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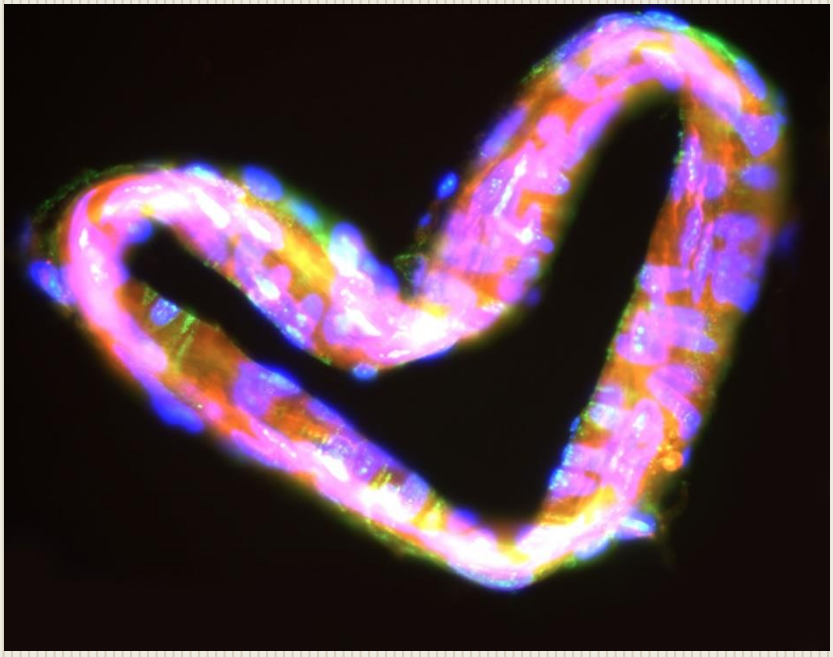
# Metabolic Signaling in the Cerebrospinal Fluid

Degree Project in Medicine



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# Metabolic Signaling in the Cerebrospinal Fluid

Degree Project in Medicine

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Programme in Medicine

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# 1. Abbreviations

| | |
|---|---|
| aCSF | artificial CSF |
| AgRP | Agouti-related peptide |
| AMA | American Medical Association |
| ANOVA | Analysis of variance |
| ARC | Arcuate nucleus |
| BAT | Brown adipose tissue |
| BMI | Body mass index |
| CART | Cocaine- and amphetamine-regulated transcript |
| CCK | Cholecystokinin |
| cDNA | complementary DNA |
| CeA | Central nucleus of the amygdala |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| CV | Coefficient of variation |
| DAPI | 4',6-diamidino-2-phenylindole |
| DPP-4 | Dipeptidyl peptidase-4 |
| ELISA | Enzyme-linked immunosorbent assay |
| EMA | European Medicines Agency |
| Ex-4 | Exendin-4 |
| FTO | Fat mass and obesity-associated protein gene |
| GABA | γ-aminobutyric acid |
| GIP | Glucose-dependent insulinotropic peptide |
| GLP-1 | Glucagon-like peptide-1 |
| GLP-1R | GLP-1 receptor |
| GLP-2 | Glucagon-like peptide-2 |
| gp130 | Glycoprotein 130 |
| GPCR | G-protein–coupled receptor |
| ICV | Intracerebroventricular |
| IHC | Immunohistochemistry |

| | |
|---|---|
| IL-1 | Interleukin-1 |
| IL-1β | Interleukin-1 beta |
| IL-6 | Interleukin-6 |
| IL-6 Rα | Interleukin-6 receptor-α |
| IL-6 $R\alpha^{lox/lox}$ | IL-6 Rα lox genotype |
| IP | Intraperitoneal |
| JAK | Janus kinase |
| LHA | Lateral hypothalamic area |
| ME | Median eminence |
| NAc | Nucleus accumbens |
| NPY | Neuropeptide Y |
| NTS | Nucleus of the solitary tract |
| ob | Obese |
| OB-R | Obese receptor |
| PC1/3 | Proprotein convertase 1 |
| PCR | Polymerase chain reaction |
| POMC | Proopiomelanocortin |
| pSTAT3 | Phosphorylated signal transducer and activator of transcription 3 |
| PYY | Peptide tyrosine tyrosine |
| SC | Subcutaneous |
| SOS | Swedish Obesity Study |
| STAT3 | Signal transducer and activator of transcription 3 |
| TNF-α | Tumor necrosis factor alpha |
| WHO | World Health Organization |
| VMH | Ventromedial hypothalamic nucleus |
| WT | Wild type |
| VTA | Ventral tegmental area |

# 2. Abstract

Obesity is a dangerous disease that affects more than 600 million individuals. Traditionally this ailment has been treated with bariatric surgery. Surgery can always be dangerous and this method does not always produce satisfactory results. Recently, several drugs have been approved to treat obesity, although so far the weight loss effect is not as impressive as with surgery. One rather effective group of drugs are analogues to the hormone glucagon-like peptide-1 (GLP-1), believed to prevent obesity acting in the brain. Another hormone, leptin, was a promising candidate for treatment of obesity but failed due to leptin resistance in obese individuals. Interleukin-6 is a protein most known for its role in the immune system; however, it can also act as a neuropeptide that decreases appetite.

Here we use high sensitivity ELISA to show that human cerebrospinal fluid (CSF) does not contain measurable levels of GLP-1 although it is produced in the brain. . We further show that the effect of leptin is not influenced by knock-down of the receptor to interleukin-6 that exists on the inside of the ventricles. Finally we show that GLP-1, leptin and interleukin-6 has the potential to activate receptors found on the inside of the ventricles. We used immunohistochemistry to identify these cells lining the ventricles as tanycytes.

Collectively these findings suggest that at least pharmacologically, glucagon-like peptide-1, leptin and interleukin-6 may exert their effect via the cerebrospinal fluid. This may be done by activating special ependymal cells lining the inner surface of the ventricles known as tanycytes.

Key words: obesity, glucagon-like peptide-1, leptin, interleukin-6, tanycytes

# 3. Introduction

## 3.1. Obesity

Obesity is defined by the World Health Organization (WHO) as a state of excessive fat mass. A common tool to evaluate if a patient is obese is called body mass index (BMI). BMI is calculated by dividing the weight in kilograms of an individual by their height in meters squared. Normal BMI is defined as 18.5 to 24.9 $kg/m^2$, overweight is defined as a BMI between 25 and 29.9 $kg/m^2$ and obesity is defined as a BMI greater than 30. (1)

Interestingly, statues of obese individuals, such as *Venus of Willendorf,* and wall carvings depicting obesity have been found all the way back to the Paleolithic era (20,000 to 30,000 BC) suggesting that obesity has been present for a long time. This also suggests that obesity once was something celebrated and desirable. (2) Obesity was especially cherished during the $17^{th}$ century in Europe where it indicated prosperity, power, and fertility (3). However, the view on obesity changed drastically in the $18^{th}$ century with the first scientific publications linking obesity with an impaired health (4). Nowadays it is not unusual to hear obese individuals say that they would rather be blind or deaf than obese (5).

Obesity is a severe medical condition linked with morbidity in almost every organ system. Most discussed is the impairment in cardiovascular health resulting in a considerably shorter life span due to the increased risk of myocardial infarction. Obese individuals also experience a higher risk for developing some kinds of cancers, e.g. colorectal cancers. (1, 6) Obesity is also linked to a wide array of non-fatal medical complications that results in an impaired life quality. Some common complications of obesity include osteoarthritis, type 2 diabetes mellitus, severe depression, asthma, obstructive sleep apnea and non-alcoholic fatty liver disease. (7)

Alarmingly, Obesity is not only a dangerous medical condition; it is also common. WHO states that since the year 1980 the prevalence of obesity has more than doubled. In 2014 more than 600 million people were obese and that number is increasing for every day. (1) Today all major health agencies, including the American Medical Association (AMA), recognize obesity as one of the largest threats to health today (8).

The cause of obesity has been discussed frantically. In summary, it all boils down to an increased energy intake compared to energy expenditure; meaning the modern individual generally eats too much and exercises too little. (1) In a world were desk jobs dominates and high calorie food are delivered within a few minutes, this should not surprise anyone. Due to this the modern environment is sometimes referred to as an "obesogenic environment". Historically the ability to store energy as adipose tissue may very well have been an advantage in times when food was scarce, and gene variants promoting this would then be inherited. This was originally proposed by Neel in 1962, and is referred to as the thrifty genotype hypothesis. (9)

Speakman later suggested that there has been selection against obesity as well. Obesity may historically have increased the risk of predation. Speakman reasons that the occurrence of cardiac and metabolic disease relating to obesity has not been the reason to genetically select lean individuals over obese. These ailments mostly occur after fertile age and may have been less important historically. In summary, an individual's genetic regulation of body fat mass may historically have been a trade-off between the need to have energy depots in case of famine and the need for agility, speed and endurance to avoid predators. Hence, Speakman suggests that the obesogenic environment does not cause obesity today. Instead the lack of selection pressure by predation may be the cause of the obesity. (10)

As discussed above, obesity has been suggested to be a genetic disease. Monogenetic obesity does exist but is extremely rare. (11) There do however exist several alleles that promote weight gain (12), for example the fat mass and obesity-associated protein gene (FTO). Genomewide association studies have shown that 16 % of humans are homozygous for the weight promoting allele of FTO . The effect on weight gain is moderate with an average weight difference of 3 kilograms between individuals that are homozygous for the weight promoting FTO allele compared to the more common allele. (13) Genome wide association studies do also suggest that the prevalence of alleles that promote weight gain has not increased during the last decades (12). Genetics may therefore be able to explain some cases of obesity but fails to explain the majority and genetics is definitely not what drives the explosive increase in obesity prevalence.

## 3.2. Treatment of Obesity

If obesity is as dangerous as described above, why does the obese patient continue to eat? The answer is of course that they eat because they feel hungry. To minimize a patients hunger there exist two treatment options: medicine and surgery.

Currently there exist three drugs that are accepted on the indication obesity in Scandinavian countries: Saxenda®, orlistat and Mysimba®. Saxenda® and Mysimba® were both approved for use in Europe by European Medicines Agency (EMA) during 2015, before that only medical weight loss therapy with orlistat was possible. All mentioned drugs have moderate impact on weight loss and need to be combined with strict diet and exercise. All drugs need to be taken on a daily basis for effect. (14)

Saxenda® is an injection that includes the substance liraglutide which is also used to treat type 2 diabetes. Liraglutide, the active substance in Saxenda, is a glucagon-like peptide-1 (GLP-1) analogue that decreases appetite and also the risk for cardiovascular disease.

Liraglutide in a lower dose (under the name Victoza®), has for many years frequently been used for patients with type 2 diabetes to increase insulin production and insulin sensitivity. The total weight loss is usually 5 to 7 % with 10 % of patients losing more than 10 % of their weight. Compared to the other weight lose medications on the market the price of Saxenda® is relatively high. Side effects are mild and usually consist of nausea. A few cases of pancreatitis have been reported. (14-16)

Orlistat is a prescription-free tablet therapy that was approved by EMA in 1998. Orlistat is the only therapy option that does not directly decrease appetite. Instead it inhibits pancreatic lipases in the gastrointestinal tract. This results in an ineffective fat absorption and an overall reduced calorie intake. The total weight loss is usually around 3 % in obese patients. As this is a relatively old therapy it is sold as generic and the price is low compared to other weight loss therapies. The side effects are relatively mild but do usually include lose stool which can be prevented with a diet rich in fibers and low in fats. (14, 17)

Mysimba® is a tablet therapy that includes bupropion and naltrexone and is not yet widely used in Sweden to treat obesity. Mysimba® regulates dopaminergic stimulation in the central nervous system (CNS) to reduce food cravings and appetite. The total weight loss is usually around 5 % in obese patients. Side effects usually include nausea early in the treatment period. Some cases of epilepsy have been reported but ends with drug withdrawal. (14)

Bariatric surgery is today the only treatment option to achieve extensive long-term weight loss. The main objective for the surgery is to bypass the stomach which makes the patient to feel full after eating very little food. The patients also get improved glucose metabolism, an effect that occurs rapidly after surgery and probably is partly independent of the weight loss. The reasons for the beneficial effects on glucose metabolism of bariatric surgery are unknown, and are subjects of intense research. The Swedish Obesity Study (SOS) shows that

patients maintain a weight loss around 20 % ten years after bariatric surgery, although the individual variation is large. (18)

Unfortunately the complications of bariatric surgery are many and at least 15 % experience acute complications that need hospital care. All patients experiences some inconvenience from the abdomen. (19) Surgical patients are also at a higher risk for developing alcohol addictions and dangerous hypoglycemia (20, 21). Additionally to the before mentioned complications patients will have to change their lifestyles drastically with everyday vitamin supplements, regular check-ups and extensive skin scarring. Sometimes surplus skin has to be removed surgically. (21)

In summary surgical treatment is today superior to medicines when it comes to total weight loss. However none of the treatment options offer a good chance for returning to a normal BMI. Surgery is also a treatment option reserved for the young and healthy with a lot of potential complications. There is a need for better treatment options of obesity.

## 3.3. Energy Homeostasis

Most adults maintain a stable BMI over time even though food intake and physical activity varies on a daily basis. This is mediated through a system that balances energy intake and energy expenditure which includes basal metabolism, thermogenesis and physical activity. This system is called energy homeostasis. Energy homeostasis is mediated mostly by peripheral signals influencing the CNS. These signals may either stimulate appetite or suppress it and either act short-term or long-term. This system both comprise of orexigenic hormones that stimulate appetite and anorectic hormones which suppress appetite but also influence nerve projections both centrally and peripherally. (22)

Short-term signals that promote hunger arise from various visual, olfactory and somatosensory stimulations. These signals are supposed to regulate the food intake on a daily

basis. Pancreatic insulin, ghrelin and saliva are for example released when an individual see a particular eye-catching meal. This leads the individual to experience hunger and food cravings. (23) Once the food is consumed the gastrointestinal tract releases anorectic hormones and stimulate the vagus nerve in order to create satiety. These hormones include GLP-1, amylin, cholecystokinin (CCK) and peptide tyrosine tyrosine (PYY) to mention a few. (24, 25)

Long-term regulation of appetite is a signal system that is able to regulate body weight over time. These signals have one goal: To maintain the same weight as previously. This preprogrammed weight is referred to as an individual's set point. However the set point can be altered but under normal physiological conditions this takes at least several months of over eating or food deprivation. (26) One of the most potent anorectic hormones for long-term satiety is leptin (27, 28). Long-term hunger is instead signaled by areas in the brain directly. One of the most important areas is the lateral hypothalamic area (LHA) which needs to be inhibited by ventromedial hypothalamic nucleus (VMH) and the arcuate nucleus (ARC) in order for an individual to experience satiety (29-32). Although it should be noted that ARC contains both neurons that stimulate satiety and neurons that stimulate hunger (33).

Centrally, signals regulating appetite are both transmitted via neuronal projections by neuropeptides and also via hormones in the cerebrospinal fluid (CSF) (32, 34, 35). Some important orexigenic neuropeptides are neuropeptide Y(NPY) and agouti-related peptide (AgRP) (36). Some important anorectic neuropeptides are cocaine- and amphetamine-regulated transcript (CART), calcitonin gene-related peptide (CGRP) and melanocyte-stimulating hormone (MSH) (32). Interleukin-6 (IL-6) is another interesting anorectic neuropeptide (37). IL-6 is both present in the brain as a neuropeptide but do also exist in the CSF along with leptin (34, 35, 37).

Energy homeostasis is not always regulated by appetite in the CNS. Energy expenditure may be increased by peripheral mechanisms. One important peripheral mechanism is the activation of brown adipose tissue (BAT). This leads to an increased basal metabolism which in turn increases energy expenditure and promotes weight loss. BAT can be activated by inflammatory cytokines such as IL-6 in diseases with a chronic inflammatory state. This is especially true for cancer patients who are prone to lose body weight and developing what is called cancer cachexia. (38) This state of extensive energy wasting is linked with a bad prognosis. Cancer cachexia could be explained as being the opposite of obesity but involving much the same processes. (39) In truth not only BAT is responsible for cancer cachexia but it is also mediated by a loss of appetite (38).

This well-developed system for energy homeostasis may make one question how it is possible for an individual to develop obesity. Obesity develops when energy homeostasis fails, just like in the example of cancer cachexia above. Both peripheral and central signals are involved, but ultimately it is central signals that decides whether an individual will increase or decrease food intake. Interestingly it is mostly the anorectic hormones of the energy homeostasis that loses their effect in obesity (40, 41). This makes it very difficult to treat obesity long-term with diet and exercise alone (42).

## 3.4. Interleukin-6

IL-6 is a small protein with a molecular weight of approximately 26 kDa. It is most known for its role as a cytokine in the immune system. IL-6 is an important contributor to inflammation when released together with proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α). This is the case in acute infections or autoimmune diseases. (43) Tocilizumab is an antibody that target the receptor of IL-6 and can be used as a treatment of autoimmune diseases (44, 45).

Low levels of IL-6 are released by adipocytes, along with low levels of IL-1 and TNF-α. This is the reason to why obese individuals continually show a state of low grade inflammation. Chronic inflammation increases the risk of morbidity. One prominent example is the insulin resistance that accompanies inflammation which makes obese individuals more prone to develop type 2 diabetes. (46) The amount of released IL-6 and inflammation correlates to an individual's BMI (47). Another condition with high levels of IL-6 in blood serum is cancer cachexia where IL-6 is thought to promote the anorectic and possibly even the muscle wasting effect (44, 48).

However, when IL-6 is released without accompanying proinflammatory cytokines it may interestingly exert an anti-inflammatory effect (49). Peripherally this is the case in working skeletal muscle as a response to physical activity and also in hepatocytes where it promotes liver regeneration (50). Another important effect of IL-6 is an increase in glucose-dependent insulin sensitivity by release of GLP-1 and inhibition of appetite in the CNS (51). Wallenius et al. showed in 2002 that mice lacking IL-6 develop obesity and leptin resistance to a much larger extent than wild type mice. (52).

In the CNS IL-6 exists both in CSF and in various regions in the brain where it acts as a neurotransmitter. Stenlöf et al. found that levels of IL-6 in human CSF are negatively correlated to fat mass. In other word; a higher BMI corresponds to a lower concentration of IL-6 in CSF. This suggests that IL-6 in CSF promotes a lean body type and is a part of normal physiology. This is interesting as peripheral IL-6 is instead increased in obese subjects. (35) In the brain IL-6 is present in areas that regulate appetite such as the parabrachial nucleus (PBN) and hypothalamic nuclei but also in other brain regions (53, 54). IL-6 is mainly produced by neurons, astrocytes, microglia and endothelial cells in the CNS (55).

Two types of receptors to IL-6 exist: a transmembrane variant and a soluble variant found in serum and CSF. The transmembrane variant is thought to be most important in mediating the effects of IL-6 and is called interleukin-6 receptor-α (IL-6 Rα). The complex of IL-6 and IL-6 Rα dimerize with glycoprotein 130 (gp130) which activates the intracellular janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT3) pathway. One step of importance is the phosphorylation of STAT3 to phosphorylated STAT3 (pSTAT3). (49, 56)

The soluble receptor to IL-6 is activated by binding to the ligand IL-6, and then binds to transmembrane gp130 in the cell membrane. While transmembrane IL-6 Rα is found in limited number of cell types, transmembrane gp130 is widely expressed on many different cell types in the body. Thus, the soluble receptor to IL-6 can via transmembrane gp130 activate many cell types in the body lacking transmembrane IL-6 Rα that cannot be activated by IL-6 alone. This trans-signaling is for instance regarded to be of importance in conditions with systemic inflammation. (49, 56)

Functional IL-6 Rα is richly present on the inside of ventricles of mice, especially localized to the third ventricle close to an area known as median eminence (ME). The ME is part of the hypothalamus and close to the ARC. Activation of so called tanycytes in the ME by the induction of pSTAT3 or activation of nerve fibers localized to the ME may be how IL-6 exerts its anorectic effects. (57) IL-6 directly injected into the ventricles of rodents has a strong anorectic effect (35, 58).

Antibodies such as tocilizumab can be used to temporary inhibit IL-6 Rα. However, in order to achieve long-term blockade of IL-6 Rα in mice it is more effective to use the Cre–lox recombination system. (37) Using this technique so called Lox-sites are introduced by transformation on both sides of the IL-6 Rα gene. Lox-sites are short DNA-fragments which

can be manipulated in several ways. There will for example be an excision of the IL-6 Rα gene if lox sites on both sides of this gene react with a TAT-CRE recombinase. (37, 59)

## 3.5. Glucagon-like Peptide-1

GLP-1 is produced peripherally in both gastrointestinal L-cells and pancreatic alpha-cells, and centrally in the nucleus of the solitary tract (NTS) of the hindbrain. It is derived from proglucagon which is a protein that is present in several different tissues throughout the human body. However, in L-cells and the brain this protein is cleaved by proprotein convertase 1 (PC1/3) into glicentin, GLP-1 and glucagon-like peptide-2 (GLP-2). (60-62) GLP-1 is a short-acting hormone with a half-life of between 1.5 to 5 minutes. This rapid degradation is due to an enzyme known as dipeptidyl peptidase-4 (DPP-4) that cleaves active GLP-1 into inactive GLP-1. (63) Long lasting analogues to GLP-1 are used to treat obesity and type 2 diabetes because of this short half-life (16).

Peripherally it is produced along with glucose-dependent insulinotropic peptide (GIP) one of only two hormones belonging to a group of hormones called incretins. Incretins are defined as being able to decrease blood sugar in a glucose-dependent manner. GLP-1 enters pancreatic beta cells along with glucose to increase the release of insulin after a meal. (60) This glucose-dependent release of insulin is one of the reasons why analogues to GLP-1 are considered relatively safe. There is in contrast to many other medicines for type 2 diabetes a very low risk of developing hypoglycemia. (64) Peripherally GLP-1 also decreases the production of glucagon in the liver and slows down gastric emptying (60) .

It is discussed whether the role of GLP-1 differs between physiological conditions and pathological conditions with much inflammation. GLP-1 is not only released from L-cells and alpha-cells as a response to macronutrients or vagus stimulation but also by stimulation of IL-6. With low physiological levels of IL-6 stimulation this results in enhanced insulin secretion

and glycemic control. This is for example the case when performing physical activity. (62) However, patients experiencing acute infections such as sepsis with high levels of IL-6 circulating in plasma may experience a 7-fold increase in GLP-1 secretion. This in turn results in hyperinsulinemia and lowering of blood glucose implicating that GLP-1 may have a key role helping the immune system fighting infections. Interestingly this increase in GLP-1 seems to be dependent on IL-6 and is not present in IL-6 knockout (KO) mice or with stimulation by other cytokines such as interleukin-1 beta (IL-1β) or tumor necrosis factor-alpha. (51)

Peripheral GLP-1 activates NTS in the hindbrain (65). NTS is also activated by illness inducing substances such as lithium chloride and to taste stimuli through various peripheral signals including vagus nerve stimulation (66, 67). NTS response to activation is a local production of GLP-1 which in turn stimulates excitatory glutamate transmission. (68-70). Neurons from NTS project to two different systems: the appetite regulatory system and the mesolimbic reward system (69, 71).

As for the appetite regulatory system, NTS sends excitatory signals to the parabrachial nucleus (PBN). PBN is a strong anorectic nucleus located to dorsolateral pons. (70) NTS projections increase the expression of CGRP in PBN. Richard et al. recently showed that IL-6 is present in the PBN. Richard et al. also injected a GLP-1 analogue directly into lateral PBN in mice and noted that the mice decreased in body weight compared to controls. Interestingly IL-6 was increased 6-fold, which is a four times higher increase than that of CGRP. This suggests that there are GLP-1 receptors (GLP-1R) in PBN and GLP-1 may exert effects directly on the area mainly by the increase of IL-6 (69).

Peripheral GLP-1 administration also turns ARC into anorectic mode by increasing proopiomelanocortin (POMC) and CART transcription while decreasing AgRP and NPY

(72). ARC does usually transmit inhibitory γ-aminobutyric acid (GABA) signals to PBN which is decreased during GLP-1 administration (72-75). This further contributes to the anorectic effects of PBN. This system is extremely powerful. Loss of all GABA transmission from ARC to PBN leads to death by starvation in rodents. (75) There also exist nerve fibers from NTS that terminate in the hypothalamus suggesting that NTS may interfere with the hypothalamic appetite system directly (76).

PBN projects with GABAergic nerve fibers to the central nucleus of the amygdala (CeA) where the sensation of experiencing satiety may be exerted (77). As a response anorectic CGRP is released into the CeA from nerve terminals (78, 79). It is today unknown whether IL-6 exerts anorectic effects directly on the amygdala but experiments are ongoing.

NTS do also project to structures in the mesolimbic reward system. These structures include the dopamine-containing ventral tegmental area (VTA) and the nucleus accumbens (NAc). Activation of these projections has been shown to decreases food-reward and food-motivated behavior in rodents, but also decreases reward of beverages such as alcohol. (71, 80) The neural circuits regulating appetite activated by GLP-1 are illustrated in Figure 1.

It is unknown whether GLP-1 exists in human CSF during physiological conditions. Studies have shown the presencee of GLP-1 in cat and rat CSF but only after cerebral implantation of encapsulated mesenchymal stem cells which is far from physiological conditions (81, 82). Only one study claims to have found GLP-1 in CSF during physiological conditions. This study was performed on rats, and GLP-1 was analyzed with a high sensitivity enzyme-linked immunosorbent assay (ELISA). However, the results remain unclear as the concentration of GLP-1 measured in CSF was just slightly above the detection limit of the assay and the results have not been replicated. (83)

To our knowledge no studies that measured GLP-1 in human CSF has been published, neither during physiological or pathological conditions. However, accumulating evidence indicates that GLP-1 analogues such as liraglutide does act by crossing the blood CSF barrier but does not enter the CSF (84, 85). DPP 4 exists in human CSF although the concentration seem to be low compared to that of serum (86).

Even though it is unclear about the existence of GLP-1 in human CSF it has been studied what happens when GLP-1 is injected in the CSF of mice. Shirazi et al. found that injections of GLP-1 analogue exendin-4 (Ex-4) into the lateral ventricle of mice led to decreased food intake. Interestingly, this effect was reversed when adding a TAT-CRE fusion protein that removed IL-6 Rα from the inside of the ventricles. Furthermore the authors discovered that intraventricular injections of Ex-4 increased the levels of IL-6 in the hypothalamus and hindbrain. It is not known if this effect could occur during physiological conditions or if it is purely pharmacological as it is unknown whether GLP-1 is present in CSF or not. (37)

Along with other proteins that are cleaved from PPG, GLP-1 binds to a G-protein–coupled receptor (GPCR). The GLP-1 receptor (GLP-1R) is made up by 7-transmembrane-spanning regions and do when phosphorylated by GLP-1 induce an intracellular messenger pathway (60). This pathway is much like the one of other GPCR pathways and does include phosphorylation of STAT3 into pSTAT3 (87). Peripherally the receptor is mostly expressed on various cells of the pancreas, liver and gastrointestinal system but is also present in cells of the lung, heart, kidney, skin and on peripheral nerves. (60) In the brain of rodents, the receptor is abundant around the ventricles, but also in specific areas of appetite regulation (88). However the GLP-1R does not seem to be present directly on the inside of the ventricles (89).

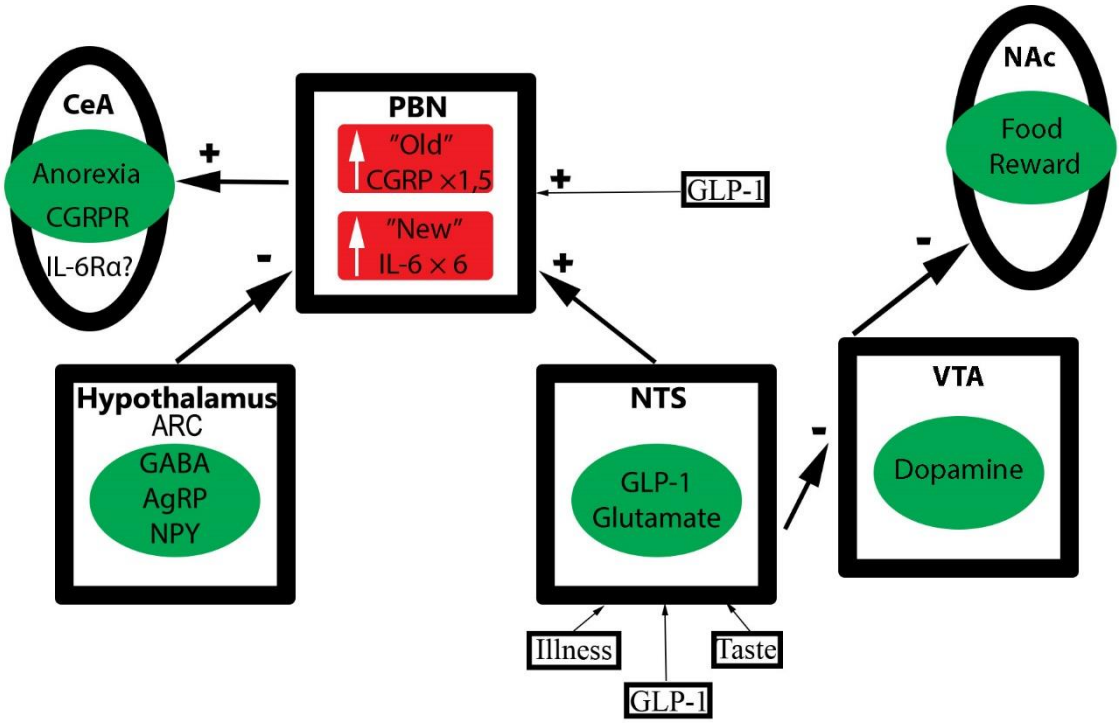


**Figure 1.** This figure illustrates the central signaling of GLP-1 that is known to date. Peripheral GLP-1 stimulates the Nucleus of the Solitary Tract (NTS) and arcuate nucleus (ARC) in hypothalamus. This increases the activity in the anorectic parabrachial nucleus (PBN) by transcription of interleukin-6 (IL-6) and calcitonin gene-related peptide (CGRP). The transcription of IL-6 in PBN is a relatively new finding (2013) compared to CGRP. The sensation of satiety resulting in anorexia and food avoidance is then generated by the central nucleus of the amygdala (CeA). NTS does also project to the ventral tegmental area (VTA) and the nucleus accumbens (NAc) which decreases food reward.

## 3.6. Leptin

Many potential medical cures for obesity has been tested during the history. However, they all failed due to lack of effect or adverse side effects. When Coleman and Friedman discovered leptin in 1994 many thought that things would finally change and a new era of treating obesity would begin (90). Leptin is translated from the obese (ob) gene in adipocytes and is then released into blood. The response to release of leptin is extremely efficient and mice consume five times more calories if a loss of function mutation occurs in the ob gene. (91, 92) Mutations in the human ob gene are rare but are associated with extreme obesity (93).

Leptin decreases appetite and inhibits decreases in energy expenditure while losing weight. Unfortunately, the serum levels of leptin also decrease with weight loss as leptin is secreted by adipocytes. The basal metabolism and thermogenesis is hence normally depressed in proportion to lower leptin levels when an individual loses weight. As a result, humans tend to maintain a stable weight over time. (94, 95) However this may partially be prevented by leptin therapy (96). This correlation between energy expenditure and leptin is not always present in obese subjects, probably as a result of leptin resistance (97). Furthermore leptin increases insulin sensitivity and is necessary for the menstrual cycle to function correctly (98, 99).

Leptin resistance is an inconvenient phenomenon that occurs in most obese individuals and renders leptin therapy useless. Leptin resistance is also the main reason for obese subjects to experience an increased hunger and to continue eating. (100) The reason for leptin resistance has not been fully elucidated and so far no one has been able to reverse leptin resistance. However, endurance exercise in rodents seems to have reversed leptin resistance partially but far from completely (101). Various theories of the origin of leptin resistance have been discussed. In summary obesity is correlated with increased levels of leptin and triglycerides which creates an inflammation in important areas of the hypothalamus regulating appetite, such as ARC. Neurons in these areas are damaged and replaced by astrocyte scar tissue as this inflammation progresses creating desensitization. A decreased transportation of leptin into the brain is also seen in leptin resistance. (102-104)

Leptin is a small protein of 16 kDa and able to cross the blood CSF barrier. The entrance to CSF is thought to be through circumventricular organs such as the ME which is in close contact to fenestrated microvessels. However, this mechanism is also decreased in obesity and further contributes to leptin resistance. (41, 105, 106) It then crosses the ME in the dorsal part of the third ventricle in order enter the brain. This is mediated by uptake of special ependymal cells known as tanycytes. Leptin tends to get stuck inside of tanycytes in leptin resistance. The

ME is closely situated to the ARC where leptin exerts its effects. (107) Leptin activates POMC in the ARC and downregulates AgRP and NPY which turns it into anorectic mode (108, 109).

The leptin receptor is also known as the obese receptor (OB-R). The OB-R is present on the choroid plexus and on circumventricular organs such as the ME. It is especially abundant on tanycytes. (106, 110) The OB-R is also expressed on neurons in appetite regulating parts of the brain such as the ARC (109). Activation of the OB-R leads to a similar intracellular signaling pathway as that of the IL-6 Rα. This includes the JAK and STAT3 pathway and phosphorylation of STAT3 into pSTAT3 (111, 112).

## 3.7. Tanycytes

Tanycytes are special ependymal cells that line the walls of the ventricles. Their primary role is to regulate the uptake of nutrients and proteins into the brain and CSF. One subdivision of tanycytes known as beta-tanycytes is primarily present in the ME. (107) They express both IL-6 Rα and the OB-R receptor on the surface. Some data suggests that leptin therapy induces the JAK and STAT3 pathway in tanycytes. (57, 107) However, the GLP-1R does not seem to be present on beta-tanycytes (89).

Tanycytes are morphologically shaped as long thin branches in order to fulfill their roles as gatekeepers of the CNS (107). Tanycytes are exposed to shear stress in areas such as the ME where it is only a few layers of cells between the CSF and bloodstream. As tanycytes need to withstand this shear stress they have a well-developed cytoskeleton which is rich in the protein vimentin. This is especially true for the beta-tanycytes located to the ME. (113) The location of tanycytes are similar in mice and humans (114, 115).

# 4. Overall Aim and Specific Objectives

The overall aim of the study was to further elucidate how the anorectic peptides IL-6, GLP-1 and leptin signal via the CSF. Specifically we hypothesize that:

a) GLP-1 is present in human CSF.

b) IL-6 Rα on tanycytes in mice are necessary for the anorectic effect of leptin.

c) Intraperitoneal (IP) administration of IL-6, GLP-1 and leptin in mice activate tanycytes in the ME.

d) Intracerebroventricular (ICV) administration of IL-6, GLP-1 and leptin in mice activate tanycytes in the ME.

# 5. Methods

## 5.1. Study Design

The study was done in three steps. The first step consisted of measuring the concentration of GLP-1 in 40 human CSF samples using a high sensitivity enzyme-linked immunosorbent assay (ELISA). This concentration was then compared to that of human serum. The second step consisted of three days of leptin therapy to 16 mice in which IL6 Rα had been reduced at the inside of the ventricles using a TAT-CRE recombinase. The parameters measured were body weight and food intake. We also performed quantitative polymerase chain reaction (PCR) on the choroid plexus to evaluate how large the reduction of the IL-6 Rα was after the TAT-CRE treatment. The third step consisted of IP and ICV injections of IL-6, GLP-1, leptin and saline in a total of 16 mice. We then used immunohistochemistry (IHC) to evaluate activation of tanycytes 15 minutes after the injections.

## 5.2. Human Cerebrospinal Fluid and Serum Analysis

We used 40 human CSF samples stored at the Mölndal Hospital in Gothenburg. Samples were collected from patients with a finished medical investigation without any pathological findings. The samples were anonymized, stored at -80°C and no more than 30 days old. 10 µl of a DPP-4 inhibitor (10µl/ml, DPP4-010, Merck Millipore; St Charles, U.S.) was instantly added to 14 of the samples upon defrosting. 50 µl of each sample were then used to assess the concentration of GLP-1. 27 of the samples, including the 14 samples with a DPP-4 inhibitor, were used to assess the concentration of active GLP-1. This was done using ELISA (EGLP-35K, Merck Millipore; St Charles, U.S.) according to the manufacturer's protocol. The remaining 13 samples were used to assess the concentration of total GLP-1, including both the active and the inactive cleaved variant of GLP-1. This was done using ELISA (EZGLP1T-36K, Merck Millipore; St Charles, U.S.) according to the manufacturer's protocol.

We also used 50 µl of blood serum samples from 27 random individuals stored in the same biobank to assess the concentrations of active GLP-1. This was to appreciate the ratio between the concentration of GLP-1 in CSF and that of serum. 14 samples were analyzed after quick addition of a DPP-4 inhibitor using the previously mentioned ELISA (EGLP-35K, Merck Millipore; St Charles, U.S.). Furthermore we analyzed total GLP-1 in serum samples. This was done in serum from the same 13 individuals we assessed total GLP-1 in CSF in, also using the same ELISA as before (EZGLP1T-36K, Merck Millipore; St Charles, U.S.).

## 5.3. Animals

This study was performed with male and female mice of the strain C57BL6. Two different genotypes were used: wild type (WT) and IL-6 Rα lox (IL-6 $R\alpha^{lox/lox}$), both from Jackson Laboratories, Bar Harbor, Maine, USA. The mice were four to six months old and kept under standardized conditions with a 12-hour light/12-hour dark cycle with free access to water and standard chow pellets (Tekland Global; Harlan, the Netherlands).

## 5.4. Drug Injections

Subcutaneous (SC), IP and ICV injections were performed on C57BL6 mice in this study. SC injections were performed in the scruff of the neck. IP injections were placed, after aspiration, in the lower left quadrant of the abdomen.

For ICV-injections mice were anesthetized with a continuous flow of 3 % isoflurane gas (Baxter; Deerfield, USA). The mice were then fixed in a model 900 stereotax (Kopf, St. Julians, UK). The head was shaved and sterilized with chlorhexidine and the epicranial aponeurosis was split with a dorsal to anterior line using a scalpel. Bregma and lambda are two specific fontanelles on the mice skull that can be visualized macroscopically. The skulls of the mice were lined straight by placing bregma and lambda in the same vertical coordinate. An injection syringe (80366, Hamilton; Reno, Nevada, U.S.) was fixed in an injection pump

(KDS-310-PLUS, GENEQ Inc, Québec, Canada) and moved 1.0 mm right and 0.3 mm posterior of bregma. A small hole was drilled in the skull at this location to allow passage of the syringe into the brain. The syringe was then slowly moved 2.3 mm ventrally and 1.5 µl of the specific substance was injected with a velocity of 0.75 µl per minute. The syringe was left inside of the ventricle for 3 minutes after the injection was finished and then withdrawn slowly. A small volume of CSF could be seen inside the injection hole as a result of successful puncture of the lateral ventricle. The epicranial aponeurosis was sutured and mice were placed in a cage on a heating pad until fully awaken.

## 5.5. Leptin Therapy in IL-6 Rα Knock-down Mice

### 5.5.1. Knock-down of IL-6 Rα Inside of the Ventricles

Knock-down by injecting TAT-CRE into a mouse with lox sites is a well-known method to remove a specific gene, in this case IL-6 Rα. locally 1,5 µl of a TAT-CRE recombinase with a concentration of 4,5 mg/ml (EG-1001, Excellgen; Rockville, U.S.) was directly injected into the right lateral ventricle of mice as described above. This was performed on a total of 32 mice: 16 WT mice and 16 IL-6 $R\alpha^{lox/lox}$ mice. The Mice were then placed in individual cages for seven days recovery before any drug treatment started. Two mice died after the ICV injections leaving 30 mice to be randomized to either leptin therapy or saline therapy. IL-6 $R\alpha^{lox/lox}$ mice that received TAT-CRE is referred to as IL-6 Rα knock-down mice.

### 5.5.2. Leptin Therapy

After seven days of recovery mice were randomized to get SC treatment with either 0.1 ml of murine leptin with a concentration of 300 ng/µl (450-31, PeproTech; Rocky Hill, U.S.) or 0.1 ml of 0.9 % saline (B. Braun; Melsungen, Germany). In total 8 WT mice got treatment with leptin and 7 WT mice got treatment with saline. Furthermore 8 IL-6 Rα knock-down mice got treatment with leptin and 7 IL-6 Rα knock-down mice got treatment with saline, thus

including a total of four groups in the experiment. Each injection was given twice a day with a 12-hour interval in-between. Injections proceeded for three days. Body weight and food intake was monitored every morning at 8 AM during the treatment period.

### 5.5.3. Gene expression analysis of IL-6 Rα

Mice were anesthetized with a 0.2 ml IP injection of a mix of Domitor®Vet (0.5 ml, 1 mg/ml, Orion Pharma, Espoo, Finland), Ketalar®Vet (0.75 ml, 50 mg/ml, Intervet, Boxmeer, Netherlands) and saline (0.75 ml, 0.9 %) four days after the start of leptin therapy. Mice were then sacrificed using decapitation. Brains were removed instantly after decapitation and placed in 0.9 % saline. The choroid plexus and frontal cortex were dissected and placed in 0.5 ml tubes (D-51588, Sarstedt; Nümbrecht, Germany). Tubes were instantly put in liquid nitrogen and stored at -80°C until analysis.

We used RNeasy® Micro Kit (QIAGEN, Hilden, Germany) to extract RNA from the choroid plexus and RNeasy® Lipid Tissue Mini Kit (QIAGEN; Hilden, Germany) to extract RNA from cerebral cortex, both according to the manufacturer's protocol. The concentration of RNA in ng/µl for each sample was determined by using Nanodrop Spectrophotometer ND-1000 (Saveen Werner; Limhamn, Sweden). We continued by using 50 ng of RNA from each sample to synthesize complementary DNA (cDNA) by the use of iScript™ cDNA Synthesis Kit (BIO-RAD; Hercules, U.S.).

Concentrations of IL-6 Rα cDNA was then assessed with a real-time polymerase chain reaction (RT-PCR) QuantStudio 7 Flex (Applied Biosystems; Foster City, U.S.). We used a fast process with a 96-well plate. Each sample was analyzed in duplicates and concentrations of cDNA were calculated as the mean value of the two wells. TaqMan Fast Advanced Master Mix, TaqMan Gene Expression Assays (Gusb (Mm00446953_m1) and IL-6 Rα (Mn01211444_g1)) and nuclease-free water were added before the analysis according to the

manufacturer's protocol (all reagents from Applied Biosystems; Foster City, U.S.). Gusb (beta-glucuronidase) is present in both choroid plexus and cerebral cortex and was used as a reference gene for both tissues. PCR was done identically for both choroid plexus and cerebral cortex.

The differences between the concentration of Gusb cDNA and IL-6 Rα cDNA were calculated for each sample to estimate the gene expression of IL-6 Rα by the ΔΔCT method (116).

## 5.6. Immunohistochemistry of Tanycyte Activation

### 5.6.1. Drug Treatments

WT mice of the strain C57BL6 was injected with either IP or ICV injections. Two mice received each treatment. For IP injections, we used either 0.1 ml of the GLP-1 analogue exendin-4 (Ex-4) with a concentration of 300 ng/µl (1933, Tocris; Bristol, UK) 0.1 ml of leptin with a concentration of 300 ng/µl (450-31, PeproTech; Rocky Hill, U.S.), 0.1 ml of IL-6 with a concentration of 500 ng/µl (216-16, PeproTech; Rocky Hill, U.S.) or 0.1 ml of 0.9 % saline (B. Braun; Melsungen, Germany). For ICV injections, we used either 1.5 µl of Ex-4 with a concentration of 300 ng/µl, 1.5 µl of leptin with a concentration of 300 ng/µl, 1.5 µl of IL-6 with a concentration of 500 ng/µl or 1.5 µl of artificial CSF (aCSF) (3525, Tocris; Bristol, UK).

### 5.6.2. Tissue preparation

15 minutes after drug injection mice were deeply anesthetized with 0.2 ml of the Dormitor-Ketalar mix as described previously. Mice were then pinned to a styrofoam board and perfused transcardially with heparinized saline (50 IU/ml) for approximately two minutes followed by ten minutes of perfusion with PFA buffer (0.1 M phosphate buffer, 4 % paraformaldehyde). The brains were carefully dissected and placed in a short-term storage

solution (0.1 M phosphate buffer, 4 % paraformaldehyde, 15 % sucrose) overnight at 4°C. The brains were then transferred to a long-term storage solution (0.1 M phosphate buffer, 30 % sucrose) and stored at 4°C until sectioning.

The brains were sectioned into 20 µm thick coronal sections within a month using a Leica CM3050S cryostat (Leica Microsystems; Wetzlar, Germany). Sections containing appetite regulating parts of the hypothalamus and the third ventricle were chosen and transferred to 2.0 ml tubes (72.694.006, Sarstedt; Nümbrecht, Germany) with tissue storage solution (0.1 M phosphate buffer, 25 % ethylene glycol, 25 % glycerol) and stored at 4°C until IHC.

### 5.6.3. Immunohistochemical staining

IHC was performed within one month after sectioning. The sections were rinsed in washing buffer (0.1 M Tris-HCl, pH 7,5, 0.15 M NaCl, 0.2 % Triton-X-100) during 15 minutes for a total of three times and then stored in 0.2 ml TNB blocking buffer (1:100, TSA System, Perkin Elmer; Waltham, U.S.) for 1 h. 3 µl of diluted primary antibodies (see Table 1) was added and the sections were incubated for two days in 4°C.

Sections were once again rinsed in washing buffer during 15 minutes for a total of three times after the incubation. Sections were transferred to wells with 1 ml of TNB blocking buffer. 3 µl of diluted secondary antibodies (see Table 1) were added and the sections were incubated for 1 h. Sections were then rinsed in washing buffer during 15 minutes for a total of three times. Finally the nuclei of the sections were stained with 1 µl 4',6-diamidino-2-phenylindole (DAPI) (1:5000, D1306, Molecular Probes; Carlsband, U.S.) and rinsed once in another washing buffer (0.1 M Tris-HCl, pH 7,5, 0.15 M NaCl).

Sections were mounted on slides (Superfrost Plus, 4951PLUS, Thermo Scientific; Carlsband, U.S.) with prolong gold antifade (P36930, Molecular Probes; Carlsband, U.S.). Mounted sections were kept away from light and stored at 4°C until analysis to prevent loss of the

fluorescence. Sections were viewed with microscope within a week and assessed in a qualitative way.

**Table 1.** This table illustrates the antibodies used for immunohistochemistry.

| Antibody | Type | Sections Treatment | Color | Cat. No. | Manufacturer |
|---|---|---|---|---|---|
| Rabbit anti-PSTAT3 | Primary | All sections | - | 9131 | Cell Signaling Technology; Danvers, U.S. |
| Chicken anti-vimentin | Primary | All sections | - | AB5733 | Merck Millipore; Billerica, U.S. |
| Goat anti-rabbit Alexa fluor 488 | Secondary | IP injections | Green | A-11008 | Molecular Probes; Carlsbad, U.S. |
| Goat anti-rabbit Alexa fluor 568 | Secondary | ICV injections | Red | A-11036 | Molecular Probes; Carlsbad, U.S. |
| Goat anti-chicken Alexa fluor 488 | Secondary | ICV injections | Green | A-11039 | Molecular Probes; Carlsbad, U.S. |
| Goat anti-chicken Alexa fluor 594 | Secondary | IP injections | Red | A-11042 | Molecular Probes; Carlsbad, U.S. |

### 5.6.4. Microscopy

All stained sections were viewed in a fluorescence microscope equipped with a camera (Leica Microsystems GmbH, Wetzlar, Germany). Sections showing the ME and with successful staining were chosen. Images of the sections were either taken with this fluorescence microscope or by using a confocal microscope to earn better resolution (LSM 700; ZEISS, Oberkochen, Germany). Images were taken for each color, merged and then assessed in a qualitative fashion.

# 6. Data Collection Procedures and Statistical Methods

The concentration of total GLP-1, active GLP-1 and active GLP-1 after addition of a DPP-4 inhibitor was measured in undiluted CSF. This was done in a total of 40 samples. The concentration of samples with a concentration of GLP-1 below the detection limit of 2 pM was set as 2 pM. We used non-parametrical Mann Whitney-U test in IBM SPSS Statistics 24® to compare this concentration to that of serum. $P<0.05$ was considered statistically significant. All data are presented as means ± SEM.

We monitored weight and food intake for three days in 8 WT and 8 IL-6 Rα knock-down mice receiving leptin therapy. We also monitored weight and food intake for three days in 7 WT and 7 IL-6 Rα knock-down mice receiving saline therapy. Weight was expressed as percentage of body weight at the start of the therapy and food intake was expressed as cumulative food intake in grams. We used two-way analysis of variance (ANOVA) in IBM SPSS Statistics 24® to assess the significance between all four groups at the third day of the experiment. The knock-down of IL-6 Rα was assessed with student's t-test in Microsoft Excel®. $P<0.05$ was considered statistically significant. All data are presented as means ± SEM.

Brain sections were stained with fluorescent colors of green, red and blue. A picture was taken of each color by filtering wavelengths. The different colored images were then merged into one so called *overlay picture* by the use of a program called ImageJ® (NIH, 2017). Both images from fluorescence microscopy and confocal microscopy were used. The IHC images were analyzed in a qualitative way without statistical methods. The analyze was not blinded.

# 7. Ethics

This study included both human samples and animal experiments. Human sampling was approved by the regional ethics committee of Gothenburg and followed the provisions of good clinical practice stated by the Helsinki Declaration. Written informed consent was obtained from all patients to utilize CSF and blood samples for research purposes. All animal procedures were approved by the local ethics committee for animal care at the University of Gothenburg and were conducted in according to the ethics committee's guidelines.

# 8. Results

## 8.1. GLP-1 Levels are too Low to be Measurable in Human CSF

The concentration of active GLP-1 (Figure 2) and that of total GLP-1 (Figure 3) was below the detection limit of 2 pM in all CSF samples. Active GLP-1 was present in 5 of the 14 serum samples (Figure 2). We found detectable concentrations of total GLP-1 in all serum samples (Figure 3).

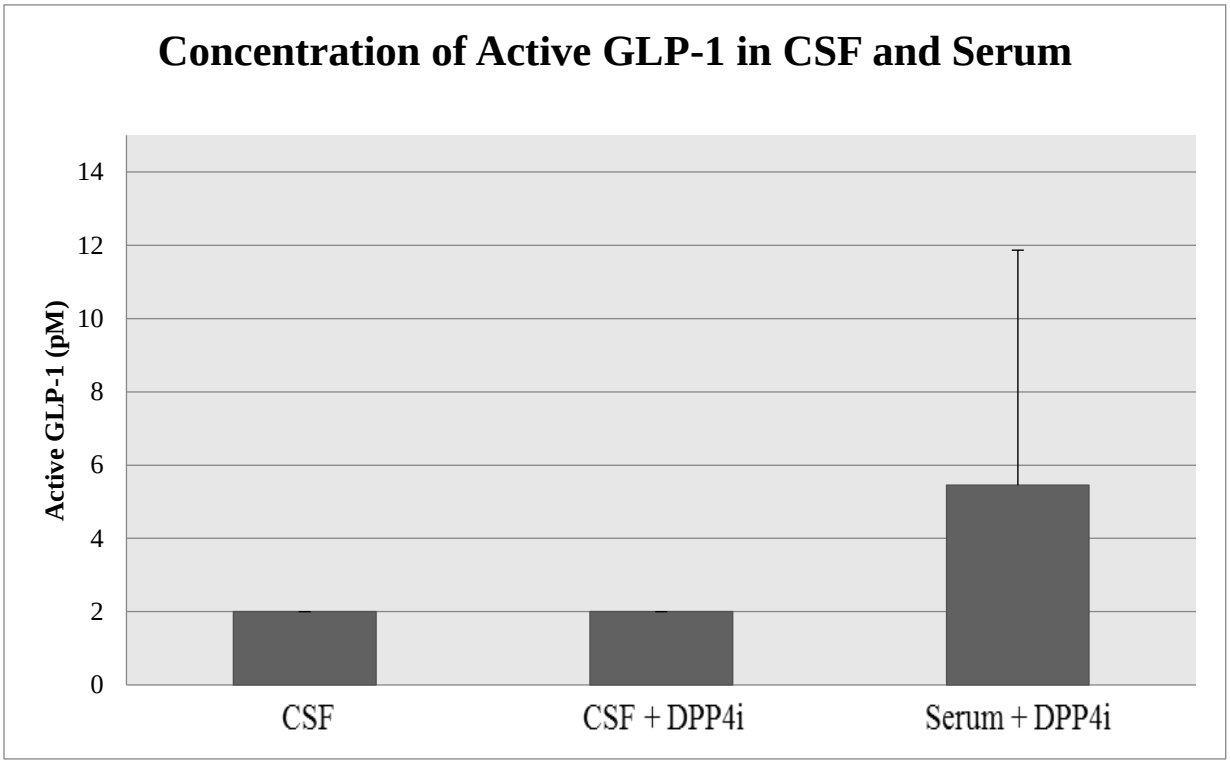


**Figure 2.** Concentration of active GLP-1 in CSF ( n=13), in CSF after the addition of a DPP-4 inhibitor (DPP4i) (n=14) and in serum after the addition of a DPP-4 inhibitor (n=14).

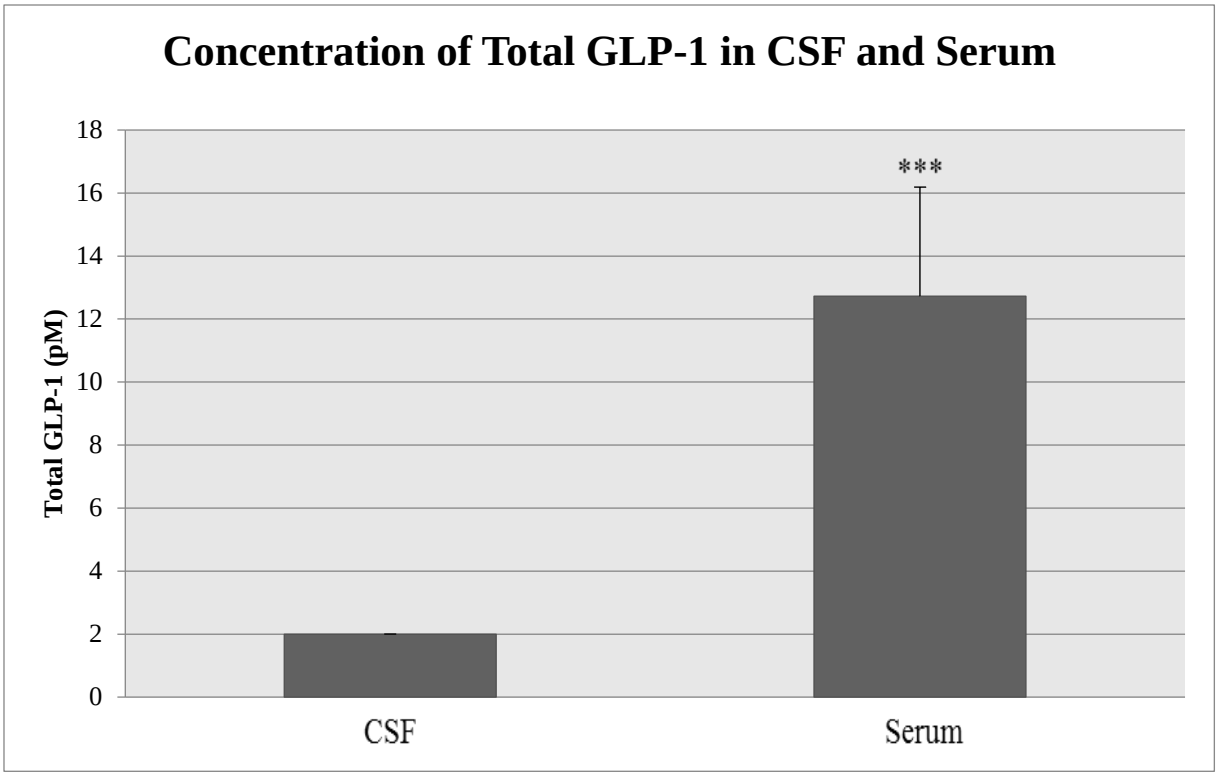


**Figure 3.** Concentration of total, i e the sum of the active and inactive forms, glucagon-like peptide-1 (GLP-1) in CSF (n=13) and in serum (n=13) from the same individuals. ***P<0.001.

## 8.2. IL-6 Rα Knock-down does not Influence the Effect of Leptin in Mice

There was no significant difference in body weight (Figure 4) or food intake (Figure 5) between IL-6 Rα knock-down (IL-6 $R\alpha^{lox/lox}$ mice injected with TAT-CRE) and control mice given leptin treatment. Leptin treatment significantly decreased bodyweight (Figure 4) and food intake (Figure 5) in both IL-6 Rα knock-down and control mice. Body weight was calculated as percentage of the body weight when the treatment started (Day 0). Food intake was calculated as cumulative food intake in grams. IL-6 Rα mRNA expression was analyzed two weeks after TAT-CRE injection and we found the knock-down of IL-6 Rα mRNA to be approximately 34 % in the choroid plexus (Figure 6). No difference was observed in IL-6 Rα mRNA in the cerebral cortex (Figure 7).

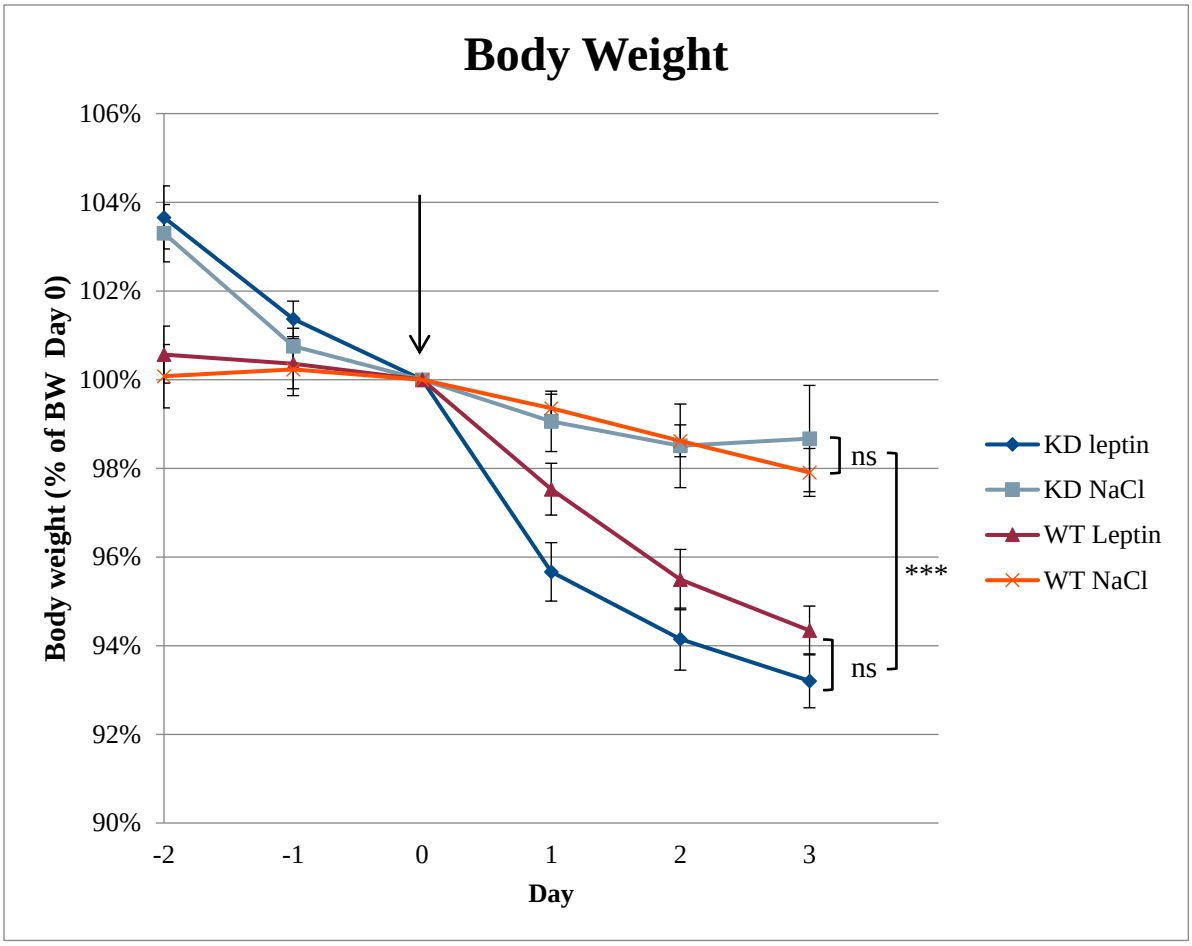


**Figure 4.** Body weight before and during injections of either saline (NaCl) or leptin were given to IL-6 Rα knock-down mice (KD NaCl, n=7; KD leptin, n=8) and WT mice (WT NaCl, n=7; WT Leptin, n=8). Injections started at day 0 and are marked by an arrow. ns=not significant, ***P<0.001

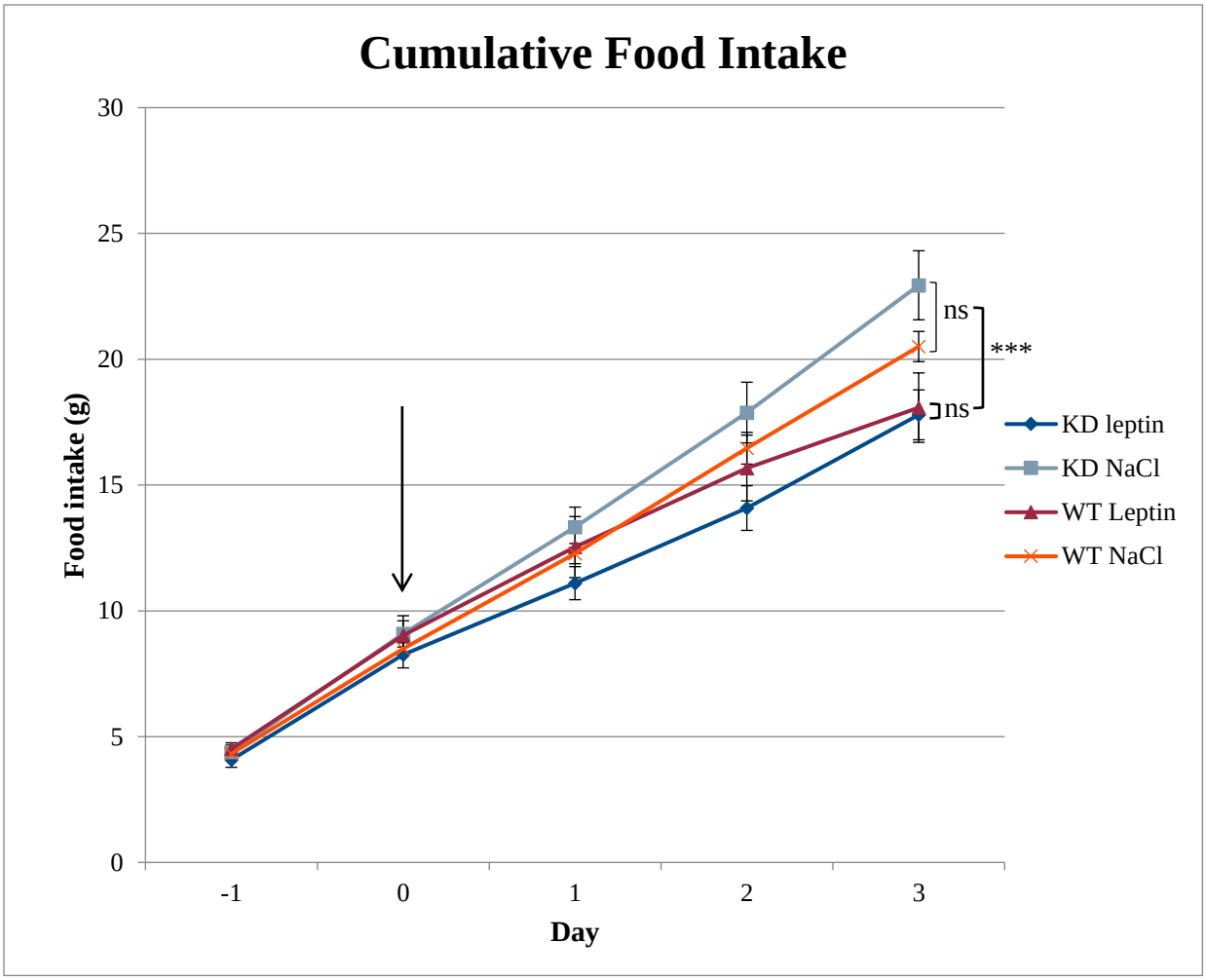


**Figure 5.** This figure shows cumulative food intake. Injections of either saline (NaCl) or leptin were given to IL-6 Rα knock-down mice (KD NaCl, n=7; KD leptin, n=8) and WT mice (WT NaCl, n=7; WT Leptin, n=8). Injections started at day 0 and are marked by an arrow. ns=not significant, ***P<0.001

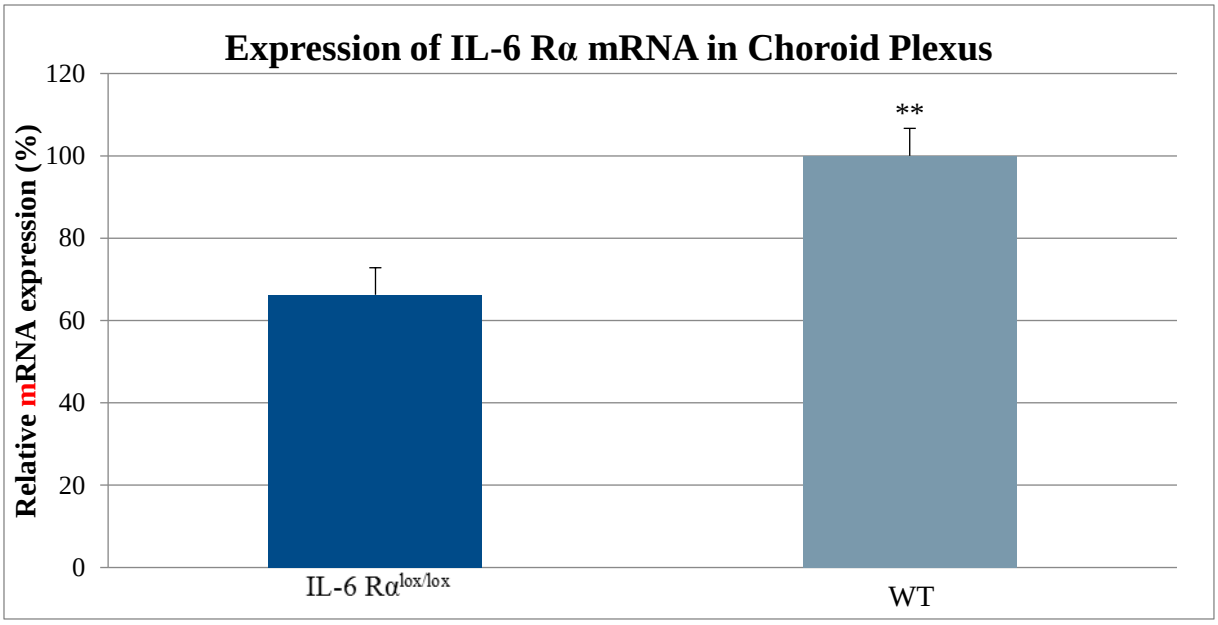


**Figure 6.** mRNA expression of IL-6 Rα in the choroid plexus of IL-6 $R\alpha^{lox/lox}$ (n=15) and WT mice (n=15). mRNA levels of IL-6 Rα in WT mice were set as 100 %. **P<0.01. IL-6 Rα mRNA expression is shown as fraction of IL-6 Rα mRNA in wild type mice.

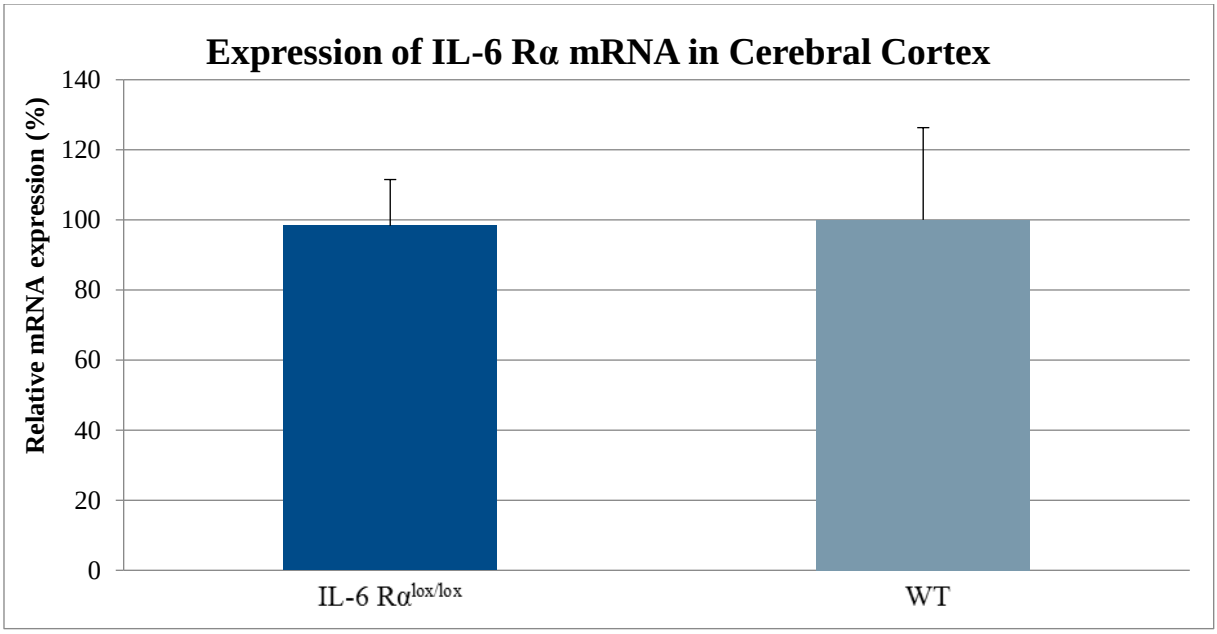


**Figure 7.** mRNA expression of IL-6 Rα in the cerebral cortex of IL-6 $R\alpha^{lox/lox}$ (n=15) and WT mice (n=15). mRNA levels of IL-6 Rα in WT mice were set as 100 %. IL-6 Rα mRNA expression is shown as fraction of IL-6 Rα mRNA in wild type mice.

## 8.3. IP Injections of Leptin Activates the Tanycytes n Mice

We compared the anatomy of mouse brain sections to that described in a renowned mouse brain atlas to locate the ME (bregma -1.70 to -2.00) (117). The area used for images are shown in Figure 8. No activation was observed after IP injection with IL-6, Ex-4 or saline (Figure 9A, B, D). However, tanycytes in the ME were activated upon IP injections with leptin (Figure 9C). Representative sections are shown in Figure 9.

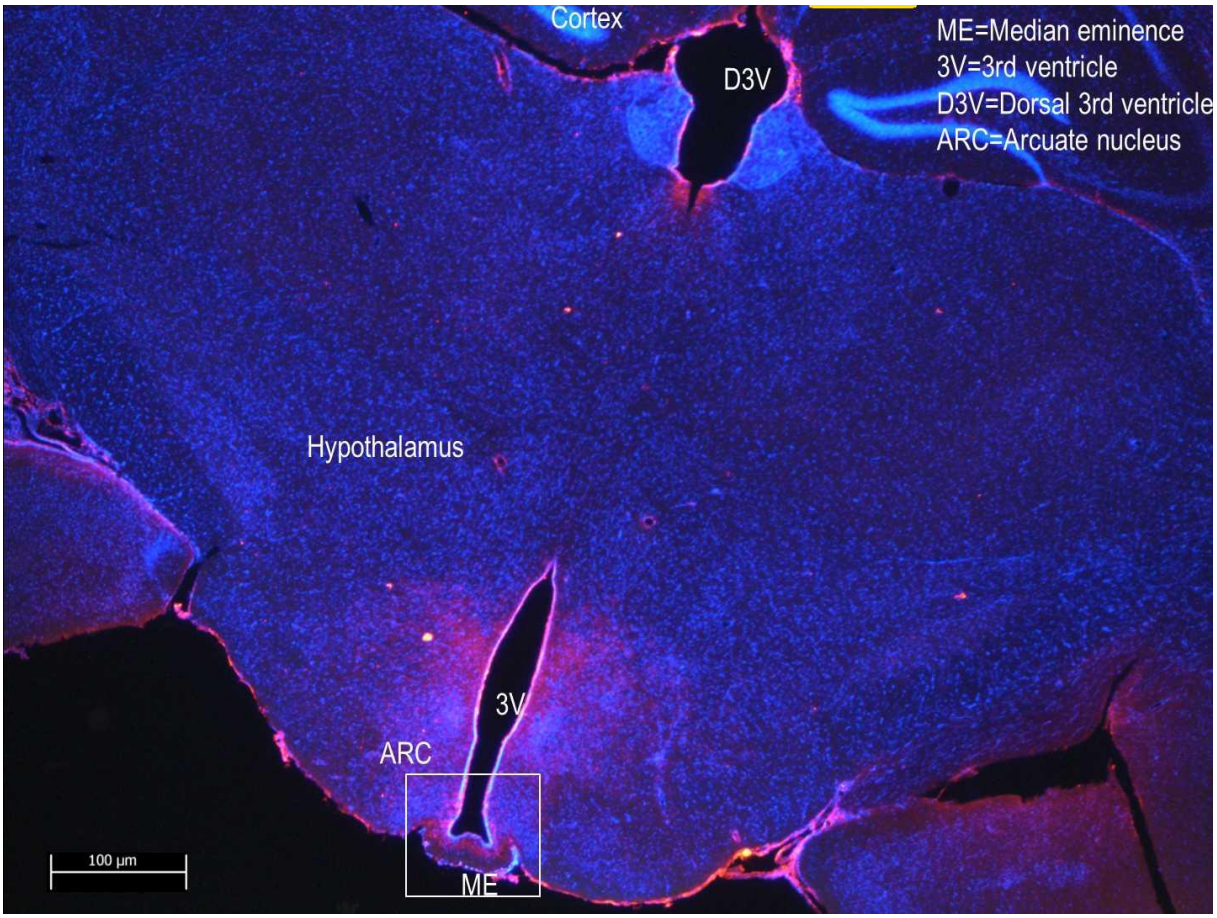


**Figure 8.** This is an overview image that shows the area we identified as the median eminence (ME). The ME is highlighted by a rectangle and is located to the most ventral part of the third ventricle (3V). The arcuate nucleus of the hypothalamus (ARC) is located close to the ME. This picture was obtained by the use of a fluorescence microscope equipped with a camera.

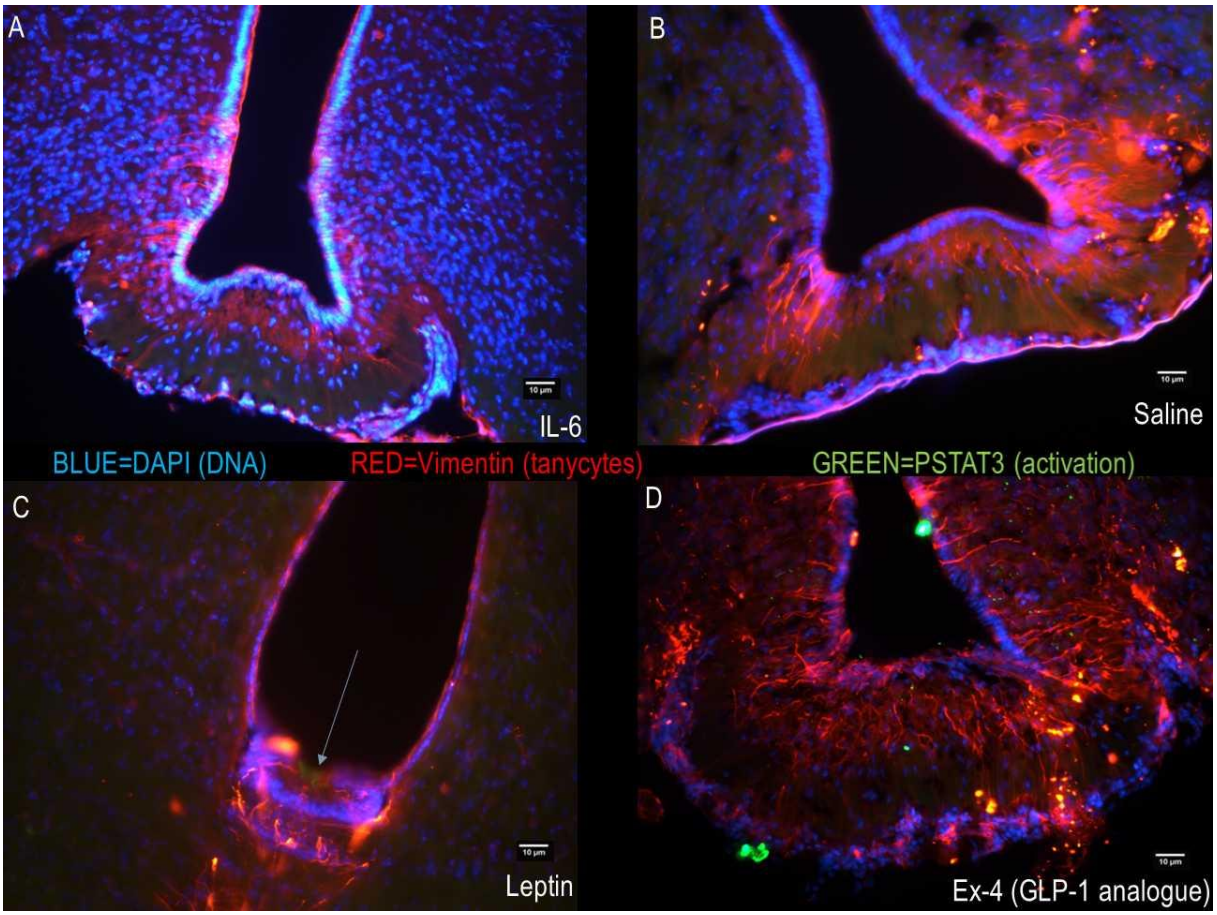


**Figure 9.** This figure shows the typical staining of the median eminence 15 minutes after IP injections with interleukin-6 (IL-6) (A), saline (B), leptin (C) or the glucagon-like peptide-1 analogue exendin-4 (Ex-4) (D). pSTAT3 is marked by an arrow and shown in green. The vimentin-rich cytoskeletons of tanycytes are shown as red stripes. Blue staining by 4',6-diamidino-2-phenylindole (DAPI) visualizes cell nuclei. These pictures were obtained by the use of a fluorescence microscope equipped with a camera.

## 8.4. ICV Injections of IL-6, Leptin and GLP-1 Activates the Tanycytes in Mice

Tanycyte cells in the ME were activated upon ICV injections with IL-6, Ex-4 and leptin (Fig. 10A, C, D). However, no activation was observed after ICV injection with aCSF (Fig 10B). Phosphorylation of STAT3 into pSTAT3 was seen after injections with IL-6, leptin and Ex-4 but not with injections of aCSF. The presence of pSTAT3 was most prominent in tanycytes in

the ME. There were also pSTAT3 present in the ARC after leptin injections. Representative sections are shown in Figure 10.

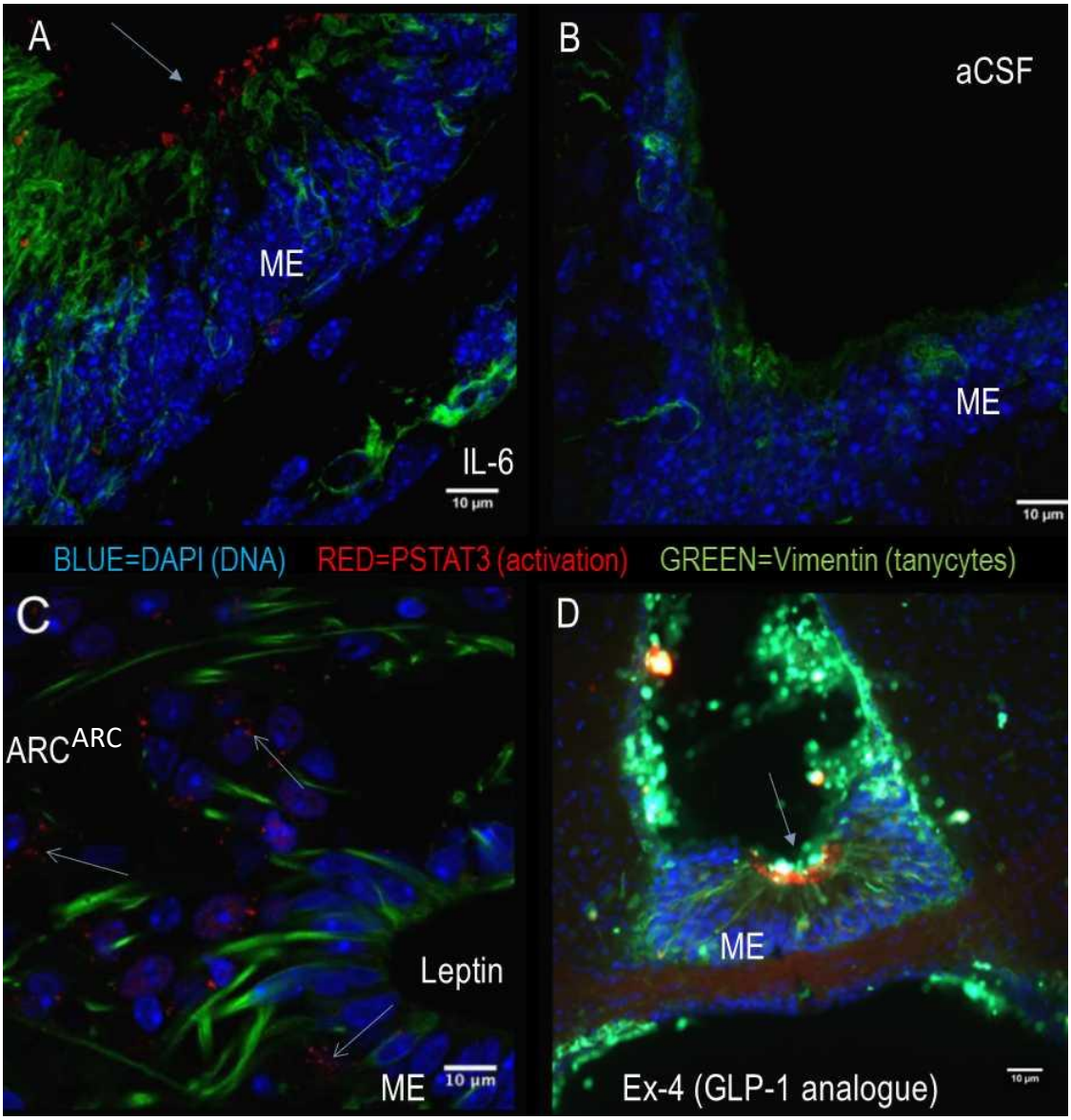


**Figure 10.** This figure shows the typical staining of the median eminence (ME) 15 minutes after ICV injections with interleukin-6 (IL-6) (A), artificial CSF (aCSF) (B), leptin (C) or the glucagon-like peptide-1 (GLP-1) analogue exendin-4 (Ex-4) (D). Presence of pSTAT3 is colored red in the figure and marked by arrows. The vimentin-rich cytoskeletons of tanycytes are shown as green stripes. Blue staining by 4',6-diamidino-2-phenylindole (DAPI) visualizes cell nuclei. Images marked A, B and C were obtained using a confocal microscope. The image marked D was obtained using a fluorescence microscope.

# 9. Discussion

## 9.1. Conclusions and Implications

Articles that address signaling of anorectic substances in the CSF have started to appear during the last years. However, there are still many aspects of the anorectic signaling in the CSF that are unknown. Here we further elucidate the signaling of the anorectic peptides IL-6, GLP-1 and leptin in CSF. We especially focus on the relation between these peptides and tanycytes. Collectively our conclusions are shown in Figure 11.

GLP-1 is not present in human CSF according to our analysis. This is surprising as Shirazi et al. found that injection of GLP-1 analogue Ex-4 directly into CSF of mice will markedly reduce body weight and food intake. GLP-1R agonists such as liraglutide do however cross the blood CSF barrier which may be an important reason to their success, but evidence is accumulating that they do not enter the CSF (84, 85). It stands to reason that a GLP-1 analogue that enters the CSF would be more potent than GLP-1 analogues used today.

It may be argued that the concentration of GLP-1 in CSF is too low to detect it using ELISA; but such a low concentration of GLP-1 are likely not physiologically relevant. The concentrations of GLP-1 detected in human serum correspond to that of other studies (51).

We measured GLP-1 in CSF from healthy individuals. GLP-1 in serum is increased 7-fold in septic patients (51). More studies are needed to determine if GLP-1 is present in CSF from patients experiencing various pathological conditions. Patients with acute infections, such as bacterial meningitis, are probably good candidates. It is also possible that the presence of GLP-1 in CSF varies between species as one study claims to have found low levels of GLP-1 in the CSF of rats (83).

We continued by evaluating the effect of leptin therapy on mice with lower levels of IL-6 Rα mRNA on the inside of the ventricles, referred to as IL-6 Rα knock-down mice. Here we

found that a knock-down to 66 % of IL-6 Rα in control mice does not affect the anorectic effects of leptin. It should be noted that the IL-6 Rα knock-down mice were continually decreasing in body weight already before the experiment started. The reason for this is unknown, but IL-6 Rα knock-down mice that received saline therapy did not continue to lose more weight than controls.

Finally we show that IP injections with leptin and ICV injections with IL-6, GLP-1 analogue Ex-4 and leptin induce pSTAT3 in tanycytes after 15 minutes. pSTAT3 is a marker for activation of cells. This experiment was performed on WT mice. We do not think the activation of IL-6 was due to the soluble receptor to IL-6 as pSTAT3 was induced at the location of IL-6 Rα. It should also be noted that there were more pSTAT3 found in ARC than the ME in mice injected ICV with leptin. This is probably due to the fast transportation of leptin over the ME. A shorter time frame between injection and perfusion may show more pSTAT3 staining in the ME or a completely different pattern, this is especially plausible for leptin injections.

Previous studies have shown that leptin activates tanycytes but this is the first evidence for activation of tanycytes by IL-6 and GLP-1(89). The activation of tanycytes by GLP-1 should probably be seen as a pharmacological effect as we in the present study show that GLP-1 does not exist in CSF, at least not in humans. More studies are needed to confirm these results and to study how tanycytes are activated by GLP-1 as they seem to lack the GLP-1R (89). A previous study show that they may interfere with IL-6 Rα but if this is directly or indirectly remains unclear (37).

Collectively our findings further elucidate the metabolic signaling in the CSF. We especially show that IL-6 and GLP-1 could be important regulators of appetite in the CSF as they activate tanycytes. More potent drugs for obesity and cachexia may be created by further investigation and application of our findings.

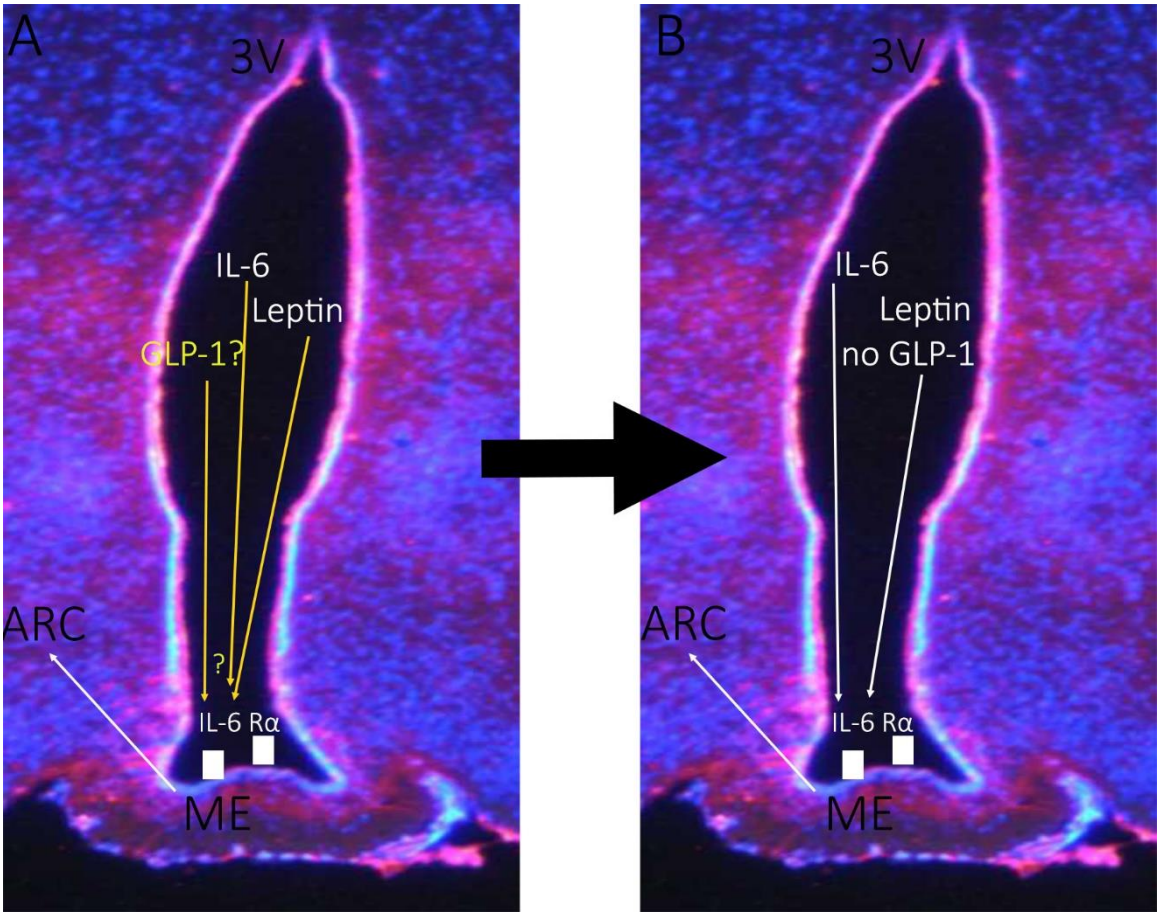


**Figure 11.** This figure summarizes our results. Unknown data are shown in yellow and known data are shown in white. (A) Before this degree project it was not known if glucagon-like peptide 1 (GLP-1) was present in human CSF. Both interleukin-6 (IL-6) and leptin has been shown to exist in human CSF in previous studies. The gateway between CSF and appetite regulating parts of the brain such as the arcuate nucleus of the hypothalamus (ARC) is thought to be the median eminence (ME). The ME contains the IL-6 receptor alpha (IL-6 Rα) and is located to the third ventricle (3V). We aimed to find out if this receptor is functional and if it is activated by IL-6, GLP-1 and leptin. (B) We did not find any GLP-1 in human CSF. Our data also suggest that leptin does not signal via the IL-6 Rα on the inside of the ventricles. Furthermore we show that the ME is reactive to IL-6, suggesting that the IL-6 Rα is functional. Finally we show that the GLP-1 analogue Ex-4 activates receptors located on tanycytes in the ME. These activated receptors could be the IL-6 Rα as tanycytes lack the GLP-1 receptor.

## 9.2. Methodological Considerations

The ELISA kits used to measure GLP-1 in human CSF were produced to measure GLP-1 in serum. This may have interfered with the result. However, the same kits have been used in studies with CSF from rodents and cats. ELISA kits for serum are commonly used to measure compounds in CSF. The ELISA kits had an intra-assay coefficient of variation (CV) of <5% and an inter-assay CV of <12.

During the experiment with leptin therapy we used IL-6 $R\alpha^{lox/lox}$ mice that were in median 4 g heavier than the controls (WT mice). This is the reason to why body weight change was expressed as percentage of body weight at the start of the experiment. Food intake was instead expressed as cumulative food intake in grams as this is the conventional visualization.

We determined the knock-down of IL-6 Rα by TAT-CRE to be 34 %. This may seem low but shirazi et al. injected Ex-4 in IL-6 $R\alpha^{lox/lox}$ mice with a knock-down of IL-6 Rα by 43 % and found a significant reduction of the anorectic effect of Ex-4 compared to controls (37). The knock-down of this technique is also thought to reduce after time and the knock-down was measured two to three weeks after the TAT-CRE injection. This means that the knock-down may have been higher during the leptin therapy. Antibodies to block the IL-6 Rα can also be used, however the effect of these are much shorter than that of TAT-CRE treatment.

Mice were treated with IL-6, GLP-1 or leptin before IHC analysis. We used concentrations previously described in publications with clear results (37, 58, 107). Images were then assessed in a qualitative fashion. We did not use cell counting to quantify our results as some slices had close to zero staining with pSTAT3-antibodies and the pSTAT3 coloration were concentrated to the dorsal part of the ME. Future studies with western blotting or GCAMP3 calcium imaging could be used to confirm our results in a quantitative fashion.

# 10. Populärvetenskaplig sammanfattning på svenska

**Titel: Hur ämnesomsättningen regleras i ryggmärgsvätska**

Fler än 600 miljoner människor har nu drabbats av den livsfarliga sjukdomen ”fetma”. Den mest effektiva metoden för att bota denna sjukdom är idag kirurgi där magsäcken kopplas bort. Detta är dock en mycket dyr och farlig metod och dessutom fungerar den inte för alla. Det finns även mediciner för att behandla fetma. Det mest effektiva läkemedlet som finns på marknaden idag liknar ett hormon som kallas glucagon-like peptide-1. Leptin är ett annat hormon som många trodde skulle bli ett effektivt läkemedel mot fetma under 90-talet, dock blev så inte fallet. Även ämnet interleukin-6 har en fetmaminskande effekt.

I denna tes har vi undersökt hur glucagon-like peptide-1, leptin och interleukin-6 signalerar i ryggmärgsvätska. Vi använde först så kallad ELISA-teknik för att avgöra om glucagon-like peptide-1 finns i mänsklig ryggmärgsvätska under friska förhållanden. Vi fortsatte med att undersöka om leptin har samma effekt på viktnedgång och födointag hos möss som saknar receptorn för interleukin-6 på viktiga platser i hjärnan. Slutligen användes tekniken immunohistokemi för att undersöka var glucagon-like peptide-1, leptin och interleukin-6 som injicerats i ryggmärgsvätska på möss aktiverar hjärnan.

Sammanfattningsvis visar våra studier att glucagon-like peptide-1, leptin och interleukin-6 kan signalera via ryggmärgsvätska. Däremot verkar inte glucagon-like peptide-1 finnas naturligt i ryggmärgsvätska hos människa. Med immunohistokemi såg vi att samtliga av dessa tre ämnen har potential att aktivera så kallade tanycytceller i hjärnan. Dessa fynd är viktiga då de förhoppningsvis kan bidra till utveckling av bättre fetmaläkemedel.

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*May we all meet again in the future!*

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