF was reduced in both the hypothalamus and the brainstem by the gut microbiota. As for GLP-1, low BDNF levels may contribute to the elevated fat mass caused by gut microbiota (Figure 7). BDNF has been reported to be induced by leptin, as well as the activation of melanocortin receptor 4 (Mc4R), and has been suggested to mediate the downstream signal of the leptin/POMC/Mc4R route in the hypothalamus and the brainstem, to suppress fat mass [133-137]. Therefore, it may seem surprising that mice with gut microbiota, which have both elevated leptin levels and up regulated POMC expression, have decreased BDNF (Figure 7). On the other hand, the gut microbiota may influence BDNF expression through another, yet unknown mechanism, separated from leptin and POMC, and the inhibitory effect of this mechanism may exceed the stimulatory effect of leptin. In line with this assumption, BDNF was not affected by leptin treatment in our own study (Paper 4).

Gut microbiota and leptin resistance

We found implications that gut microbiota may contribute to leptin resistance, which may be a result of the elevated circulating leptin levels found in mice with gut microbiota. Leptin treatment caused only a minor weight reduction in mice with gut microbiota, while the same dose dramatically decreased the body weight of germ free mice. This indicates that the leptin responsiveness was reduced in the presence of gut microbiota. This is supported by the fact that, the low levels of NPY and AgRP mRNA found in the presence of gut microbiota, was not further reduced by

leptin, as was the case in germ free mice. Thus, we speculate that the gut microbiota contributes to reduce leptin sensitivity with respect o body fat mass, possibly as a consequence of elevated fat mass and subsequent elevated circulating leptin levels. Gut microbiota does not however seem to induce total leptin resistance, as we found NPY and AgRP expression to be decreased and POMC and CART expression to be increased in the presence of gut microbiota, reflecting the elevated leptin levels. Thus, this indicates that leptin still is able to regulate the expression of NPY/AgRP and POMC/CART in the hypothalamus. However, this regulation is not sufficient to decrease the gut microbiota induced adiposity (Figure 7).

It is possible that leptin resistance is the primary factor, and that increased fat mass and serum leptin levels are altogether secondary to leptin resistance. Of note, is the fact that both BDNF and GLP-1 expression were decreased in mice with gut microbiota, although the synthesis of both these neuropeptides seem to be induced by leptin [131, 135].

In mice with gut microbiota, the expression of SOCS-3 was elevated. SOCS-3, together with protein-tyrosine phosphatase 1B (PTP-1B) and ER-stress, has been proposed to be an important factor in the development of leptin resistance, and has been found to restrain leptin intracellular signaling, by reducing the phosphorylation of STAT-3 (Figure 8)[138]. Thus, we speculate that SOCS-3 may mediate the reduced leptin sensitivity caused by gut microbiota, possibly even specifically, as we did not find PTP-1B or ER-stress to be regulated by gut microbiota (Figure 8).

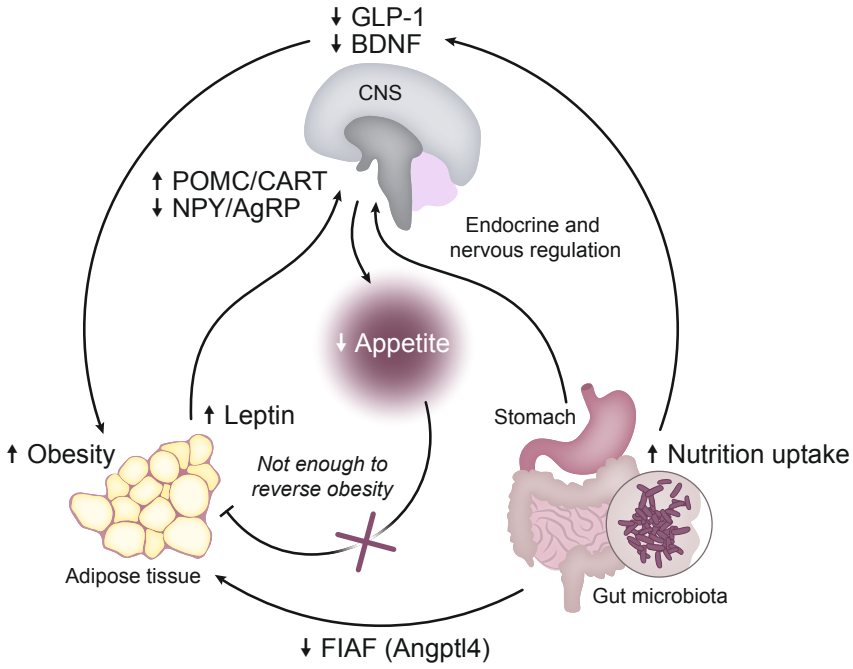


Figure 7. Gut microbiota may increase fat mass through neuropeptides in the hypothamaus and brain stem.
Based on our results from germ free mice, we speculate that gut microbiota, through yet unknown components, decreases the expression of GLP-1 in the brainstem and BDNF in both the hypothalamus and brainstem. This may increase fat mass, which in turn will result in increased leptin levels, and subsequent increase in hypothalamic POMC and CART expression, as well as decreased NPY and AgRP expression. Elevated leptin and POMC/CART along with reduced NPY/AgRP may explain the decreased appetite found in mice with gut microbiota. However, the decrease in appetite is not enough to decrease obesity. We speculate that gut microbiota may reduce leptin sensitivity, making obesity induced changes in leptin and downstream neuropeptides insufficient to reverse the increase in fat mass.

The fasting induced adipose factor (FIAF) has been found to be downregulated by gut microbiota, which may also contribute to elevated fat mass.

AgRP, agouti-related peptide; Angptl4, angiopoietin-related protein 4; BDNF, brain-derived neurotrophic factor; CART, cocaine and amphetamine regulated transcript; FIAF, fasting induced adipose factor; GLP-1, glucagon-like peptide-1; MCH, melanin-concentrating hormone; POMC, pro-opiomelanocortin.

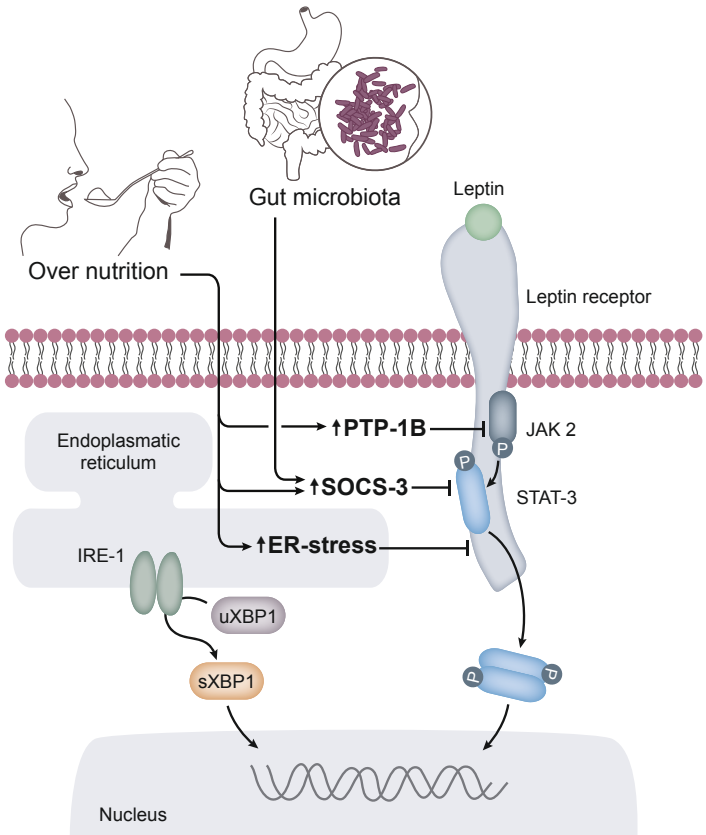


Figure 8. Gut microbiota may reduce leptin sensitivity through elevated SOCS-3. Leptin signaling involves the binding of leptin to the leptin receptor, which triggers JAK-2 to induce phosphorylation of STAT-3. Phosphorylated STAT-3 then dimerize, becomes activated, and enters the cell nucleus. In the nucleus STAT-3 binds to DNA and acts as a transcription factor, and the leptin signal is brought forward.

As the hypothalamic expression of SOCS-3 was elevated in mice with gut microbiota, we speculate that SOCS-3 mediates the reduced leptin sensitivity caused by gut microbiota. SOCS-3 is known to restrain intracellular leptin signaling by inhibiting the phosphorylation of STAT-3. Other factors implicated in leptin resistance are PTP1B and endoplasmatic reticulum (ER) stress. ER-stress, PTP-1B and SOCS-3 have been suggested to increase by over nutrition (Mori 2004, Elchebly 1999, Wisse 2009). However, neither PTP1B nor ER-stress was affected by gut microbiota (Paper 4).

ER-stress induces the formation of spliced (s) XBP-1 from unspliced (u) XBP-1 through IRE-1. The presence of ER-stress can therefore be measure by measuring the presence of sXBP-1.

IRE-1, inositol-requiring protein 1; JAK-2, janus kinas 2; PTP-1B, protein-tyrosine phosphatase 1B; SOCS-3, suppressor of cytokine signaling 3; STAT-3, signal transducer and activator of transcription 3; XBP-1, x-box binding protein 1.

General discussion

IL-6 in obesity

Where are we standing today in our knowledge of IL-6 in relation to obesity? Well, more than a decade after the first publication that IL-6 knockout mice become obese and after numerous articles reporting metabolic effects of IL-6, it is safe to say that IL-6, indeed is involved in regulation of metabolism. To what extent IL-6 is involved, if IL-6 is mostly beneficial or deleterious, and the underling mechanisms are, however, less clear.

Strong evidence for possible favorable effects of IL-6 in regard to metabolism is the fact that IL-6 is released from muscles, in high quantities, in response to exercise (Figure 3) [107]. As exercise by itself has beneficial effects on obesity and obesity related conditions such as insulin resistance, it has been suggested by Pedersen and co-workers, that IL-6 may contribute to the beneficial effects of exercise [139]. Indeed, the only published study on the effect of IL-6 knockout on exercise, support this notion [58]. A recent study by Marc Donath indicates that exercise induced IL-6 release from skeletal muscle, in turn enhances the release of peripheral GLP-1 from L-cells in the gut and α-cells in the pancreas. The GLP-1 can then mediate beneficial effects on glucose metabolism [140].

Contradictory, however, is the general belief that obesity is associated with a chronic low-grade inflammation, including elevated IL-6 levels, which has been suggested to promote insulin resistance [141, 142]. This controversy is a subject of debate, but it has been pointed out that from a physiological point of view it seems rather paradoxical that a working muscle would release a factor that inhibits insulin signaling [59]. Indeed, IL-6 knockout mice display insulin resistance in addition to increased obesity, reported by two separate studies, which provide strong evidence that IL-6 could act as an anti-obesity factor and has beneficial effects on insulin resistance [36, 143]. Moreover, IL-6 seems to prevent development of liver steatosis [143]. One study could however not detect these properties of IL-6 [144]. The reason for this discrepancy in not known, but based on our acquired knowledge from working with IL-6 knockout mice, and the ubiquitous role of IL-6 in immune-function , one may speculate that the metabolic phenotype of IL-6 knockout mice is susceptible to outer circumstances such as infections or microbial gut flora. In human genetic studies, variability in the IL-6 gene is associated with adiposity, which further supports a role of IL-6 in obesity [55, 56].

As IL-6 is produced and released from adipose tissue, and serum levels of IL-6 correlates with adipose tissue mass and adipocyte volume [64, 65] it is tempting to compare IL-6 with leptin, and draw the conclusion that IL-6, as leptin, could act as an adipostatic factor communicating fat mass status to the brain. To what extent circulating IL-6 could enter the brain, or if it is locally produced within the brain, or maybe a combination is not known, but it is rather clear that centrally administered IL-6 could influence energy homeostasis by increasing energy expenditure and body temperature, eventually leading to decreased body fat. In contrast to the positive correlation between fat derived serum IL-6 and fat mass, IL-6 in cerebrospinal fluid (CSF), negatively correlates with fat mass. Moreover, many lean individuals have higher IL-6 levels in the CSF than in serum [145]. Taken together, these data indicate that IL-6 can be produced within the brain in healthy individuals. Clear is also the fact that endogenous IL-6, directly affects the expression of fat suppressing neuropeptides in PVN-neurons (Paper 2), and that IL-6 directly could act on several other hypothalamic neurons known to produce energy balance regulating peptides. Even though our results indicate that IL-6 acts on several hypothalamic nuclei, functional studies are needed, to investigate the actual involvement of these neuronal circuits in the anti-obese effects of IL-6.

Beside affecting central energy balance regulation, IL-6 may also in part contribute to decreased fat mass by inducing lipolysis and and fat oxidation [58, 59]. These effects of peripheral IL-6 are most likely not exerted via the brain.

IL-6 may have deleterious effect. Indeed, IL-6 has been proposed to be involved in the progression of several diseases and metabolic disturbances, which has been associated with elevated serum IL-6 levels [47]. Thus, released IL-6 may have diverse function under different circumstances. We speculate that low levels of endodgenous IL-6, seen during normal physiological conditions, may have favorable effects, while elevated IL-6 levels accompanied by other factors (e.g. TNF-α) during pathophysiological states can have deleterious effects. During exercise, for instance, such release of accompanied factors such as TNF-α is absent [107].

Many of the proposed actions of IL-6 are demonstrated in patients with Castleman's disease. A disease, which in most cases seem to be due to elevated IL-6 levels. Common symtoms of Castleman's disease are fever, loss of appetite, weight loss and low levels of red- and white blood cells, which all may be caused by the overproduction of IL-6. These symptoms may be reversed by IL-6Rα blockade, discussed below [146].

Much of the controversy regarding IL-6 still stands, and if IL-6 is good or bad is still a matter of debate. However, such categorization, which could be perceived as rather artificial, may not necessarily lead to understanding the physiological action of IL-6. The issue of beneficial or deleterious effects by IL-6 may simply be a matter of adjoining circumstances.

Future perspectives

IL-6 in the CNS outside the hypothalamus

In addition to the hypothalamus, the brain stem is an important area of the brain involved in energy homeostasis. The brainstem receives input from the gut through vagal afferents which communicate the nutritional status of the gut [132]. Therefore, it would be of interest to investigate if IL-6 directly could act on the brain stem to influence energy balance. So far, we have identified IL-6Rα to be present in the brainstem, including the NTS and area postrema, and it has been reported that IL-6 microinjected to the NTS can influence cardiovascular control [147]. In addition, mRNA levels of IL-6Rα and gp 130 has been detected in the NTS [147]. Thus, it seems likely that IL-6 could act on the NTS, and it might be speculated that IL-6 can inhibit energy expenditure via this route. Interestingly, systemic IL-1β has been found to stimulate CRH neurons in the PVN through noradrenergic neurons in the NTS that project to the PVN. The noradrenergic release in the PVN is blocked by endogenous opiods, which can be induced by allopregnanolone. Thus, allopregnanolone may be responsible for inhibition of the HPA-axis by opiods in late pregnancy [148]. It would be interesting to investigate if IL-6, alone or in conjunction with IL-1 could act on noradrenergic neurons in the NTS, and increase CRH expression through this pathway.

Central GLP-1 is produced from pro-glucagon which, within the CNS, is produced by neurons in the NTS [131]. Central GLP-1 is a potent inhibitor of feeding that reduces body weight [149]. Peripherally derived GLP-1 is produced by α-cells in the pancreas, and intestinal L-cells, and is not believed to act on the brain due to its very short half-life [140, 150]. Both endogenous and administered IL-6 has been found to stimulate secretion of GLP-1 from intestinal L-cells and pancreatic alpha-cells [140]. Hence, two out of three cell types known to produce GLP-1, have to date been shown to be stimulated by IL-6. Therefore, it would be of interest to investigate if also GLP-1 producing neurons in the NTS, the third cell type, are stimulated by IL-6.

One important aspect of energy balance is the motivational force of consuming food. Lately, the view of seeing obese individuals as being addicted to food has got more in focus, and the role of the reward systems of the brain in relation to obesity has been more extensively investigated [151]. One important node, which integrates homeostatic, satiety and reward-related inputs, is the lateral hypothalamic area [6]. In humans, we found IL-6Rα to be exclusively located in MCH neurons, which predominantly are restricted to the LHA. This opens up the possibility that IL-6 is involved in the interactions between reward and maintenance of energy balance. Whether IL-6 directly could influence brain nuclei involved in reward, such as ventral tegmental area (VTA) and nucleus accumbance, through the IL-6Rα could become a matter of future investigation. To date, there is little information about the

abundance of IL-6Rα in these reward-related nuclei. However, there are indications that central IL-6 could affect dopamine release in the nucleus accumbance [152].

IL-6 has been reported to influence memory and learning, as well as depression-like behavior, characteristics known to involve the hippocampus. Indeed, stress increases IL-6 expression in the hippocampus [153]. In the regulation of energy homeostasis, the role of the hypothalamus and brain stem is to, a large part, to detect satiety, adiposity and hunger signals. The processing and translation of these signals, to ultimately give rise to a decision to eat or not, may depend on other "higher" brain sites involved in the control of behavior. It has been suggested that the hippocampus can contribute to this "higher" control of food-intake [154]. Thus, it would be interesting to investigate possible relationship with IL-6 and hippocampal driven energy balance related behavior.

Possible pharmaceutical targets

Although endogenous IL-6 has been found to have beneficial effects on metabolism, including body weight and glucose metabolism, treating obese and diabetic individuals with low dose of IL-6 may still not be advantegous. IL-6 is a potent stimulator of the immune system, and adverse side-effects caused by a too aggressive immune response are very likely. However, the idea may not be ruled out completely. It is possible that tailoired IL-6 treatment, directed at specific sites in the CNS, may bypass some of the unfavorable immune-related effects of IL-6. Indeed, in the hypothalamus we found the IL-6Rα to be localized solely on neurons and not on astrocytes or microglia, and many of these neurons produce peptides known to regulate energy homeostasis. As the location of IL-6Rα was much more specific in humans than in mice (Paper 3), it might be possible to get more specific biological effects with less side effects of IL-6 treatment in humans. In addition, it has been suggested that the pro-inflammatory and anti-inflammatory effects of IL-6 are related to trans-signaling or classic-signaling respectively. The metabolic effects of IL-6 may then mostly be related to the classic-signaling, involving the membrane bound IL-6Rα [39]. Even though our results indicate that IL-6 may signal through classic-signaling on MCH containing neurons in the human CNS, one cannot exclude that human IL-6 exert trans-signaling in other cells of the human CNS.

Interestingly, IL-6 is closely related to the neuropeptide CNTF, known to pharmacologically suppress fat mass. CNTF has been subjected to intense investigation as the anti-obesity drug Axokine® tested in phase 3 studies in humans, is a modified version of CNTF [155] CNTF and IL-6 not only share the common gp130 receptor subunit, but CNTF may also, in combination with LIF receptor, utilize the IL-6Rα in addition to the CNTF receptor subunit to elicit downstream signaling. Thus, indicating that CNTF and IL-6 may share intracellular pathways, and through those, elicit similar biological effects. Unfortunately, in this context, CNTF has broad neu-

rotrophic properties which could possibly lead to unwanted side effects, and CNTF has also has been reported to activate inflammatory genes in the brain [156, 157], which may limit the use of CNTF as a therapeutic drug [155]. The biological significance of the wide spread distribution of IL-6Rα in the murine hypothalamus is not known, and many functions of IL-6 in the hypothalamus remain to be investigated, but it is clear that IL-6 increase energy expenditure when administered centrally [57]. IL-6 may share the beneficial effects of CNTF on energy homeostasis in part through the same hypothalamic routes, as CNTF has been found to influence both NPY and POMC neurons in the arcuate nucleus [155], two neuronal populations we found to have IL-6Rα. However, unlike what is reported for CNTF, our group has not found any substantial and sustained anti-obesity effect by peripheral treatment with IL-6 in high doses (Wernstedt et al, unpublished results).

Tocilizumab

Many diseases have been coupled to IL-6 and elevated IL-6 levels, not at least many autoimmune related conditions. In many case the blockade of IL-6 signaling has been successful in treating such conditions. Initially, IL-6 itself was subjected to blockade by neutralizing antibodies, but it was soon discovered that treatment with neutralizing IL-6 antibodies led to dramatic systemic increase in IL-6 levels, due to impaired clearance of antibody-associated IL-6. Thus, investigators sought to block the IL-6Rα instead, and developed tocilizumab, a humanized IL-6R-specific monoclonal antibody, which prevents IL-6 from binding the IL-6Rα. Tocilizumab has been reported to be effective in reducing disease activity in patients with rheumatoid arthritis and Castleman disease [47].

Judged from what we know about IL-6 in relation to metabolism, it would not be surprising if tocilizumab affect metabolism. Indeed, tocilizumab has

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