IL-6 and GLP-1 in body fat regulating parts of the CNS in healthy mice

Fredrik Anesten

Department of Physiology/Endocrinology, Institute of Neuroscience and Physiology at Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, 2016

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ISBN 978-91-628-9724-6 (print)
ISBN 978-91-628-9725-3 (epub)
E-published at http://hdl.handle.net/2077/41238

Printed by INEKO AB, Gothenburg, Sweden

“I may not have gone where I intended to go, but I think I have ended up where I needed to be.”
― Douglas Adams, The Long Dark Tea-Time of the Soul

Populärvetenskaplig sammanfattning

Fetma är ett växande problem i västvärlden idag. Förmågan att lagra extra energi som fett, vilket under evolutionens gång har varit en överlevnadsfördel, har i vårt moderna samhälle istället blivit en belastning. Forskare har kommit en bit på vägen till att lösa fetmans gåta, men det återstår troligen en lång sträcka innan vi helt förstått oss på dess komplexitet.

Det har gjorts försök att hitta en så kallad ”fetma-gen” som leder till att vi utvecklar fetma. Upptäckten av genen för leptin, ett hormon som insöndras av fettväv och stimulerar mättnadskänsla, ledde forskare till att tro att fetmans gåta var löst. Tyvärr visade det sig att fetma medförde en minskad känslighet för leptin och därav visade det sig vara ineffektivt som botemedel. Hittils verkar den största delen av fetma orsakas av ett samspel mellan ett flertal olika gener och den födorika miljö vi i västvärlden lever i.

Den här avhandlingen fokuserar på ett hormon och en cytokin som tillsammans förhoppningsvis kan ge oss en liten bit av det pussel som fetma är. GLP-1 är ett hormon som huvudsakligen insöndras från tarm och bukspottkörtel efter måltid men som även tillverkas i hjärnstammen. Dess analog Ex4 används idag som läkemedel mot typ2-diabetes där en av bieffekterna hos vissa patienter är en marginell viktnedgång. Min avhandling studerar de mekanismer via vilka EX4 och GLP-1 verkar i hjärnan för att minska födointag.

GLP-1 i hjärnan verkar enligt våra studier ha ett nära samverka med cytokinen IL-6, vilken är mest känd för sin roll i inflammation och autoimmuna sjukdomar. Det har tidigare visats att möss som saknar IL-6, så kallade IL-6 knock-out möss, utvecklar fetma i vuxen ålder. Enligt våra data samspelar IL-6 och GLP-1 i hjärnan för att minska födointag och därmed minska kroppsvikten.

Tanycyter är en celltyp som bekläder botten av tredje ventrikeln i hjärnan. Dessa celler fungerar som en sorts dörrvakter och reglerar vad som passerar från blodet och vidare in till cerebrospinalvätskan. De har nyligen varit i fokus eftersom det har visats att de är viktiga vid transport av leptin. Här visar vi att de även uttrycker receptorn för IL-6, IL-6Ra, och möjligen skulle kunna spela en roll för dess effekter i hjärnan.

List of Papers

This thesis is based on the following papers:

Paper 1. **Glucagon-like peptide 1 receptor induced suppression of food intake, and body weight is mediated by central IL-1 and IL-6**
Rozita Shirazi, Vilborg Pálsdóttir, Jim Collander, Fredrik Anesten, Heike Vogel, Fanny Langlet, Alexander Jaschke, Annette Schürmann, Vincent Prévot, Ruijin Shao, John-Olov Jansson, and Karolina P. Skibicka
Proc Natl Acad Sci U S A. 2013 Oct 1;110(40):16199-204. doi: 10.1073/pnas.1306799110. Epub 2013 Sep 18

Paper 2. **Preproglucagon (PPG) neurons in the hindbrain have IL-6 Receptor α (IL-6Rα) and show Ca2+ influx in response to IL-6**
Fredrik Anesten, Marie K Holt, Erik Schéle, Vilborg Pálsdóttir, FrankReimann, Fiona M Gribble, Cecilia Safari, Karolina P. Skibicka, Stefan Trapp, John-Olov Jansson
Manuscript

Paper 3. **GLP-1 Receptor Stimulation of the Lateral Parabrachial Nucleus Reduces Food Intake: Neuroanatomical, Electrophysiological, and Behavioral Evidence**
Jennifer E. Richard,* Imre Farkas,* Fredrik Anesten,* Rozita H. Anderberg, Suzanne L. Dickson, Fiona M. Gribble, Frank Reimann, John-Olov Jansson, Zsolt Liposits, and Karolina P. Skibicka
* J.E.R., I.F., and F.A. contributed equally to this work.
Endocrinology. 2014 Nov;155(11):4356-67. doi: 10.1210/en.2014-1248. Epub 2014 Aug 13.

Paper 4. **Functional interleukin-6 receptor-α is localized on tanycytes at the base of the third ventricle**
Fredrik Anesten, Tina Bake, Erik Schéle, Vilborg Pálsdóttir, Teodor Swedung-Wettervik, Björn Meister, Karolina P. Skibicka, Julian Mercer, John-Olov Jansson
Manuscript

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Abbreviations

AgRP – Agouti-Related Peptide

AP – Area Postrema

ARN – Arcuate Nucleus

CCK – Cholecystokinin

CGRP – Calcitonin gene-related peptide

CRF – Corticotrophine releasing factor

CSF – Cerebrospinal Fluid

DARPP-32 – Dopamine- and cAMP-regulated phosphoprotein

Dpp-4 – Dipeptidyl Peptidase-4

Ex4 – Exendin-4

Fgf-10 – Fibroblast growth factor-10

FTO – Fat mass and obesity-associated protein

GLP-1 – Glucagon-like peptide-1

IL-1 – Interleukin-1

IL-6 – Interleukin-6

IL-6Rα – Interleukin-6 receptor alpha

LepR – Leptin receptor

LHA – Lateral hypothalamic area

LiCl – Lithium chloride

lPBN – Lateral parabrachial nucleus

MC4R – Melanocortin 4 receptor

MCH – Melanin concentrating hormone

mPBN – Medial parabrachial nucleus

NO – Nitric oxide

NPY – Neuropeptide-Y

NTS – Nucleus of the solitary tract

PFA - Paraformaldehyde

POMC – Pro-opiomelanocortin

PPG –Pre-proglucagon

PVN – Paraventricular nucleus

RT-PCR – Real-time polymerase chain reaction

Sim-1 – Single minded homolog 1

SNS – Sympathetic nervous system

SO – Supraoptic nucleus

TRH – Tyrotropin releasing hormone

TSH – Thyroid-stimulating hormone

YFP – Yellow fluorescent protein

Background

Obesity

Obesity, classed as a state with abnormal or excess fat accumulation that may lead to an impairment of health, is a growing problem worldwide, although the details have been debated. Two main theories have been proposed: One is that the recent plateau in prevalence of worldwide obesity is temporary and it will continue to increase. Alternatively, we have now reached the peak of obesity [1, 2]. Obesity is commonly seen as a by-product of our sedentary western lifestyle, but the likelihood of developing obesity also features a substantial genetic aspect.

A substantial amount of research has been put into finding "obesity genes". Although quite a few good candidates have emerged, these account for a very small part of the obesity epidemic. Genes coding for the fat-derived satiety peptide leptin and its receptor have been identified as the cause for morbid obesity in some patients, but they account for a very small part of those suffering from obesity. Likewise, mutations in the MC4R gene have been found to cause obesity, but these mutations are also rare.

Leptin itself deserves to be further expanded upon. After its discovery, leptin was thought to be the key to solving the problem of obesity [3-5]. Mice lacking the gene coding for leptin, so called ob/ob mice, developed a morbid obesity that was reversible by administration of leptin. However, it was also seen that normal mice (mostly the C57 BL strain) on high fat diet that developed obesity were less prone to lose weight after leptin administration.

Unfortunately, studies on human patients did not show any beneficial effects to leptin treatment either, and it has been stated

that obese people are resistant to leptin. As a hormone produced by fat tissue, leptin is secreted in levels that correlate to the fat mass of the individual. Therefore, obese people have naturally higher leptin levels in serum than lean people. Due to leptin resistance, the higher levels of leptin do not give the expected weight loss. This has caused a shift in research aim to finding the underlying mechanisms of leptin resistance [6].

As a contrast to the monogenetic causes for obesity described above, a sizeable part of obesity is believed to have polygenetic origins. This can be explained as an evolutionary advantage. In times when food availability was plentiful, it was an advantage to be able to store more fat for the inevitable times of low food availability. Thus, genes that allowed its bearer to gain weight gave an advantage in survival over individuals that lacked such genes. However, in a western society where food is plentiful and readily available, these genes instead act to their bearers' disadvantage in that they are more prone to develop obesity.

Researchers have found that some of the obesitity-associated genes, such as FTO, on their own are not associated with more than a modest weight difference in the range of a few kilograms. However, an individual with several of these obesity-promoting genes will most likely have a propensity for developing obesity in a food-rich environment.

Obesity-regulating parts of the brain (hunger/satiety centers)

Hypothalamus

Located below the thalamus and close to the brainstem, the hypothalamus is important for regulation of basal body functions. Made up of several different nuclei (groups of neuronal cell bodies), one of the most important functions of the hypothalamus is metabolic regulation.

One part of the hypothalamus thought to be involved in regulation of body fat mass is the bilateral ARN. The ARN hold distinct adjacent populations of NPY/AgRP positive neurons and POMC positive neurons, key players in the fat regulating central melanocortin system [7-9].

Both NPY/AgRP and POMC positive neurons project to the PVN and the LHA where NPY and AgRP exert modulatory fat-promoting effects.

In the PVN of the hypothalamus, CRF has been shown to have metabolic effects, as central administration of CRF inhibits food intake and stimulates energy expenditure, possibly via activation of the SNS [10]. Another PVN derived neuropeptide, TRH, may regulate energy homeostasis not only through effects on thyroid function but also through other central effects, e.g to enhance thermogenesis and decrease feeding [11, 12]. It has also been suggested to be a downstream modulator of leptin signaling in the brain [13].

Oxcytocin, a peptide produced by the magnocellular neurons of the PVN and SO may also inhibit feeding and obesity when administered centrally [14, 15]. Peripheral oxytocin may also act to suppress feeding via the vagus nerve [16]. The result of surgical

lesions as well as depletion of the crucial PVN master gene Sim-1 indicates that an important function of the PVN as a whole is to suppress obesity. IL-6 appears to stimulate the expression of the anti-obesity peptides CRF and oxytocin via direct actions on the IL-6ra in these neurons [17].

Within the hypothalamus, the LHA is considered an obesity-promoting center. Tumors in the LHA may cause anorexia in humans and in experimental rat models lesions of the LHA cause anorexia while electrical stimulation induces food intake. The neuropeptides MCH and orexin are highly abundant and predominantly restricted to the LHA in rodents, raising the possibility that these neuropeptides play a role in the body fat regulating properties of the LHA.

Studies indicate that MCH promotes obesity, at least in rodents [18, 19]. This is in line with the pro-obesity effects of the LHA as a whole. Conversely, orexin suppresses body fat by increasing energy expenditure more than food intake as judged from studies in knockout mice. We have obtained evidence that IL-6ra is present on neurons containing orexin and MCH in rodents and on neurons containing MCH in humans [20].

Tanycytes

Tanycytes are specialized glial cells located mainly at the bottom of the third ventricle wall. They have processes that reach deep into the parenchyma of the hypothalamus, reaching into the ARN and the median eminence. These cells have been proposed to act as gatekeepers, regulating which substances that passes into the brain parenchyma and which that do not [21-23].

In line with their function as gatekeepers of the median eminence, tanycytes have also been indicated to form a link between the CSF

and the hypophyseal porta system. Hormones secreted into the CSF are thought to be then transported via tanycytes to the portal vasculature of the pituitary gland [24]. This may indicate that tanycytes have some control over the pituitary gland.

Tanycytes might also have a hemodynamic function as indicated by the finding that they respond to NO, a highly active free radical and potent vasodilator. The finding that tanycytes respond to NO might hint at a potential function in altering cerebral blood flow [25].

It has recently been shown that tanycytes regulate the transport of leptin into the brain [21, 26]. The LepR shares similarities with class-1 cytokine receptors, a family of receptors that include IL-6rα, utilizing the same JAK/STAT signaling pathway [27]. It might be speculated that IL-6 is transported by tanycytes into the brain parenchyma in a similar fashion as leptin. On the other hand, the fact that IL-6, unlike leptin, has a much more potent anti-obesity effect when given ICV compared to systemically may argue against this assumption [28-30].

Another potential function for tanycytes is in neuroplasticity where they have been indicated to form a diet-responsive neurogenic niche[31]. It has been found that tanycytes have the potential to generate new neurons and that blocking this neurogenesis may lead to alterations in metabolic activity in mice. A subpopulation of the hypothalamic tanycytes has been found to express FGF-10, a neuronal growth factor. It has been shown that these neurogenic tanycytes have the potential to generate both neurons and astrocytes in vivo[32].

Traditionally, anti-vimentin antibodies have been used in immunohistochemistry to stain tanycytes. Vimentin, a cytoskeletal protein, is expressed in many cells that are not tanycytes.

Therefore, we have also used anti-DARPP-32 antibodies in paper 4. It has been shown that this antibody is confined to tanycytes in the brain and as such gives a more specific staining [33-36].

Hindbrain

Nucleus of the Solitary Tract

The NTS is located in the caudal brainstem close to the central canal. It is the more prominent of two known sites in the brain where GLP-1 neurons are found, the other being the intermediate reticular nucleus also located in the caudal brainstem.

The NTS receives sensory afferent input from the lungs, the blood vessels, the heart and the gut. It has a significant role in integrating GI-derived signals that are mechanically or physiochemically mediated by the vagus nerve and other cranial nerves [37].

Glucose and intestinal lipids are among the vagally mediated, meal-related nutritional signals that act in the brainstem. However, intestinally produced factors that mediate meal-sensing act via vagus as well [38]. One of these factors is CCK, which plays a major role in meal termination when bound to its appurtenant receptor in the vagus nerve. Receptors for leptin, and the stomach-derived hunger hormone ghrelin, are also expressed on the vagus nerve [39].

Signals reaching the NTS from different parts of the body are then transferred to further sites in the CNS. NTS, together with AP, mediate afferent information to hypothalamic nuclei such as PVN, VMH, DMH and LHA.

Parabrachial Nucleus

Projections from the NTS reach nuclei of importance for energy balance outside the hypothalamus such as the lPBN. Some of these projections appear to be GLP-1 positive as seen in fig X and they may even reach cells that stain positively for IL-6Ra.

The parabrachial nucleus is situated in the pons of the hindbrain on the border to the midbrain, laterally of the fourth ventricle and close to the superior cerebellar peduncle. It consists broadly of two different subnuclei; the mPBN and the lPBN of which the lPBN is of most interest to this thesis. The mPBN receives gustatory signals via the NTS, relaying these to the ventral posteromedial nucleus of the thalamus.

The chief known function of the lPBN is anorexigenic and it receives input from the NTS. Projections from the lPBN reach important centers for energy balance such as the medial and lateral hypothalamic nuclei and also the amygdala. Neurons containing NPY/AgRP in mice exert a tonic inhibition of the lPBN to allow the animals to eat [40]. It was shown by Palmiter that diphteria toxin induced depletion of PPY/AgRP caused severe anorexia [41]. It was also shown by Palmiter et al that this effect depends on GABA as depletion of NPY and/or AgRP is insufficient to block the inhibition of food intake [42, 43]. In contrast, when the entirety of the neurons, including GABA, was depleted, the animals lost interest in eating altogether.

A key player in the lPBN is CGRP [44]. In humans, this peptide can act as a potent vasodilator and increased levels have been reported in patients suffering from migraine and hypertension. In mice, it is present in the lPBN and studies have shown that stimulation of the CGRP neurons leads to a reduction in food intake. Moreover, CGRP neurons seem to be necessary for the

anorexigenic effects of appetite-suppressing agents such as LiCl [45]. Interestingly, this effect seems to persist even in the presence of intact NPY/AgRP/GABA neurons. Therefore, stimulation of orexigenic NPY/AgRP/GABA neurons does not seem to be sufficient to inhibit the anorexigenic effect of LiCl and CGRP. CGRP projections from the lPBN reach the CeAc of the amygdala, and seem to be important for the anorectic effect of these neurons.

Interleukin-6

General

Interleukin-6 is a 26 kDa protein that is involved in many biological activities. It was first discovered as an important factor in B-cell stimulation and was thus first called B-cell stimulatory factor.

One can view IL-6 as akin to the Roman god Janus, with his two faces, in that it appears to be able to have both beneficial and deleterious effects on metabolism [46]. Released together with other classic pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-6 appears to have deleterious effects on metabolism [47, 48]. One example is the increase in insulin resistance thought to be mediated via IL-6 released from adipose tissue in obesity [49, 50]. On the other hand, if released together with hormones such as glucagon-like peptide-1 (GLP-1), IL-6 appears to have positive effects on metabolism [51, 52].

After IL-1B, IL-6 is the most important endogenous mediator of fever [53]. It is a necessary component for induction of fever, though paradoxically IL-6 alone cannot induce fever. TNF-α, a potent inducer of fever, could not induce this febrile response in the absence of IL-6 [48]. The role of IL-6 in acute inflammation is to stimulate recruitment of neutrophils and eventually monocytes and T-cells. It is also important in differentiation of T-cells, mainly Th17 that is of great importance for the pathology of autoimmune diseases [54]. Tozilucimab, a selective antagonist for IL-6Rα, has been successfully used in for example the treatment of rheumatoid arthritis [55].

IL-6 also has anti-inflammatory properties, linked to the configuration of its receptor complex. The IL-6 receptor is a dimer made up of the IL-6Rα and gp130. While IL-6Rα is only expressed

in certain cells, gp130 is much more ubiquitous. There is also a soluble form of the IL-6 receptor which, after binding IL-6, can act on cells expressing gp130 (but not transmembrane IL-6Rα) via a process called trans-signaling [56]. It has been proposed that the anti-inflammatory effects of IL-6 use classic signaling pathway in cells that express IL-6Rα whereas the pro-inflammatory effects of IL-6 utilize the trans-signaling pathway [56, 57].

Metabolic effects

The role of IL-6 in metabolism appears to be two-fold[58]. Firstly, IL-6, along with TNF-a, have been regarded as two of the major factors for induction of inflammation. Inflammation is regarded as an important factor in the development of obesity and insulin resistance. It has also been shown that acute administration of IL-6 increases insulin resistance in mice [59-61].

As mentioned above, IL-6 has potent pro-inflammatory effects but along with them also potent anti-inflammatory effects. One such anti-inflammatory feature is seen in the IL-6 released from skeletal muscle during exercise [59-62]. This IL-6 release appears not to be coupled with induction of inflammation but instead to function as a communicator of information about metabolic states across the body [59, 61].

Apart from its beneficial actions in skeletal muscle, IL-6 also has been strongly indicated as a promotor of liver regeneration after hepatectomy or liver damage [63-65]. The other side of the coin is that IL-6 may play a role in growth of liver cancer, at least in males [66]. IL-6 may also act as an anti-steatosis factor and inhibits gluconeogenesis [67]. Increased hepatic glucose production and steatosis of the liver, are two important factors linked to development of obesity [68].

Role in obesity

IL-6 is produced in adipose tissue in correlation to its size, in skeletal muscle during exercise, and in immune cells. IL-6 is also abundant in the CNS. The IL-6 receptor IL-6Rα, has been shown to be expressed in a major part of fundamental energy-regulatory hypothalamic nuclei [17, 20, 69]. It is suggested that IL-6, in cooperation with the related cytokine IL-1, partly prevent obesity by inhibiting the production of the orexigenic neuropeptide NPY in the ARN. Co-expression of IL-6Rα with the anorectic neuropeptides CRH, AVP, TRH and oxytocin has also been seen in the anti-obesity center PVN [17]. Further, IL-6Rα is also located in the LHA and is co-expressed with the orexigenic peptide MCH. Here, IL-6Rα might act by suppressing on MCH-production [20].

IL-6 exerts a tonic weight-suppressing effect in the CNS of healthy mice. Studies that have been performed in IL-6 knock-out mice have shown that these animals develop a mature –onset obesity from about 6 months of age [29, 70]. The typical phenotype in IL-6 -/- mice is decreased energy expenditure and increased leptin resistance, while they do not seem to increase their food intake significantly [30, 71]. Therefore a decrease in energy expenditure seems to drive the mature onset obesity.

The weight-suppressing effects of IL-6 have been assumed to be exerted at a central level, as supported by the fact that ICV-injections, but not peripheral injections of IL-6, increases energy expenditure and decreases body fat in rodents [72].

As discussed above, mice lacking IL-6 develop mature onset obesity [29]. Conversely, cachexia, the wasting seen in terminal cancer patients, seems to depend partially on IL-6 release[73, 74].

Thus, lack of IL-6 appears to promote obesity, while high levels of this cytokine seem to promote wasting. Interestingly, IL-6 levels in CSF appear to correlate inversely with fat mass in humans [72].

IL-1

Background

IL-1 is one of the classic pro-inflammatory cytokines together with IL-6 and TNF-a. IL-1 was chemically identified already in 1972, and discovered as a pyrogen in 1977 [75, 76]. IL-1 plays an important role in the induction of fever[77] and is part of the IL-1 family of cytokines, a group including IL-1β which is studied in paper 1.

The ARN is one of the few nuclei in the rat brain that has been shown to express IL-1RI mRNA [69, 78]. Moreover, intravenous injection of IL-1β induces c-Fos expression in rat ARN neurons [79]. The results of some studies indicate that IL-1 treatment exerts several effects on the ARN that may reflect the pathophysiological role of IL-1 during inflammation. Much less is known about the physiological role of low endogenous IL-1 activity in individuals without inflammation.

Metabolic Effects

Like leptin and IL-6, IL-1 seems to exert a tonic weight-suppressing effect in the CNS. Studies performed in rodents, which were genetically depleted of IL-6 or the IL-1-receptor 1 (IL-1R1), have shown that these animals develop mature –onset obesity [28, 29, 71, 80, 81]. Phenotypes observed in these mice include decreased locomotor activity, reduced energy expenditure, decreased use of fat compared to carbohydrates, and leptin insensitivity. IL-1 also appears to mediate the central effects of leptin as blocking its receptor, IL-1R1, abolishes the leptin-induced elevation of body

temperature and reduction in food intake in rodents as reported in an early publication by Rothwell et al [82].

Interactions with IL-6 and GLP-1

It has been speculated that since both IL-6 -/- and IL-1R1 -/- mice develop mature onset obesity, the phenotype of mice lacking both IL-6 and IL-1R1 might show an earlier onset of obesity or more severe obesity. It does appear that these mice display an early onset obesity [83]. However, both IL-1 and IL-6 are important in induction of inflammation and this might leave these mice with a markedly increased sensitivity to infection.

IL-1 appears to also play a role in mediating the effects of central GLP-1[51]. Working as a downstream mediator of the reduction in food intake exerted by Ex4 it appears to, together with IL-6; mediate the central effects of this drug (Paper 1).

GLP-1

General

GLP-1 is an incretin derived from the transcription product of the pro-glucagon gene [84]. Under the influence of the enzyme prohormone convertase 1 (PC1), pro-glucagon is cleaved to produce GLP-1. If instead the enzyme PC2 is present, the result is a cleavage to glucagon. The biggest source of GLP-1 in the body is the ileal L-cells, who secrete GLP-1 in the presence of nutrients in the intestine during and after a meal [85]. In the brain, GLP-1 is produced only in the NTS and the IRN of the hindbrain [37]. The population of GLP-1 neurons in the IRN, while seemingly less dense than that in the NTS, has not been the subject of much study; instead the main focus has been on the neurons of the NTS. As discussed above, it has been shown that these NTS neurons

project far and wide in the brain, reaching many of the important body-fat regulating centers such as the lateral PBN, ARN, and PVN. Many of these projections seem to contain GLP-1.

Effects on glucose metabolism

GLP-1 release after meal induces insulin release from pancreatic b-cells and decreases glucagon release from pancreatic a-calls. The latter effect is contributing to increased insulin sensitivity. GLP-1 and its analogues may also promote survival of b-cells. Not surprisingly, GLP-1 receptor agonists such as Ex4 are widely used in treatment of type 2 diabetes [86-89].

Role in obesity

GLP-1 is rapidly degraded by the enzyme dpp-4 and has a very short half-life in serum [87]. Consequently, to study the effects of GLP-1 on obesity and other metabolic parameters, GLP-1 analogues that are less easily degraded by dpp-4 with have been useful. One such analogue is Exendin-4 (Ex-4), first found in the saliva of the gila monster, a lizard. As mentioned above, Ex4 has been used clinically as a treatment for diabetes type 2 [86, 88]. In some patients Ex-4 and other long-acting GLP-1 analogues also promotes moderate weight loss but the mechanisms for this action, has been essentially unknown.

Interactions between IL-6 and GLP-1

It has been shown that IL-6 and GLP-1 interact peripherally to increase insulin sensitivity and promote insulin secretion[52]. It seems that IL-6 increases the levels of GLP-1 secretion from pancreatic α-cells and ileal L-cells, and that these effects may mediate the stimulation of insulin secretion by IL-6. We have

recently shown that IL-6 and GLP-1 also interact centrally, but in the opposite way [51].

It seems as if GLP-1 and more specifically the GLP-1 analog Exendin-4 (Ex-4) increases levels of IL-6 mRNA in the hypothalamus and the brainstem. Mice and rats injected with Ex-4 lose weight and reduce their food intake. These effects possibly depend on IL-6, as pre-treating these animals with neutralizing antibodies against IL-6 attenuate these effects (Paper 1).

Methodological Considerations

Immunohistochemistry

Immunohistochemistry is a method where you add antibodies that bind specifically to the protein(s) you wish to study, for example in fresh tissue. These antibodies can be either monoclonal or polyclonal and both approaches come with their own advantages and disadvantages [90, 91].

For monoclonal antibodies, the staining itself usually gives a cleaner picture, as they consist of one specific clone of antibodies directed against one specific epitope. The staining that you attain with a monoclonal antibody is also likely to be consistent between different batches. However, using monoclonal antibodies have the risk of giving a false absence of staining because it dependent on the availability of one single epitope in each slide that the antibody might not reach in that particular slide.

Polyclonal antibodies on the other hand are directed against several epitopes. Immunohistochemistry with polyclonal antibodies usually result in less specificity with risk of false positive staining, as they contain many antibody types directed against different epitopes. These antibodies are also more batch-dependent as the staining patterns may change between different batches of antibodies. However, due to the fact that polyclonal antibodies are directed against several epitopes they have a higher likelihood of success.

Thus, a good approach to immunohistochemical staining would be to start an experiment with a polyclonal antibody against your target protein and investigate the staining pattern it gives. Once satisfactory staining has been attained, a monoclonal antibody with higher specificity can be used as confirmation.

Immunohistochemistry is most commonly performed on either fresh or perfused tissue and both these methods have advantages and disadvantages. Perfusion, commonly with PFA, gives a clearer view of the architecture of the cell whereas snap-freezing of fresh tissue aids visualization of cell membranes.

Verification of antibodies

Western blot can be used to verify the specificity of antibodies used in IHC. A lack of bands in Western does not necessarily indicate that the antibody is non-specific [92]. The method of preparation of proteins for Western blot might change configuration of the antigen. Loss of epitopes to bind might be a result. On the other hand, the presence of a band of the expected size is a good verification of the specificity of the antibody.

Another way to verify antibodies is co-staining two monoclonal antibodies against the same substance directed against two different parts of the molecule. If these two antibodies stain the same structures and cells, it is likely that intended molecule is visualized. This approach was used to verify the IL-6Rα staining seen in this thesis. In that case, one antibody was directed against the extracellular part and one against the intracellular part of the IL-6Rα [20].

At times however, it is difficult to obtain specific antibodies against a substance of interest. This has been a problem with GLP-1 antibodies. It can be circumvented by creating reporter mice, for example the Venus mouse that is described below.

Venus Mouse strain - a prepro glucagon (PPG) cell reporter mouse

A reporter mouse can be used to visualize the cells that express a certain gene, in this study the PPG gene. A construct is made consisting of the promotor of the gene, e g the PPG promoter, coupled to a fluorescing gene, e.g. YFP. Two basic approaches can be used to generate a genetically modified reporter mouse such as the Venus mouse used in this thesis. The first approach is to inject the genetic construct (e.g. the PPG promoter – YFP encoding gene) into a mouse embryo with the caveat that this genetic construct can then integrate anywhere in the genome. The second approach is to modify a specific part of the genome in an embryonic stem cell with a homologous sequence attaining a homologous recombination. This approach is more commonly referred to as a knock-in as the gene sequence targeted by homologous recombination (e g the PPG encoding gene) is lost in one allele.

The venus mouse is an example of the first approach and the five different founder animals were generated from three different BAC strains (one from mouse and two from rat). In place of the PPG promoter, a region that is highly conserved, a fluorescent YFP-Venus region was inserted [85]. The Venus mouse has been used extensively although mainly in the periphery where it has been well-validated [52]. One potential problem for us is that the model is less extensively validated in CNS-applications [93, 94]. As mentioned above, the PPG gene transcript can be spliced into many different mRNAs and proteins including glucagon and GLP-1. Of interest to this thesis is that glucagon is much less expressed than GLP-1 in the brain, leading us to assume that the fluorescence described in paper 2 and 3 originates from GLP-1 producing neurons. The fluorescence observed in this thesis also

corresponds to the anatomical location of the GLP-1 neurons according to the literature, i e mostly in the NTS.

Confocal Microscopy

The most important difference between a confocal microscope and a standard fluorescence microscope is the spatial filtering of light that is used to eliminate out-of-focus light in thicker specimen such as tissue slices. This is achieved via the pinhole, a key component that allows only light from the focal plane to traverse it. As shown in Fig 1, light originating above or below the focal plane is filtered out and does not reach the detector[95].

This principle is of great importance in determining possible co-localization of fluorescence. In a regular fluorescence microscope two fluorophores might appear to be co-localized. However, that might actually be because these two overlapping fluorophores are situated at different z-levels in the tissue. With the use of fluorescence microscopy a difference in z-level does not become apparent and this might lead the researcher to believe in a false positive co-localization. The same tissue seen in a confocal microscope would look quite different as out-of-focus light is filtered. Thus, it eliminates the false positive co-localization that stems from out-of-focus fluorescence[96].

Confocal microscopes feature a good z-axis resolution and this can be used to determine co-localization on single-cell level. By using a method called focal stacking, multiple images with different z-coordinates, but the same x- and y- coordinates, are obtained as the objective is moved in the z-axis. Thus, by focusing on the lower parts of a single cell and eventually moving your way up to the upper parts, it is possible to acquire a three-dimensional

representation of a cell. This is ideal for studies of co-localization, as it results in a more complete view of a cell.

By making use of the z-stack that has been obtained in the above mentioned method, it is also possible to orthogonally view a cell. By looking at one focal plane from three different directions (x, y and z) and focusing on a specific region of interest, it is possible to study the potential co-localization between two fluorophores in more detail.

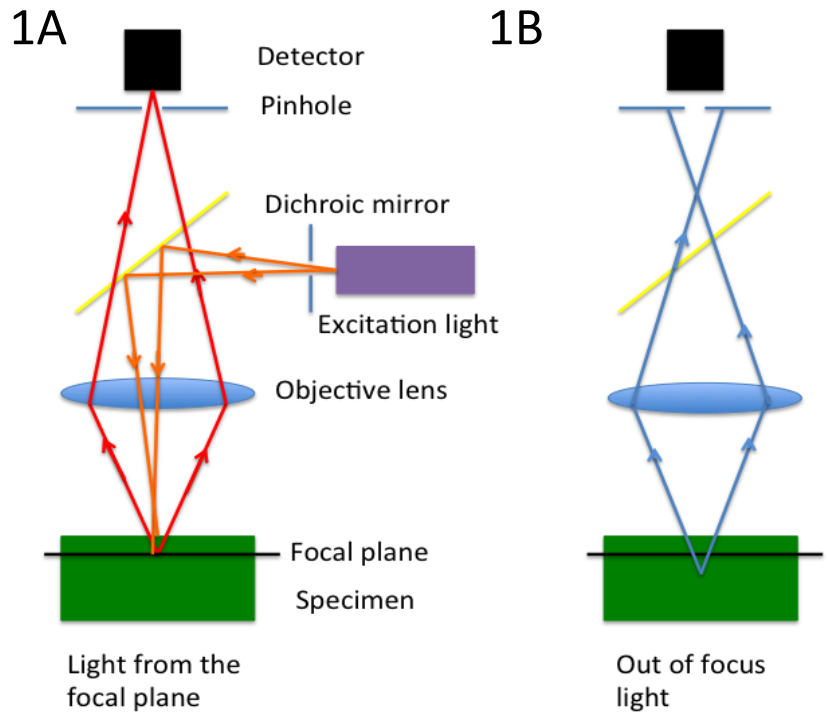


Figure 1: Fig A shows the basic principle of confocal microscopy. Light from the focal plane is directed through a pinhole to a detector. Fig B shows that light from planes that are out of focus do not pass the pinhole and is therefore not picked up by the detector.

Electrophysiology

Loose patch-clamp

Loose patch-clamp electrophysiology was used in paper 3 for measurement of action currents in the neurons of the external lPBN after application of the GLP-1 receptor agonist Ex-4 or antagonist Ex-9. A basal firing rate is first recorded after which the substance of interest is applied and the firing rate change recorded [97].

A major advantage of using the patch-clamp technique on single cells is that you can exclude all environmental factors from the cell. Hence, you get to study the cell itself and not for example confounding uncontrolled synaptic inputs or hormonal influences. Both the internal and external milieu of the cell is possible to control and as such the method is very useful for testing the influences of, for example, drugs on the cell.

The major advantage detailed above, however, can also become a major disadvantage. As the cell is removed from its normal milieu, several factors that might influence how, for example, a drug might work in vivo are excluded. It is possible that certain synaptic inputs or hormones influence the workings of the cell in vivo, but these factors will not be present in a patch-clamp.

2

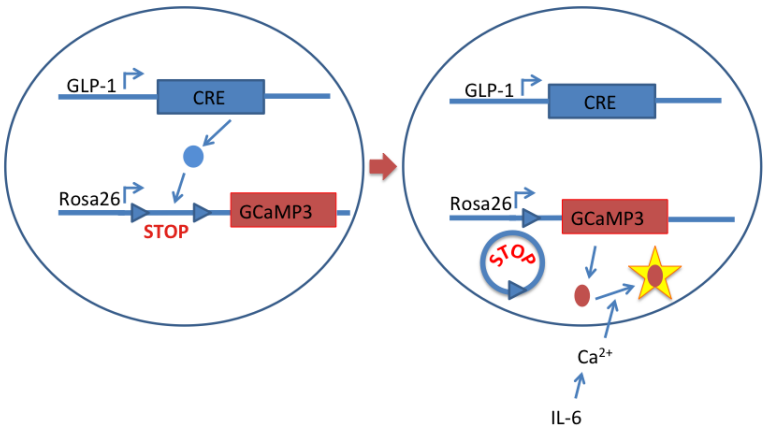


Figure 2: A GLP-1 promoter-CRE mouse is paired with a Rosa26 promoter-GCAMP3 mouse. The Rosa26 promoter is active in most cell types. In cells with active GLP-1 promoter, the CRE protein cleaves out the stop sequence before the GCaMP3 gene. This results in cells in which the GCaMP3 gene is translated into a protein that gets fluorescent in the presence of Ca^{2+}.

GCaMP3

To attain mice for the GCaMP3 method used for the electrophysiology in paper 2, GLP-1promoter-Cre mice were crossed with Rosa26promoter-GCaMP3 mice as described in Figure 2. GCaMP3 utilizes a modified green fluorescent protein that is activated by high calcium concentration. Calcium binds to calmodulin, a high calcium-affinity protein, and this protein then binds GCaMP3 and activates the fluorescence. This approach is especially relevant in neurons as Ca^{2+} generates signals that control key functions in these cells. Furthermore, these signals originate in well-defined subcellular compartments such as the

nucleus where Ca^{2+} can regulate the transcription of genes [93, 98].

Ca^{2+} influx into a cell is under the control of voltage-gated calcium channels (VGCCs). On a basic level, they can be divided into high- and low-voltage-activated channels but further subdivisions into classes depending on which cell type they are expressed in is also common[99]. However, Ca^{2+} can also enter the cytosol from intracellular compartments such as the endoplasmatic reticulum [100].

GCaMP3 is part of a family of genetically coded Ca^{2+} indicators that have been continuously developed to improve their spectral parameters and/or fluorescent response. Early versions of these indicators, such as GCaMP, gave a quite weak fluorescent response, but each new development has led to a stronger response. Another problem with the early GCaMPs was that their expression under physiological temperature was quite poor, but it was possible to overcome this by modifications of the GCaMP molecule.

Comparison of GCaMP3 to patch-clamp

The chief criterion for evaluating GCaMPs is the relationship between fluorescence and number of action potentials fired. In earlier versions of GCaMPs such as GCaMP, it was found that its signal often fails to capture spiking response kinetics, and that it can miss even high instantaneous rates of activity if those are not sustained. However, these problems have been overcome in later versions of GCaMPs such as GCaMP3, which has been used in paper 2 of this thesis [101]. Indeed, it has been shown that there is a high correspondence between intensity of fluorescence of GCaMP3 and action potential firing rate [102, 103].

GCaMP3 shows a quite high basal fluorescence but also a quite robust change in fluorescence after stimulation. To accurately measure this, the difference between basal fluorescence and fluorescence after stimulation has to be measured. This is similar to the difference in firing rate between baseline and after stimulation in patch-clamp electrophysiology that was used in paper 3.

RT-PCR

RT-PCR can be used to measure the expression of genes of interest in a specific tissue [104]. mRNA is first extracted from the tissue; in this thesis meaning the hypothalamus, hindbrain or PBN. The extracted mRNA is then used as a template for complementary DNA (cDNA). Probes specific to the gene of interest are then added to the cDNA along with a master mix containing nucleotides for extension of DNA. These probes contain both a fluorescent dye and a quencher to prevent this fluorescence. During the extension phase of the PCR reaction, the dye and the quencher drift further apart which lifts the inhibition and causes the dye to fluoresce. This fluorescence can then be measured, and a higher intensity of fluorescence will then correspond to a higher expression of the gene of interest.

In this thesis, the expression of the gene of interest was compared to the expression of a reference gene. These reference genes are ideally housekeeping genes with stable expression in both the treated and the untreated groups. Calculations were then made using the expression of the gene of interest relative to the reference gene in both groups. Thus, the papers in this thesis do not contain absolute numbers of expression.

General Discussion

Paper 1 and 2

Paper 1 shows a possible interaction between the GLP-1 analogue Ex4 with IL-6 and IL-1β. A blockade of either IL-6 or IL-1B separately attenuates the effect of Ex4 on 22 h food intake when administered i.c.v. These findings lead to the proposition that both cytokines are needed to mediate the suppression of 22 h food intake by Ex4. However, there appears to be some redundancy in the system as an inhibition of both cytokines, but not cytokine only, led to an effect on food intake after 4h.

Paper 2 builds on the findings of paper 1, further studying the interactions between IL-6 and GLP-1 by studying the presence of IL-6Rα on PPG-expressing neurons in the NTS. Immunohistochemical studies show that there is IL-6Rα on these neurons which allows for a possible way for IL-6 to influence GLP-1. Furthermore, these neurons appear to respond with Ca^{2+}-influx to administration of IL-6.

A general mechanism found in biological systems is the negative feed-back loop. It can be exemplified by the TSH release from the pituitary which stimulates the release of T3 and T4 which in turn act on the pituitary as a negative signal to reduce the release of TSH, which in turn reduces the release of T3 and T4. The end result is fairly constant levels of T3 and T4.

Another biological mechanism, found commonly in inflammation and immune response, is feed-forward. The interaction between GLP-1 and IL-6 can, in light of the data presented in this thesis, can be thought of as such a feed-forward system.

In paper 1, we discuss how injection into the lateral ventricle of the GLP-1 analogue Ex-4 increases mRNA levels of IL-6 in the hypothalamus and hindbrain. In paper 2, we further explore the interaction. By showing that IL-6Ra is indeed present on GLP-1 neurons in the NTS and that these cells when cultured respond to IL-6 by an influx of calcium into the cytosol, probably mirroring an activation of the GLP-1 producing cells. However, it should be pointed out that we have not seen any regulation of GLP-1 expression in NTS by IL-6 (not shown). To sum up the results presented above, GLP-1 analogues increase IL-6 mRNA levels and IL-6 in turn stimulates GLP-1 producing neurons in the NTS.

In the periphery, IL-6 also seems to activate GLP-1 producing L-cells in the gut and alpha-cells in the pancreas, resulting in more GLP-1 release, and thereby insulin release [52]. It may be postulated that IL-6 and GLP-1 inside and outside the brain of healthy individuals, stimulate each other and act in tandem to exert beneficial metabolic effects, such as reduced food intake and body fat mass.

3

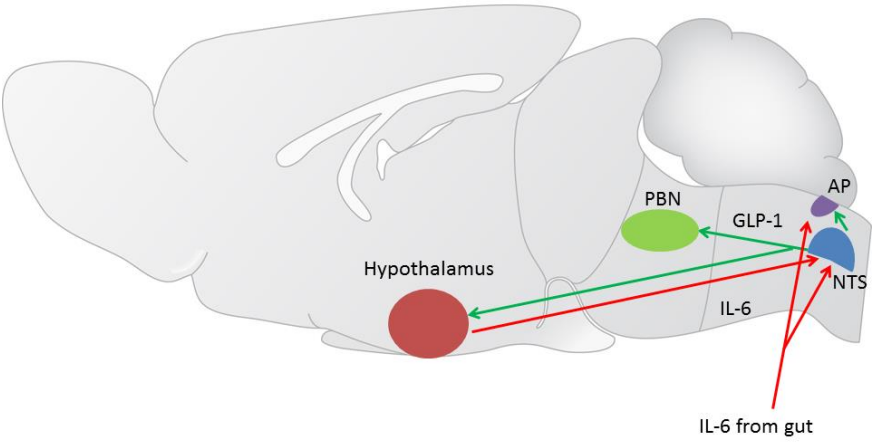
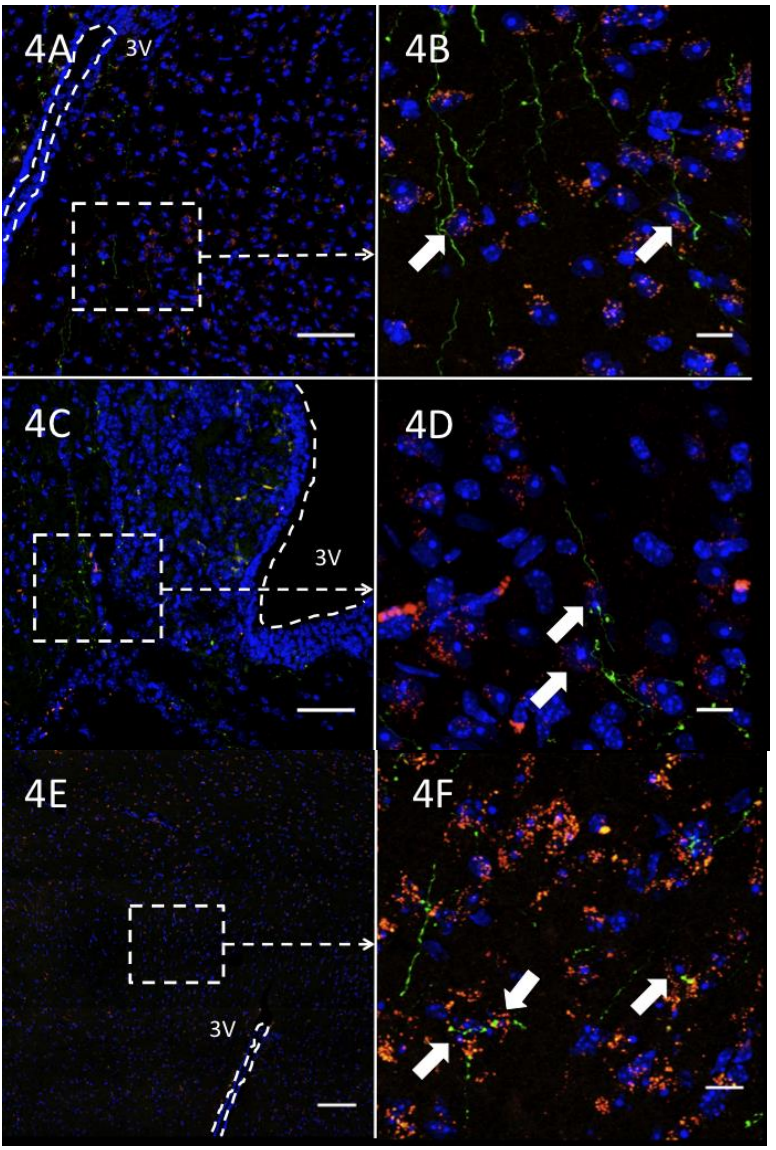


Figure 3: Schematic representation of how IL-6 and GLP-1 might affect different nuclei in the rodent brain. Green arrows represent GLP-1 and red arrows represent IL-6.

Of note is that GLP-1 containing fibers from the NTS seem to reach cell bodies in several hypothalamic nuclei of importance in energy balance, such as the ARN, PVN and DMN (Fig 4). Some of these cell bodies also stain positively for IL-6Rα, revealing a further possible site for cross talk between IL-6 and GLP-1. Examples are shown in Fig 4A, D, F. The proportions of IL-6Rα positive cell bodies in different hypothalamice nuclei that have adjacent GLP-1 containing fibers is shown in Fig 4G. Some cells in the ARN, PVN and DMN may respond both to GLP-1 originating from the ascending fibers, and to IL-6 via receptor binding. Therefore, IL-6 and GLP-1 may act in parallel on some cell bodies. As mentioned above, IL-6 may mediate effects on GLP-1 and GLP-1 neurons may

be stimulated by IL-6. In summary, there are several possible interactions between IL-6 producing and GLP-1 producing neurons.



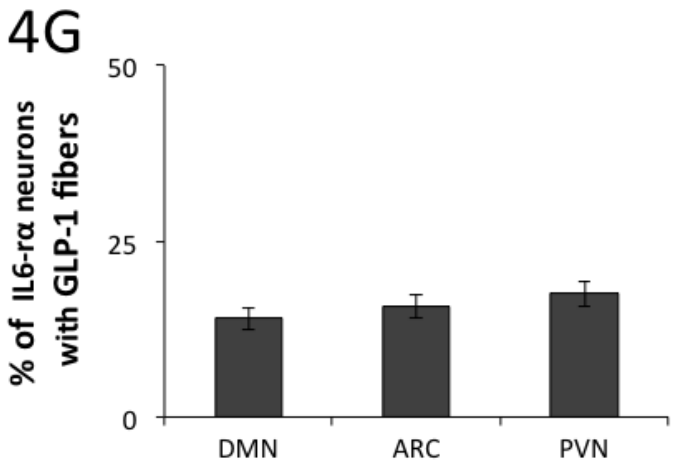


Figure 4: Representative confocal micrographs of the DMN (A-B), ARN (C-D) and PVN (E-F) showing GLP-1 fibers (green) close to IL-6Ra positive cells (red). Cell nuclei are stained with DAPI (blue). The percentage of IL-6Ra positive cells that receive GLP-1 fibers is quantified in (G).

Paper 3

The role of IL-6 and GLP-1 in the PBN

The PBN is a key player in the maintenance of body weight and food intake. Inhibitory inputs from the hypothalamus reach the PBN their absence leads to starvation [41]. However the PBN also receives excitatory input, the nature of which has been largely unknown. In paper 3, we hypothesize that this input may be in the form of projections from GLP-1 neurons in the NTS. We show that GLP-1 fibers from the NTS reach the lPBN where they lie in close apposition to CGRP-neurons. Injections of Ex-4 into the lPBN reduced food intake and decreased body weight in rats.

GLP-1 containing fibers (probably emerging from the NTS) are found close to both CGRP- and IL-6Ra-expressing cells. However, there appears to be no co-localization between CGRP and IL-6Ra (Fig 5).There is a possibility that both CGRP and IL-6 in the lPBN are of importance for the effects of GLP-1 on food intake, but further studies are needed.

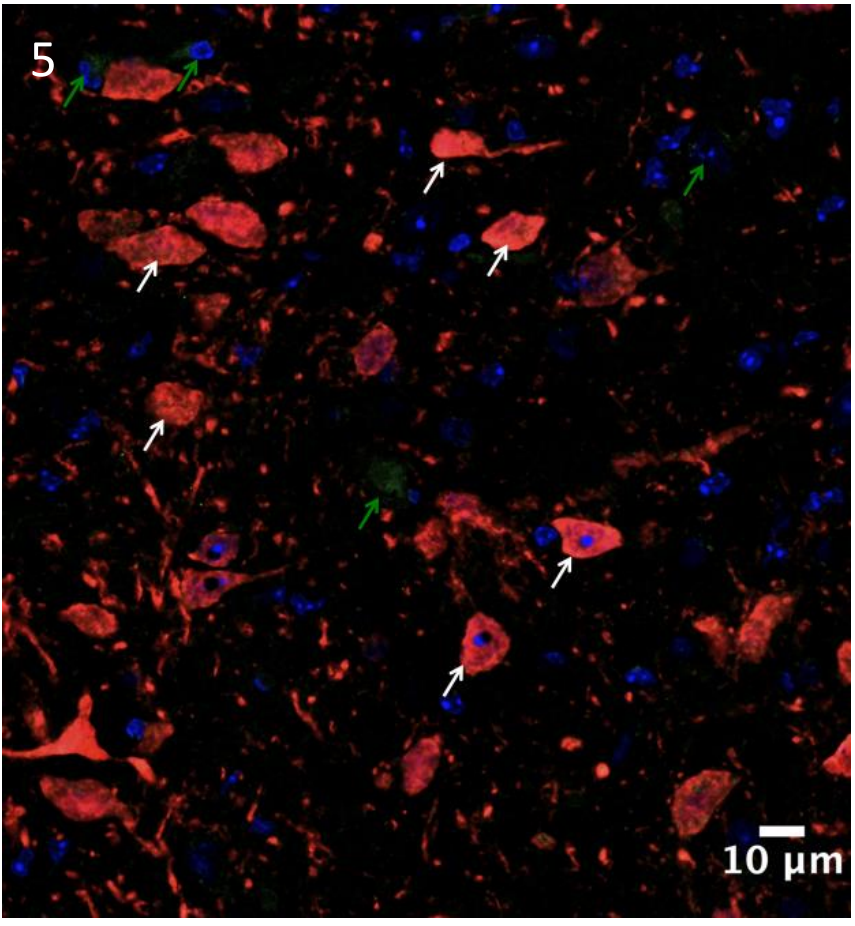


Figure 5: Representative confocal micrograph of the lPBN showing CGRP (red) and IL-6Rα (green). Cell nuclei are stained by DAPI (blue). White arrows show CGRP-positive neurons and green arrows show IL-6Rα positive neurons. There appears to be no co-localization between cells expressing CGRP and IL-6Rα.

Paper 4

As indicated by immunohistochemical staining, IL-6rα is present on the luminal side of tanycytes. It has previously been shown that IL-6 levels in CSF are inversely correlated with fat mass. Moreover, in some individuals the IL-6 levels were considerably higher in the CSF than in the blood circulation, suggesting local production of IL-6 in the CNS rather than filtration of from the blood to the CSF over the blood brain barrier. However, it has been unknown if IL-6 in CSF exerts any biological function, and, if so, on which cells those functions could be exerted.

Our findings indicate that IL-6 in the CSF may act on IL-6Rα located on the luminal side of tanycytes at the bottom of the third ventricle close to the median eminence (ME). The immunohistochemical staining was strongest at this part of the ventricle system. Else, there were only a few tanycytes expressing IL-6Rα interspersed with ciliated ependymal cells present in the third ventricle wall, the fourth ventricle, and the lateral ventricles. It might be speculated that this is the primary site of IL-6 transport into the brain parenchyma. Supporting this hypothesis, the processes originating from tanycytes stretch further into the hypothalamus, reaching the ARN. This nucleus is of importance for metabolic control and acts as a relay between the central nervous system and the periphery. The ARN contains cell populations that co-stain for IL-6rα together with neuropeptide-Y (NPY). This neuropeptide is known to promote obesity and to co-localize with agouti-related peptide (AgRP). As such, it is possible that the IL-6 acting on these receptors is transported from the CSF to these cells via tanycyte processes.

The decreased IL-6 levels in CSF found in obesity will likely lead to a decreased signaling via the IL-6rα present on tanycytes. IL-6

being an obesity-suppressing neuropeptide, this decrease in signaling may contribute to maintenance of an obese state.

Previous studies indicate that leptin is transported across tanycytes from the blood circulation in the media eminence to the third ventricle-. This tanycyte mediated leptin transport like leptin signaling, was found to be impaired in mice with diet-induced obesity (DIO). The leptin receptor (Lepr) shares similarities with class 1-cytokine receptors, such as IL-6rα, utilizing the same JAK-STAT signaling pathway. As such, we had reason to believe that IL-6rα signaling might also be impaired in DIO mice, as IL-6^{-}/$^{-}$ mice display a leptin-resistant phenotype. However, when injecting DIO mice with IL-6 icv there seemed to be no differences in pSTAT3 activity at any of the timepoints investigated. It may be that the chronically lowered levels of IL-6 in CSF that can be seen in obesity might contributes to the higher body weight. There seems to be no IL-6 resistance as such, as the relation between body weight and IL-6 levels in CSF is the opposite of that seen for leptin (Strenlöf K et al).

It might be speculated that there is a way for IL-6 to be transported from CSF into the brain parenchyma via tanycytes located at the bottom of the third ventricle. Via the processes located on these cells, it may be further transported to nuclei important for metabolism, such as the arcuate nucleus, where IL-6rα is present. This mechanism thus provides one possible way for IL-6 levels in CSF to influence maintenance of body weight. This hypothesis could be tested by injection of labeled IL-6.

Future Perspectives

Immunohistochemical studies of IL-6 itself

An astute reader might have noticed by now that the immunohistochemical staining in this thesis are focused solely on the IL-6Ra. This however, begs the question of where in the brain IL-6 itself is located and in which cells. Immunohistochemical staining for IL-6 itself has been a difficult task. Countless hours have been spent testing one antibody after the other only to see the same pattern and intensity of staining in both wild type and IL-$6^{-}/^{-}$ mice. However, it has recently been shown that there now exists an antibody against IL-6 that appears to give close to no staining in IL-$6^{-}/^{-}$ mice [105].

An alternative way to proceed would be to create a reporter mouse similar to the Venus mouse model used in this thesis. We have obtained such a reporter mouse and by studying it we are set to investigate in which cells IL-6 is produced in the brain. An advantage of such a reporter mouse is that it can be used to verify the anti-IL-6 antibodies that we have previously tried. Should one of these antibodies display a staining pattern that matches the staining in the reporter mouse it might lead us to believe that the antibody is actually staining IL-6. Work in our lab is now ongoing to repeat the immunohistochemical staining of paper 2, 3 and 4 only this time with IL-6 instead of IL-6Rα.

Would IL-6 work as a treatment for obesity?

While it might, courtesy of the role it has been shown to play in for example cancer cachexia and our finding that mice lacking IL-6 develop mature onset obesity, it is important to keep in mind that this cytokine can have potent effects on the immune system. One example is the rare Castleman disease, a lymphoproliferative

disorder which leads to elevated levels of cytokines in blood, not least IL-6 [106]. In severe cases this disease can lead to intense inflammation, organ failure and death.

Moreover, IL-6 seems to play a role in several autoimmune diseases not least because of IL-17 production. The drug tozilucimab, a potent blocker of IL-6Ra, has been used with good effect to treat these diseases, suggesting that IL-6 is necessary to develop many of the symptoms. It should be emphasized that IL-6 alone in the absence of other cytokines might not give symptoms associated with immune stimulation, as suggested by the increase in IL-6 release from working skeletal muscle during exercise [59-61].

Taking into account the above mentioned dangers of IL-6 administration; an alternative approach would instead be to find the substances that interact with IL-6 in its positive effects on metabolism. As described previously in this thesis, GLP-1 appears to be one such substance. Although the GLP-1 analogues used clinically to treat diabetes type 2 lead to only a moderate weight loss, there might be other substances at play that could modulate, especially stimulate, the responses to GLP-1 analogues. Especially, factors related to the IL-6 system may have such potential, but further studies are needed to investigate this.

Another problem with IL-6 analogues as a treatment for obesity is that there are indications that they act within the blood brain barrier [28, 29]. The assumption of an effect within the CNS is in line with the finding that IL-6 in serum from white adipose tissue is increased in obesity [72], an effect that obviously does not prevent the development of obesity. On the other hand IL-6 has recently been reported to contribute to browning of WAT during cancer cachexia [107], suggesting that this cytokine sometimes

can exert effects potentially associated with suppressed obesity also outside of the blood brain barrier.

Another way to exploit the anti-obesity effects of IL-6 would be to identify the downstream targets of the effects of IL-6. If such targets can be found, it might be possible to influence the anti-obesity effects of IL-6 without also affecting its' pro-inflammatory properties.

Acknowledgements

I would like to thank everyone that has contributed to my thesis. Without you, this book would never have been written.

I would especially like to thank:

My supervisor **John-Olov Jansson**, for your endless enthusiasm and for encouraging me to proceed with my ideas. It's been a long journey but you've supported me every step of the way.

Karolina Skibicka, my co-supervisor, for your priceless insight in the world of research and for taking the time to listen to my problems; both scientifical and non-scientifical.

Carina Mallard, my co-supervisor, for your help getting me started on my Ph.D and letting me borrow all the fantastic machines on your floor.

Vilborg Pálsdóttir, for always being there to help me with whatever troubles I encountered. You're a fantastic person and I'm glad to have worked together with you.

Erik Schéle, for teaching me the secrets of immunohistochemistry and for your laid-back attitude that's helped me overcome some of the stress that comes with research. It's been a pleasure to work with you.

Sara Svahn, for pushing me when I'm being a hopeless procrastinator. For all the fikas we've had and teaching me the proper use of "compared with".

Rozita Anderberg, for being great company and always bringing a smile to my lips. I've really appreciated working and sharing an office with you.

Jennifer Richard, for all the laughs and always being a positive influence. Also for safekeeping all the things I misplace, like perfusion tubes.

The rest of the Skibicka group, especially **Kim** for fun discussions about immunohistochemistry.

Ruijin Shao, for all the talks we've had about research and anything under the sun. You've never failed to brighten my day.

Sofia Särnsten, for being my buoy when I'm helplessly lost in the seas of bureaucracy.

Everyone else at the physiology department, **Heike**, **Christina**, **Anna**, **Tina** and the rest of my lovely co-workers for making work feel a little less like work and for being patient with my sometimes loose relationship with time.

My former and current students, **Teodor, Cecilia** and **Edwin** for putting up with my way of explaining things and getting the job done despite my somewhat loose way of handling protocols.

The "**Junta**" for fikas, crossword puzzles and discussions. Special thanks to **Anna** for completing the "formerly black hair dye users" office.

Everyone at the pharmacology department, **Fredrik, Daniel, Daniel, Erik, Robert** and **Nina** for making sure I had a social life with game nights and lunches.

The **Circulation** group for the lovely after works and quizzes.

Jakob, for being an amazing friend and always being there for me. It was a blessing to meet you during our studies.

Arvid, Jacob and **Alexandra,** for reminding me that there is a life outside of science as well. I'm lucky to have you as friends.

Mum, dad and my amazing sister **Charlotte** for always being there for me. You've always tried your best to help and support me, no matter what. I'm very grateful for that.

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