Gravitostat: A Homeostatic Regulator of Body Weight

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Cover illustration: "Neuroendocrinology" by Igor Milanović

To those who share my blood and those who
chose to share my path.

SVANENMÄRKET

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Sapere aude[1], ad prosperitatem humanitatis [2].

[1] The phrase "Sapere aude" (Dare to be wise) originates from 'Horace's Epistles' (20 BCE, I.2.40). It gained profound philosophical significance through Immanuel Kant's 1784 essay 'What is Enlightenment?' (Beantwortung der Frage: Was ist Aufklärung?), where he transformed its meaning into a fundamental principle of enlightenment. Kant emphasized that true enlightenment requires not only the intellectual courage to think independently but also the moral fortitude to express one's convictions.
[2] "Ad prosperitatem humanitatis" (Towards the prosperity of humanity).

ABSTRACT

This project focuses on elucidating central and peripheral physiological mechanisms behind load-induced body weight reduction. These pre-clinical works are a continuation of previous studies in obese humans and Diet-Induced Obese (DIO) rodents, which demonstrated that increased weight load leads to a significant reduction in biological body weight and food intake. Based on these findings, our research group hypothesized that the observed body weight reduction is due to a homeostatic mechanism which we termed "the Gravitostat." This mechanism is activated by increased load, which in turn is likely to stimulate weight sensors in the lower extremities. These are then likely to send signals to integrating centers in the brain to reduce appetite.

This thesis encompasses mapping studies performed in the brain and spine of weight-bearing DIO mice to identify regions involved in load-induced weight loss, as well as an exploration of alternative methods for load application. Since load-induced weight loss is a relatively acute process, we additionally explored potential alterations in water balance and showed that water and sodium levels are unaffected in weight-bearing rats. This strengthened our previous findings that fat loss is the primary mechanism for body weight reduction in weight-bearing rodents. Finally, we developed a method which allows animals to recover from the surgical procedure used to increase load, by implanting a fillable capsule. This allowed us to fill capsules with wolfram granulate and increase the load once the rodent has recovered from the surgical trauma. We also utilized subcutaneous implantation of capsules on rodents' back, which seems to induce less surgical trauma than intraperitoneal implantation.

The main findings in this thesis include the identification of a group of neurons activated by increased load in the medial Nucleus of Solitary Tract (mNTS) and the dorsal horn (DH) of the Lumbar Spine (LS) in mice. More specifically, methods such as immunohistochemistry and RNAscope were employed for closer identification of load-activated neurons, such as Norepinephrine (NE) containing cells in the brainstem. The effects of capsaicin in the DH lead us to speculate that the nerves transmitting information from the hindlimbs contain TRPV1-channels. However, the identity of the cells receiving these projections remains to be identified.

In conclusion, these findings have established a strong foundation for future studies to identify other potential regions involved in load-induced weight loss. Our results potentially pave the way for developing effective preventative measures for obesity, as well as pharmacological targeting of regions involved in this process. This research contributes to our understanding of body weight regulation mechanisms and may lead to novel approaches in obesity management.

Keywords: obesity, physiology, neuroscience, brainstem, NTS, spine, dorsal horn, mapping

SAMMANFATTNING PÅ SVENSKA

Projektet fokuserar på att klarlägga centrala och perifera fysiologiska mekanismer bakom hur kroppsvikt reduceras av belastning. De prekliniska arbeten som presenteras här är en fortsättning på tidigare studier på överviktiga människor och gnagare. Vad vi har sett i de studierna är att ökad viktbelastning leder till en betydande minskning av både kroppsvikt och födointag. Baserat på dessa fynd har vi utvecklat hypotesen att förändringarna i kroppsvikt och födointag beror på en homeostatisk mekanism som vi kallar "Gravitostat." Mekanismen aktiveras sannolikt av ökad belastning, vilket sannolikt stimulerar viktsensorer i de nedre extremiteterna. Sensorerna skickar sedan troligen signaler till aptitkontrollerande centra i hjärnan för att minska aptiten.

Avhandlingen omfattar kartläggningsstudier utförda i hjärnan och ryggraden hos viktbärande DIO-möss för att identifiera regioner involverade i belastningsinducerad viktnedgång, samt en undersökning av alternativa metoder för belastningsapplicering. Eftersom belastningsinducerad viktminskning är en relativt akut process, undersökte vi potentiella förändringar i vattenbalansen och visade att vatten- och natriumnivåer är opåverkade hos viktbärande råttor. Det stärkte i sin tur våra tidigare fynd att fettförlust är den primära mekanismen för viktminskning hos viktbärande gnagare.

Vidare utvecklade vi en metod som låter djuren återhämta sig från det kirurgiska ingreppet som används för att öka belastningen, genom att operera in en fyllbar kapsel. Det gör det möjligt för oss att fylla kapslarna med wolframgranulat och öka belastningen när gnagaren har återhämtat sig från det kirurgiska traumat. Vi använde också subkutan implantation av kapslar på gnagarnas rygg, vilket verkar orsaka mindre kirurgiskt trauma än intraperitoneal implantation.

Huvudfynden i denna avhandling inkluderar identifieringen av en grupp neuroner som aktiveras av ökad belastning i mediala nucleus tractus solitarius (mNTS) och dorsalhornet (DH) i lumbalryggen hos möss. Mer specifikt har metoder som immunohistokemi och RNAscope använts för närmare identifiering av belastningsaktiverade neuroner, såsom norepinephrin (NE)-innehållande celler i hjärnstammen. Effekterna av capsaicin i DH fick oss att spekulera i att nerverna som överför information från bakbenen innehåller TRPV1-kanaler. Dock återstår det att identifiera vilka celler som tar emot dessa projektioner.

Sammanfattningsvis har våra fynd etablerat en stark grund för framtida studier för att identifiera andra potentiella regioner involverade i belastningsinducerad viktminskning. Våra resultat banar potentiellt väg för utveckling av effektiva förebyggande åtgärder mot fetma, samt farmakologisk målsökning av regioner involverade i denna process. Min forskning bidrar till vår förståelse av kroppsviktreglerande mekanismer och kan leda till nya angreppssätt inom fetmabehandling.

SAŽETAK NA SRPSKOM

Ovaj projekat je usmeren na razjašnjavanje centralnih i perifernih fizioloških mehanizama koji su u osnovi redukcije telesne mase indukovane dodatnim težinskim opterećenjem. Ova pretklinička istraživanja predstavljaju nastavak prethodnih studija na gojaznim ispitanicima i eksperimentalnim glodarima koje su pokazale da povećano opterećenje težinom dovodi do značajne redukcije telesne mase i unosa hrane, kao i da je za ovaj proces neophodno prisustvo osteocita u koštanom tkivu donjih ekstremiteta. Na osnovu ovih nalaza, naša istraživačka grupa je postavila hipotezu da se uočena redukcija telesne mase odvija putem homeostatskog mehanizma nazvanog "Gravitostat". Ovaj mehanizam se aktivira dodatnim težinskim opterećenjem, koje verovatno stimuliše senzore težine u donjim ekstremitetima, a koji potom signaliziraju integrativnim centrima u mozgu da smanje apetit.

U ovoj tezi su obuhvaćene studije mapiranja neurona u mozgu i kičmenoj moždini miševa podvrgnutih težinskom opterećenju, kao i alternativne metode koje smo razvili za aplikaciju dodatnog opterećenja. S obzirom da je redukcija telesne mase izazvana dodatnim opterećenjem relativno akutan proces, ispitali smo potencijalne promene u homeostazi telesnih tečnosti i demonstrirali da nivoi vode i natrijuma ostaju nepromenjeni kod pacova sa dodatnim težinskim opterećenjem. Ovaj nalaz potvrđuje naše prethodne rezultate da je redukcija masnog tkiva primarni mehanizam smanjenja telesne mase kod gojaznih glodara izloženih dodatnom težinskom opterećenju.

Takođe, razvili smo metodologiju koja omogućava eksperimentalnim životinjama oporavak od hirurškog postupka implantacije kapsule koja se može naknadno puniti. Ovaj pristup omogućava punjenje kapsula volframovim granulatom i povećanje opterećenja nakon potpunog oporavka životinja od hirurške intervencije. Paralelno je razvijena metoda za supkutanu implantaciju težinskih kapsula u lumbalnoj regiji, koja rezultira manjom hirurškom traumom u poređenju sa intraperitonealnom implantacijom.

Ključni nalazi ove teze uključuju identifikaciju populacije neurona aktiviranih povećanim težinskim opterećenjem u medijalnom jedru solitarnog trakta i dorzalnom rogu (DR) lumbalne kičmene moždine kod miševa. Primenom imunohistohemijske metode i RNAscope tehnologije za vizuelizaciju tkiva, ustanovljeno je da neuroni aktivirani povećanim težinskim opterećenjem eksprimiraju norepinefrin.

Efekti kapsaicina u DR sugerišu da aferentna nervna vlakna koja prenose informacije iz donjih ekstremiteta eksprimiraju TRPV1 kanale. Međutim, identitet ćelija aktiviranih u DR, kao i njihovih projekcija, ostaje predmet budućih istraživanja.

U zaključku, prezentovani nalazi predstavljaju solidnu osnovu za dalja istraživanja usmerena ka identifikaciji dodatnih regiona uključenih u redukciju telesne mase indukovanu dodatnim težinskim opterećenjem. Takođe, ovi rezultati potencijalno otvaraju put ka razvoju efikasnih preventivnih strategija za gojaznost i farmakološkom targetiranju relevantnih regiona. Ovo istraživanje značajno doprinosi razumevanju mehanizama regulacije telesne mase i može voditi ka razvoju novih terapijskih pristupa u tretmanu gojaznosti.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Reduction of body weight by increased loading is associated with activation of norepinephrine neurones in the medial nucleus of the solitary tract**.
Zlatkovic, J, Gasull, AD, Hägg, D, Font-Gironès, F, Bellman, J, Meister, B, Palsdottir, V, Ruud, J, Ohlsson, C, Dickson, SL, Anesten, F, Jansson, J-O.
Journal of Neurondocrinology 2023; Vol, 35, Issue 12; e13352

II. **Increased weight-bearing load reduces biological body weight while sodium and water balances are mainly unaffected.**
Zlatkovic, J, Bellman, J, Hägg, D, Magnusson, M, Ohlsson, C, DiBona, G, Anesten, F, Jansson, J-O.
Manuscript, 2024.

III. **Weight reduction by artificial load requires sensory signaling in the dorsal horn of the lumbar spine.**
Anesten, F, Zlatkovic, J, Hägg, D, Ohlsson, C, Jansson, J-O.
Manuscript, 2024.

Content

ABBREVIATIONS

ARC	Arcuate Nucleus
AgRP	Agouti-Related Peptide
AP	Area Postrema
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BDNF	Brain-Derived Neurotrophic Factor
BMR	Basal Metabolic Rate
CCK	Cholecystokinin
CVD	Cardiovascular Disease
CeVD	Cerebrovascular Disease
CGRP	Calcitonin-Gene Related Peptide
CNS	Central Nervous System
CRF	Corticotropin-Releasing Factor
DAB	Diaminobenzidine
DAPI	4′,6-Diamidino-2-Phenylindole
DbH	Dopamine Beta-Hydroxylase
DIO	Diet Induced Obesity
DMH	Dorsomedial Hypothalamic Nucleus
DVC	Dorsal Vagal Complex
EAT	Exercise Activity Thermogenesis

FosB	Marker for chronic neuronal activity	PNS	Peripheral Nervous System
GABA	Gamma-Aminobutyric Acid	PCOS	Polycystic Ovary Syndrome
GLP-1	Glucagon-Like Peptide 1	PVN	Paraventricular Hypothalamus
GIP	Gastric Inhibitory Polypeptide	POMC	Proopiomelanocortin
GI	Gastrointestinal	PBN	Parabrachial Nucleus
HDL	High-Density Lipoprotein	PYY	Peptide-YY
HFD	High Fat Diet	RMR	Resting Metabolic Rate
HPT	Hypothalamic-Pituitary-Thyroid Axis	SC	Subcutaneous
IL-6	Interleukin-6	SON	Supraoptic Nucleus
IP	Intraperitoneal	SES	Socioeconomic Status
IR	Insulin Resistance	T2D	Type-2 Diabetes
LDL	Low-Density Lipoprotein	TH	Tyrosine-Hydroxylase
LH	Lateral Hypothalamus	TNF-α	Tumor Necrosis Factor - A
NTS, mNTS	Nucleus Of The Solitary Tract, medial	TDEE	Total Daily Energy Expenditure
MS	Metabolic Syndrome	TRH	Thyrotropin-Releasing Hormone
MC4R	Melanocortin 4 Receptor	TEF	Thermic Effect Of Food
MCP-1	Monocyte Chemoattractant	TRPV1	Vanilloid Receptor 1
NEAT	Non-Exercise Activity Thermogenesis	VMH	Ventromedial Hypothalamus
NPY	Neuropeptide Y	WAT	White Adipose Tissue
NREE	Non-Resting Energy Expenditure	WHO	World Health Organization
PB	Phosphate Buffer	α - MSH	α-Melanocyte Stimulating Hormone

1 INTRODUCTION

Obesity is a condition characterized by excess energy storage in adipose tissue. According to the World Health Organization (WHO), obesity is defined by specific Body Mass Index (BMI) criteria. For obesity, this means BMI equal to or exceeding $30kg/m^2$. Extreme obesity is defined as BMI > $40kg/m^2$ [1]. Pathological accumulation of adipose tissue poses a severe risk for the development of metabolic diseases, such as diabetes and cardiovascular disorders, and further contributes to increased morbidity and mortality rates [2].

1.1 EPIDEMIOLOGY AND PREVALENCE

Statistical reports by WHO show a concerning upwards trend in obesity prevalence over the past few decades. At the global level, adult obesity rates have almost doubled, increasing from 7% in 1990 to 16% in 2022, while childhood obesity rates show more than a fourfold increase, from about 2% in 1990 to over 8% in 2022 [3].

1.2 ETIOLOGY AND PATOPHYSIOLOGY

1.2.1 CAUSATIVE FACTORS

The causes of obesity have been a topic of debate for many years. Due to advances in research we now understand that it is multifactorial; including genetic components, environmental influences as well as lifestyle choices, or several of them combined.

More than 500 genes have been reported to be related to obesity, and many of these are expressed in the brain. Some key genetic factors include monogenic or polygenic mutations on genes coding for leptin (LEP) and its receptor (LEPR), the melanocortin 4 receptor (MC4R), propiomelanocortin (POMC), prohormone convertase 1 (PCSK1), fat mass obesity associated gene (FTO) and brain-derived neurotrophic factor (BDNF) [4]. Additionally, several obesity-related syndromes, caused by chromosomal aberrations, have been described. One such example is Prader-Willy syndrome (PWS) which is caused by deletion on paternal 15q11-q13 chromosome. This in turn leads to alterations in the development of neuronal and endocrine systems [5]. Even though there are more obesity-related syndromes, such as Alstrom syndrome, Fragile X Syndrome (FXS), Bardet-Biedl Syndrome, Albright Hereditary Osteodystrophy, WAGR Syndrome, Cohen Syndrome, Smith-Magenis Syndrome

and Kallmann Syndrome, it is important to note that these are rare and complex conditions that include obesity among a multitude of larger systemic disorders [4].

It is worth mentioning the impact of broader factors such as socioeconomic status (SES) [6], school education and urban infrastructure [7] in the development of obesity, as they directly influence the pattern of otherwise well-known lifestyle factors that are important for overall health such as a healthy diet and physical activity [8,9]. Accordingly. the highest percentage of obese individuals have a sedentary lifestyle [10], an additional factor in reduced energy expenditure and a significant health threat [11]. However, it is also of importance to emphasize the role of the immediate environment, as children tend to adopt habits from their home. Statistics show that children of obese parents are more likely to be obese as well, indicating complex interplay between genetic and environmental components in obesity development [12,13].

Obesity can also be a manifestation of mental health issues. Emotional over-eating is a significant factor for obesity development. Some individuals use food to cope with negative emotions such as life dissatisfaction, stress, anxiety, depression and loneliness [14]. It is important to note that the relationship between mental health and obesity is bi-directional. Thus, obesity itself can lead to mental health issues [15,16].

Various endocrine disorders can also lead to obesity. For example, hypothyroidism, a condition caused by insufficient production of the thyroid hormones triiodothyronine (T3) and thyroxine (T4), leads to reduced resting metabolic rate (RMR) and therefore decreased energy expenditure, as well as impaired thermogenesis [17,18]. In addition, Cushing's syndrome, a condition characterized by elevated levels of circulating cortisol, can lead to excessive fat accumulation in fat depots of the face, upper back and abdomen [19]. In women, Polycystic Ovary Syndrome (PCOS) as well as menopause have been highly associated with obesity, however the mechanisms behind this are not yet fully elucidated [20,21].

Certain types of medications can also lead to obesity such as antipsychotics, antidepressants and hormonal birth control [22–24]. The mechanisms behind the weight gain associated with these medications is an active area of research, with both peripheral and central processes shown to be affected [25–27].

1.2.2 PATOPHYSIOLOGICAL CONSEQUENCES OF OBESITY

1.2.2.1 METABOLISM

Metabolic Syndrome (MS), also known as Insulin-resistance syndrome, represents a group of obesity-related conditions that pose a high risk for developing more severe diseases such as Diabetes Mellitus Type 2 (T2D) and Cardiovascular Disease (CVD). MS is characterized by excess adiposity in the abdominal area, hypertension, elevated blood glucose, triglycerides, LDL (bad cholesterol) and low HDL (good cholesterol) [28,29]. At the cellular level, MS manifests as an inflammatory state due to increased secretion of pro-inflammatory adipokines such as interleukin-6 (IL-6), tumor necrosis factor-a (TNF-a), monocyte chemoattractant (MCP-1) and resistin as well as decreased secretion of an anti-inflammatory marker – adiponectin [30,31]. Taken together, these conditions show why obesity, and therefore MS, is a high-risk factor for developing T2D, CVD as well as cerebrovascular disease (CeVD) [29].

1.2.2.2 NERVOUS SYSTEM

In the CNS, obesity-related, chronic, low-grade systemic inflammation can also manifest as "neuroinflammation". This state has been associated with structural and functional changes within hypothalamus and striatum in children [32]. In adults, neuroimaging studies have demonstrated that obesity is related to broader changes in the brain such as reduced cortical thickness, diminished integrity of white matter and altered patterns of brain connectivity and these changes in the brain can mimic the clinical manifestation of Alzheimer's Disease [33].

The neurological impact of obesity extends beyond the central nervous system to include peripheral nerve damage. Clinical evidence shows that obesity alone can cause peripheral neuropathy even in individuals with normal blood glucose levels [34]. This neuropathy preferentially affects small- and medium-sized nerve fibers and is particularly associated with central obesity, as measured by waist circumference. The pathophysiology involves multiple converging mechanisms, including lipid signaling, inflammation, and calcium signaling pathways [35].

1.2.2.3   REPRODUCTIVE HEALTH

Obesity has a significant impact on reproductive health of both men and women. In women, obesity can increase the risk of infertility by up to 78%, and can contribute to ovulatory dysfunction and menstrual irregularities [36,37]. Even though direct effects of obesity on fertility are not fully elucidated, research indicates that a chronic low-grade inflammation, as well as insulin resistance (IR) and disruption of hypothalamic-pituitary-adrenal axis have a negative impact on reproductive health [38–40]. Previously, PCOS has been described as one of the causative factors of obesity. However, the relationship between PCOS and obesity appears to be bi-directional. Therefore, obesity can contribute to the development of PCOS and further contribute to risks of pregnancy complications and problems with fertility. It has been reported that obesity negatively affects fertility treatments, as it is correlated with reduced oocyte quality, lower implantation rates and altogether lower pregnancy success rates [41]. Additionally, obese women on fertility treatments seem to require higher doses of gonadotropin [42,43]. Furthermore, obese women have higher risks of developing severe complications in pregnancy such as gestational diabetes, eclampsia and pre-eclampsia as well as postpartum depression [37].

Obese men are 42% more likely to have low sperm count and 81% increased odds for azoospermia [44,45]. Possible mechanisms of fertility issues in men with high adiposity include reduced testosterone levels, increased estrogen production and compromised function of hypothalamic-pituitary-gonadal axis [46]. Further, pro-inflammatory cytokines produced by adipose tissue may impair sperm function [47].

1.2.2.4   CARCINOGENESIS

Recent studies have revealed that obesity is a risk factor for developing certain types of cancers, including Esophageal adenocarcinoma, Gastric cardia carcinoma, Colorectal carcinoma, Hepatocarcinoma, Pancreas-, Endometrial-, Ovarian- and Postmenopausal breast cancer [48]. Some of the latest studies have also found associations between obesity and leukemia, non-Hodgkin lymphoma and bladder cancer [49].

1.3 PHYSIOLOGICAL MECHANISMS OF FAT REGULATION

The basic principle of a body weight equilibrium is founded on the balance between energy intake (calories in) vs. energy expenditure (calories out). However, human metabolism is complex and includes many hormonal processes which affect not only appetite, and therefore directly energy intake, but also metabolism or utilization of the consumed food [50].

1.3.1 ENERGY HOMEOSTASIS

The amount of energy an individual person requires is defined as Total Daily Energy Expenditure (TDEE). Its two main components are the resting energy expenditure (REE), which refers to the Basal Metabolic Rate (BMR) and non-resting energy expenditure (NREE) which includes non-exercise activity thermogenesis (NEAT), thermic effect of food (TEF) and exercise activity thermogenesis (EAT) [51–54]. Depending on age and activity levels, most women need between 1600-2200 calories, while men need around 2000-3000 calories per day [55].

In order for an individual's weight to remain stable, their energy intake should be equal to their TDEE. When intake continuously exceeds the amount of TDEE, caloric surplus is created leading to an increase in body weight, predominantly body fat. Additionally, energy expenditure greater than caloric intake leads to weight loss and this is the fundamental principle used when designing a weight-loss programs for obese individuals. However, some factors need to be considered due to metabolic adaptations.

Caloric restriction should typically not exceed more than 20-30% of TDEE for most people, as the daily caloric intake should not be lower than a person's BMR. Excessive caloric deficit poses a significant risk for metabolic slowdown, muscle mass loss and hormonal disruptions [56–59]. While there is emerging evidence that moderate energy restriction can improve fertility in female mice [60], it is worth to note that severe restrictions which lead to large weight loss has been known to exert negative effect on reproductive health in women [61,62]. From an evolutionary standpoint, it appears as if these adaptations happen to prevent further weight loss, as the human body requires a certain amount of body-fat for basic functions, including reproductive health [56,63]. A general recommendation is to maintain physical activity and/or

implement "diet breaks" to avoid this weight-loss stagnation due to metabolic adaptation [64–66]. These energy balance principles, while straightforward in theory, are complex in practice due to individual variations in metabolism, activity levels, and physiological adaptations.

1.3.2 ENDOCRINE REGULATION

1.3.2.1 PERIPHERAL

Insulin is a peptide hormone produced by β-cells in the Langerhans islets of the pancreas [67]. It is released in the serum as a response to increased glucose levels in the blood. Insulin's main function is anabolic, as it serves as a "key" for glucose transport into muscle cells, adipose tissue and liver cells, where it facilitates cellular glucose uptake and storage [68]. Additionally, insulin promotes protein synthesis, inhibits protein breakdown, stimulates lipogenesis and suppresses lipolysis. A crucial role of insulin is energy storage, as it coverts excess glucose into glycogen in the liver and muscle tissue. When glycogen stores are full, excess glucose is converted into triglycerides for long-term energy storage in adipose tissue [69]. Interestingly, insulin has a complex role in appetite regulation, as it acts on certain brain parts responsible for regulating appetite behaviors [70]. In obese individuals, insulin signaling becomes progressively dysregulated; a condition termed insulin resistance (IR). This metabolic dysfunction occurs when muscle, liver, and adipose tissues become less responsive to insulin's regulatory signals. As a result, pancreatic β-cells compensate by producing increasingly higher amounts of insulin, creating a vicious cycle of hyperinsulinemia. Muscle cells lose their ability to efficiently retrieve and metabolize glucose, forcing excess glucose to be converted and stored as triglycerides in adipose tissue. Consequently, this impaired insulin sensitivity not only promotes fat accumulation but also accelerates metabolic dysfunction, increasing the risk of type 2 diabetes and other metabolic disorders [71–73].

Leptin is an adipocyte-derived peptide hormone. It is released in the serum proportionally to the amount of adipose tissue and has a role in long-term energy homeostasis [74,75]. Leptin exerts its main functions in the CNS, particularly in the hypothalamus. In healthy individuals, increased leptin levels can lead to reduction in appetite via several mechanisms such as inhibition of orexigenic Agouti-related protein (AgRP) neurons and activation of anorexigenic Pro-opiomelanocrotin (POMC) neurons in the Arcuate Nucleus (ARC) of hypothalamus [76]. However, leptin's effectiveness changes dramatically in obesity, as obese individuals are hyperleptinemic and develop "leptin resistance"

[77]. Therefore, hypothalamic neurons do not respond to circulating leptin anymore. Given this finding, leptin has been proposed as an anti-starvation hormone, rather than anti-obesity hormone. When body fat levels are low so are leptin levels. This can lead to greater food intake, and therefore weight gain [78,79].

1.3.2.2 GASTROINTESTINAL (GI)

Ghrelin is a peptide hormone secreted from the stomach. It is also known as the "hunger" hormone, and is the only known circulating hormone that stimulates appetite. Discovered in 1999, ghrelin has a crucial role in energy regulation through multiple mechanisms [80,81]. In the CNS, ghrelin regulates food intake primarily by activating orexigenic neurons within the ARC and Paraventricular Nucleus (PVN), with particularly strong effects on AgRP/NPY neurons [82–85]. Its secretion follows a distinct daily pattern - levels rise before meals (and are elevated during fasting) and fall after eating, suggesting a role in meal initiation and energy balance [86–88]. Of note, obese individuals appear to have a blunted postprandial suppression of ghrelin, potentially contributing to overconsumption [88,89]. Beyond appetite regulation, ghrelin affects body weight and adiposity through dual metabolic pathways: it promotes lipid accumulation by enhancement of glucose utilization and lipogeneses within white adipose tissue (WAT), while simultaneously suppressing the thermogenic activity of brown adipose tissue (BAT) [90]. Interestingly, ghrelin's role extends beyond metabolism, impacting areas such as reward-seeking behavior, learning, memory, and adaptive stress responses [91–93]. Its complex physiological functions make it a critical hormone in maintaining energy balance and adaptive metabolic responses.

Cholecystokinin (CCK) is another GI peptide hormone involved in appetite regulation through both peripheral and central mechanisms. It is secreted from the proximal small intestine as a postprandial response to ingestion of lipids and proteins [94]. In the periphery, CCK's satiating effects are predominantly mediated through CCK1 receptors (CCK1R) on vagal afferent neurons, where it reduces gastric emptying and triggers digestive functions [95,96]. The central mechanisms primarily involve the Nucleus of the Solitary Tract (NTS), where vagal afferents directly terminate and integrate both hormonal and neural CCK signals [97]. Within the NTS, CCK activates second-order neurons that project to higher brain centers involved in appetite regulation, including the paraventricular nucleus (PVN) and lateral hypothalamic (LH) area [98]. Notably, leptin can enhance vagal sensitivity to CCK, creating a synergistic

interaction that amplifies satiety signaling at the NTS level, leading to more potent appetite suppression [99,100]. Beyond satiation, CCK also stimulates digestive enzyme release and gallbladder contraction, coordinating the digestive response to meals [101]. It is important to note that in obesity, vagal responsiveness to CCK becomes reduced, which then reduces its appetite-reducing function and potentially leads to further weight gain [102,103].

Glucagon-like peptide-1 (GLP-1) is a GI peptide hormone primarily produced by intestinal L-cells where it has a quick postprandial release [104]. This hormone has several metabolic roles via peripheral and central mechanisms. Within the digestive tract, it "slows down" the digestion process via inhibition of gastric emptying, acid secretion as well as GI motility [105]. In the pancreas, GLP-1 potentiates glucose-dependent insulin secretion from the β-cells and simultaneously suppresses glucagon release from α-cells. Besides pancreatic islets, GLP-1 has a role in appetite suppression as it can be synthesized locally in the brainstem and then bind to Glucagon-like peptide-1 receptors (GLP-1R) expressed throughout various CNS regions. Particularly important such regions are the NTS of the brainstem, and the PVN of the hypothalamus through which GLP-1R activation leads to reduced food intake and body weight [106,107]. Unlike previously described hormones, GLP-1's function is preserved in obese individuals, and therefore, GLP-1 has been particularly valuable in development of therapeutic interventions for T2D and obesity [107].

Other gut-derived peptides which have anorexigenic effects include intestinal, L-cells derived Peptide YY (PYY), as well as K-cells derived Glucose-dependent insulinotropic peptide (GIP) [108,109]. Most of GI hormones work synergistically to regulate energy balance, and their levels rise postprandially, and proportionally to nutritional intake [110][111].

1.3.3 NEUROENDOCRINE CONTROL

1.3.3.1 HYPOTHALAMUS

The **hypothalamus** lies below the thalamus, surrounding the third cerebral ventricle. It is one of the main brain areas involved in regulating energy homeostasis, as its numerous nuclei drive energy intake as well as energy expenditure. Beyond the ARC, PVN, and LH, other key regions include the Supraoptic Nucleus (SON), Dorsomedial Hypothalamus (DMH), and Ventromedial Hypothalamus (VMH) [112].

As mentioned earlier, there are two distinct types of neurons in the ARC, orexigenic AgRP/NPY neurons; and anorexigenic POMC neurons. Both populations integrate peripheral signals (including insulin, ghrelin and leptin), and central signals (including NPY, GABA, serotonin and melanocortin) to regulate energy homeostasis [112–116].

PVN has a major role in energy homeostasis via direct neuronal communication with ARC, as it is receptive to orexigenic signals from AgRP neurons, where the release of NPY leads to promotion of feeding behaviors via Y1 and Y5 receptors in the PVN. Additionally, PVN is involved in integrating appetite-suppressing POMC-mediated signaling, such as α-melanocyte stimulating hormone (α-MSH) which binds to MC3/4R receptors in the PVN [117,118].

PVN neurons also produce neuroendocrine peptides such as corticotropin-releasing factor (CRF), which appears to have both autocrine and paracrine properties, as it is involved in both stress response and modulation of food intake, body weight and energy expenditure [119,120]. Thyrothropin-releasing Hormone (TRH) is also produced in the PVN, and not only has a function via effects on the thyroid gland through the hypothalamo-pituitary-thyroid (HPT) axis, but has also been implicated in direct modulation of feeding behaviors and energy expenditure [121], possibly via a connection to AgRP and POMC neurons in the ARC [122]. Moreover, oxytocin has been shown to exert anti-feeding effects via magnocellular neurons in the PVN and SON, as well as some parvocellular neurons in the PVN through which gastric reflexes are regulated [123].

Additionally, PVN is involved in energy homeostasis via intricate communications with the NTS of the hindbrain. For example, NTS A2 noradrenergic projection to the PVN have been shown to modulate feeding, as PVN NE levels are increased in energy deficiency and decreased when food is available [124].

Notably, DMH integrates various inputs from ARC, including projections from NPY and POMC neuronal populations, and forwards inputs to PVN and LH [125].

As a part of hypothalamic energy-regulating systems, LH is implicated in weight regulation as it contains melanin-concentrating hormone (MCH), a neuropeptide which when released promotes feeding behavior [126]. Moreover, LH also contains orexin/hypocretin- (OX) and Neurotensin- (Nts) neurons, which coordinate complex behaviors implicated in energy balance. OX

neurons stimulate food intake, wakefulness and locomotor activity [127,128]. Nts neurons suppress feeding and can increase or reduce physical activity, depending on neural circuit activation [129–131].

Finally, VMH is an area with a multifaceted role in energy homeostasis, as it contains glucose-sensing neurons which respond to extracellular glucose levels and modulate the activity of the sympathetic nervous system. This area also expresses receptors for some of the peptides described earlier, such as insulin, NPY, OX and leptin [132].

1.3.3.2   HINDBRAIN

The **hindbrain** is located in the caudal part of the brain and is divided in three parts:

-Medulla oblongata
-Pons
-Cerebellum

The hindbrain regulates vital functions, such as breathing, heart rate, sleep and balance but it also regulates energy metabolism via the Dorsal Vagal Complex (DVC). This complex consists of three areas; the Nucleus of the Solitary Tract (NTS), Area Postrema (AP), Dorsal Motor Nucleus of the Vagus (DMNX). Additionally, the more rostrally located Parabrachial Nucleus (PBN) also plays a role in energy metabolism [133,134].

The **NTS** is located in the dorsolateral medulla oblongata and it serves as an important relay station for afferent sensory input from the vital organs; lungs, heart and blood vessels, and also from the gut. Additionally, NTS integrates vagus nerve-mediated signals from the GI tract, and then sends that input forward to brain areas responsible for hypothalamic processing, such as in meal termination in the example of CCK [135–137]. Within this nucleus, a few known appetite-regulating neuropeptides are expressed. The caudal NTS area contains GLP-1 producing neurons, which further project to the mesolimbic reward system, and are shown to regulate food intake and energy balance [138,139]. Furthermore, recent studies have shown that activation of Prolactin-releasing hormone (PRLH) containing cells that also express Calcitonin-Gene Related Peptide receptor (CGRP-R) leads to feeding reduction and weight-loss in obese rodents [140]. Major projection sites for NTS PRLH include DMH, PVN, and PBN [141,142].

The lateral part of the **PBN** is mainly involved in anorexigenic processes, some of them mediated via CGRP. Although CGRP is mainly known for its vasodilating properties, and implicated in pain transmission as well as migraine, activation of these neurons also leads to reduced food intake [143–145].

1.4 CURRENT THERAPEUTIC APPROACHES

Obesity management extends beyond lifestyle modifications such as increased physical activity and reduced calorie consumption. While these changes are fundamental, more intensive interventions are sometimes necessary. Bariatric surgery stands out as a particularly potent treatment, capable of inducing substantial weight loss ranging from 14% to 32%, depending on the specific surgical technique employed [146,147]. However, this approach is not without its drawbacks. As with any major surgical procedure, bariatric surgery carries inherent risks. Moreover, its irreversible nature contrasts sharply with pharmacological treatments, which can be discontinued if needed. Bariatric surgery may also lead to significant side effects that can impact quality of life. These include dumping syndrome, alterations in bone metabolism, and potential psychiatric complications, including an increased risk of suicidal tendencies [148–151]. These factors underscore the need for careful consideration before opting for surgical intervention.

In the realm of pharmacological treatments for obesity, options are evolving. Glucagon-like peptide 1 (GLP-1) receptor agonists, with Semaglutide as a notable example, currently represents the most effective single-drug therapy available. While Semaglutide can induce an average 17,3% of weight loss during 68 week treatment, it appears that one year after stopping participants regained around 11,6% of their weight loss [152], indicating net loss of total of 5,6% compared to their baseline weight. On the other hand, Tirzapetide which combines GLP-1 receptor agonism with Gastric Inhibitory Peptide (GIP), leads to remarkable 20,9% mean reduction in body weight during 36 weeks of treatment in non-diabetic obese individuals [153].

Even though GLP-1 receptor agonists demonstrate significant efficacy in reducing food intake and fat mass, their effects are notably less pronounced than those achieved through bariatric surgery. It's important to note that GLP-1 receptor agonists, like all medications, are associated with potential side effects. Some of most commonly reported adverse effects include nausea and other gastrointestinal disturbances [154]. However, recent evidence demonstrates that long term use of GLP-1 receptor agonists severely increases risks of

depression, anxiety and suicidal thoughts [155]. Despite these drawbacks, the reversible nature of pharmacological treatments offers a significant advantage over surgical options, allowing for treatment cessation if necessary.

1.5 THE GRAVITOSTAT

As we currently understand it, there does not seem to be a consensus about what the key anti-obesity mechanism is. In 2018, Jansson et al. published a groundbreaking study demonstrating that weight load in rodents leads to reduction in body weight and food intake [156]. This finding aligns with the hypothesis that, in the physiological attempt for the body to prevent severe obesity, when the threshold for the dangerous body weight is reached, this excessive weight increases the load on the weight bearing, lower extremities. This added weight in turn acts on osteocytes and sends afferent signal to activate satiety centers in the brain. This leads to a reduction in food intake and, consequently, body weight reduction. A similar system might exist for dangerously low body weight. This has been proposed as the 'dual hypothesis of homeostatic body weight regulation,' where two separate but complementary mechanisms - the leptin system and the Gravitostat - regulate body weight independently [157]. While leptin acts as an endocrine signal from adipose tissue, the Gravitostat functions through mechanical loading of osteocytes, providing distinct pathways that converge on the brain's integrating centers to regulate energy homeostasis. However, the mechanisms through which this load-induced body weight reduction occurs remained to be elucidated.
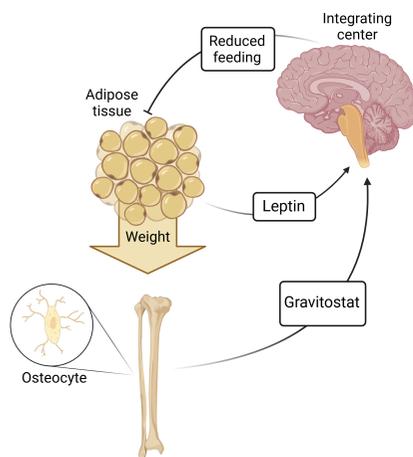

Figure 1: Illustration of the Gravitostat loop, adapted from Erik Schéle's original design.

2 AIMS

The overall aim of this study was to understand physiological mechanisms involved in body weight and food intake reduction mediated by artificial implantation of increased load in rodents.

Paper I: Investigation of central areas activated by increased load. By using FosB, a marker for neuronal activation, our goal was to determine which brain areas were activated by increased load. Additionally, we also aimed to identify substances produced by activated neurons and examine their functional involvement by intervention studies.

Paper II: To develop alternative methods for investigation of specific effects of increased load, independent of surgical trauma. By reducing the unspecific effects of surgery, we aimed to make it possible to isolate the physiological effects of increased load. Moreover, we also sought to examine if load-induced weight loss in rodents could be attributed to changes in fluid dynamics.

Paper III: To investigate involvement of peripheral sensory nerves in the transmission of the proposed Gravitostat signal. We also sought to determine the possible connection between osteocytes and the brain through which load regulates body weight and food intake. Additionally, we sought to intervene in this pathway to attenuate the signal in the dorsal horn of the lumbar spine.

This thesis encompasses three different studies which have explored neurophysiological mechanisms of action through which artificial load leads to the loss of biological body weight, as well as reduced food intake.

3 METHODOLOGY

3.1 ANIMALS

Male C57BL/6J mice (10-15 weeks of age) and Sprague-Dawley rats (20 weeks of age) were used in this study. Prior to experimental procedures, animals underwent dietary intervention with high-fat diet (HFD, 60% kcal from fat) for 6-10 weeks to establish diet-induced obesity (DIO). The obesity threshold was defined as body weight reaching 40-50g for mice. All animals were housed under controlled laboratory conditions with 12-hour light/dark cycle (lights on at 07:00), ambient temperature maintained at 21°C, and relative humidity of 50-60%. Animals had free access to water and were housed in standard ventilated cages with appropriate enrichment.

3.2 WEIGHT LOADING TECHNIQUES

3.2.1 INTRAPERITONEAL (IP)

Mice and rats described above underwent weight manipulation surgery using custom-manufactured fillable capsules (60 × 10 × 10 mm for rats, 25 × 8 × 10 mm for mice). Experimental animals received capsules filled with wolfram granulate (Edstraco AB, Sweden) to approximately 15% of their body weight, while control subjects carried minimally weighted empty capsules ~2.5% body weight (~1.2g).

Figure 2: IP model schematic

Surgical procedures were performed under isoflurane anesthesia (2-3% in oxygen). Animals received pre-operative analgesia via subcutaneous Rimadyl (carprofen, 5 mg/mL, 1 mL/kg) and 0.5% NaCl solution. A midline abdominal incision was made to access the peritoneal cavity, with capsules positioned anterior to intestines to minimize visceral contact. The peritoneum was closed with absorbable sutures, and skin was secured with surgical staples or metal clips.
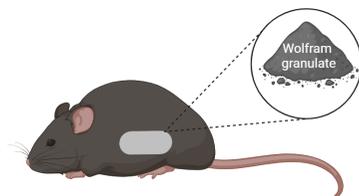
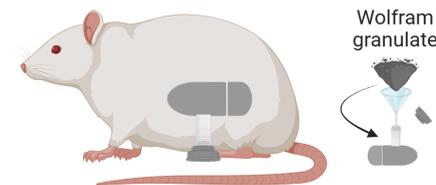### 3.2.2 IP INNOVATIVE CAPSULE TECHNIQUE

Figure 3: IP 2-step model schematic

A novel two-step loading methodology was developed to minimize surgical trauma. Specialized capsules weighing 10.76 g and a size of 60 × 10 × 10 mm featured a threaded external tube extending from the peritoneal cavity through the skin. This capsule enabled post-surgical loading with minimal additional intervention in rats. The initial surgical phase involved implanting empty capsules with a specialized nut positioned between the peritoneum and skin. A two-week recovery period allowed physiological stabilization before capsule loading. During the second phase, animals were briefly re-anesthetized, and capsules were filled with wolfram granulate to create differential weight conditions (adding 15% of body weight to Load group). The external tube, sealed with a threaded cap, allowed precise in vivo loading without additional surgical trauma. This approach enabled more precise investigation of acute physiological changes associated with increased loading.

3.2.3 SUBCUTANEOUS (SC)

An alternative weight manipulation technique utilized bilateral capsule implantation in the subcutaneous area of the lower back. Custom-manufactured capsules (18 × 8 × 8 mm) were used to create precise weight differences. Surgical procedures mirrored the IP method, with animals anesthetized using isoflu-

Figure 4: SC model schematic

rane and receiving subcutaneous Rimadyl analgesia. A small incision was made to create subcutaneous pockets, with two capsules positioned bilaterally under the skin to ensure uniform weight distribution and minimal tissue disruption. Experimental subjects were implanted with capsules filled to approximately 15% of their body weight, while control subjects carried minimally weighted empty capsules weighing 0.7g (~2.5% of average body weight). Incisions were closed using surgical staples, prioritizing minimal invasiveness and animal welfare.
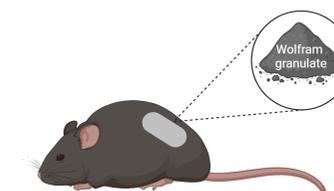
3.3 POST-OPERATIVE CARE

Following surgical procedures, animals were carefully monitored during recovery from anesthesia on temperature-controlled heating pads until fully conscious and exhibiting normal alertness and mobility. Once adequate recovery was confirmed, animals were returned to their home cages where they had unrestricted access to HFD and water. Regular post-surgical monitoring continued for 48 hours, with particular attention to wound healing, animal behavior, and overall wellbeing. This standardized post-operative care protocol was implemented across all surgical interventions to ensure optimal recovery and consistent experimental conditions.

3.4 MEASUREMENT TECHNIQUES

3.4.1 BODY WEIGHT

Body weight was tracked using a high-precision digital scale, with measurements performed consistently at the same time of day (typically at 7:00 a.m. when lights turned on) to minimize diurnal variations. The biological body weight was calculated by subtracting the weight of the implanted capsule from the total body weight. Measurements were conducted at regular intervals and recorded with a precision of 0.1 grams. To ensure accuracy, animals were weighed under standardized conditions, minimizing potential variations due to environmental factors or handling stress**.**

3.4.2 FOOD INTAKE

Food intake was quantified through a gravimetric method. Pre-weighed high-fat diet (HFD) pellets were placed on individual cage floors, with approximately 16-18 grams initially provided (4-5 pellets). At consistent daily intervals (typically at 7:00 a.m. when lights turned on), remaining food was carefully collected and re-weighed. The difference between initial and remaining food weight represented the precise 24-hour food consumption for each individual animal. This method allowed for accurate tracking of daily nutritional intake, accounting for potential variations in individual animal metabolism and feeding behavior.

3.4.3 METABOLIC CAGES

Metabolic cage trials were conducted using specialized Tecniplast metabolic cages, enabling comprehensive physiological parameter tracking. Key

parameters measured included measurements of water intake and urine excretion. Subsequent measurements of sodium intake and excretion were calculated using specialized formulas based on calculated water and food intake. Urine was collected in containers with water-saturated mineral oil to prevent evaporation, and samples were stored at -20°C for subsequent electrolyte analysis using ion-selective electrodes at the Sahlgrenska University Hospital's Clinical Chemistry lab.

3.5 NEUROLOGICAL INTERVENTIONS

3.5.1 STEREOTAXIC BRAIN INJECTIONS

Stereotaxic brain delivery of neurotoxin saporin (Advanced targeting Systems, Carlsbad, Ca, USA) conjugated with anti-dopamine-B-hydroxylase antibodies was used as a method of targeted ablation of noradrenergic (NE) neurons in the Nucleus of the Solitary Tract in the brainstem. Coordinates for injection sites were identified using the Allen Mouse Brain atlas (AP: -7.5 mm, ML: ±0.3 mm, DV: -4.8 mm from bregma). Prior to surgery, mice were anesthetized with injections of Sedastart (Omnidea, Stockholm, Sweden) and subcutaneously injected with carprofen (5 mg/mL Rimadyl®, 1 mL/kg) and 0.5% NaCl solution. The skull was fixated in the stereotaxic frame with bilateral ear bars, ensuring horizontal positioning. Fur covering the skull was shaven and central skin incision was made from between the eyes to neck region. Neck muscles were gently dissected and retracted bilaterally with forceps to prevent tissue entangling during skull drilling.

Following skull exposure, bilateral holes were carefully drilled at the predetermined coordinates. A Hamilton micro syringe (10 μl capacity) was filled with either the neurotoxin solution or vehicle control. The syringe was initially positioned at bregma as a reference point, then moved to the target coordinates. The needle was slowly lowered through the drilled openings to reach the NTS region. At each injection site, 1 μl of either sterile 0.15 M NaCl (vehicle) or neurotoxin solution (5 ng in vehicle) was delivered using an automated micropump over a 2-minute period. This slow delivery rate ensured minimal tissue disruption. The neurotoxin solution was prepared fresh from frozen stock aliquots within 2 hours before surgery to maintain optimal efficacy.

Post-surgical care began with administration of Sedastop (Omnidea, Stockholm, Sweden) to reverse anesthesia. Vital signs were carefully monitored during initial recovery. Animals were placed on a temperature-controlled heating

pad until fully conscious and ambulatory. Following initial recovery, mice were returned to their individual home cages. They were maintained under standard laboratory conditions with unrestricted access to high-fat diet (HFD). A two-week recovery period was implemented before any experimental procedures to ensure complete healing and optimal neurotoxin action.

3.5.2 INTRATHECAL CAPSAICIN INJECTIONS

Intrathecal interventions were designed to investigate spinal neurochemical signaling pathways with minimal systemic interference. Prior to surgery, mice were anesthetized with isoflurane (2-3% in oxygen). For precise targeting of the injection site, the lumbar region was shaved, and the L3-L4 vertebral level was carefully identified through manual palpation. A small midline skin incision provided clear visualization of the underlying vertebral structures. The capsaicin solution was freshly prepared immediately before injection by dissolving the compound in a vehicle mixture containing Tween-80, 99% ethanol, and sterile NaCl. Using controlled microinjection techniques, capsaicin (1 µg/µl) was delivered directly into the intrathecal space targeting spinal cord sensory circuits. This neurochemical agent was specifically selected for its properties to selectively activate and subsequently desensitize distinct neuronal populations. Post-surgical care included careful monitoring of the animals on a temperature-controlled heating pad until full recovery. This intervention protocol enabled detailed examination of neuronal activation patterns and their potential contribution to load-induced physiological responses.

3.6 ANALYTICAL APPROACHES

3.6.1 IMMUNOHISTOCHEMISTRY (IHC)

3.6.1.1 TISSUE PREPARATION
For immunohistochemical analysis of brain and spinal tissue, mice were deeply anesthetized and underwent transcardial perfusion with heparinized saline (50 IU/ml) followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB). The brain and spinal tissue were carefully extracted and post-fixed in 4% paraformaldehyde solution containing 15% sucrose in 0.1M PB overnight at 4°C. Tissues were then transferred to 30% sucrose solution in 0.1M PB for cryoprotection until sectioning. Using a Leica CM3050S cryostat (Leica Microsystem, Wetzlar, Germany), coronal sections (30 µm thickness) were obtained and stored in cryoprotectant solution (25% ethylene glycol, 25% glycerol, 50% 0.1M PB) until further processing.

3.6.1.2 FLUORESCENT IMMUNOSTAINING
The immunostaining procedure began with thorough rinsing of tissue sections in wash buffer (0.1M TrisHCl, pH 7.5, 0.15M NaCl). Non-specific binding was prevented by blocking sections for 1 hour in 5% normal goat serum containing 0.2% Triton-X-100. Primary antibodies (Table 1) were applied and sections were incubated overnight at 4°C. Following thorough rinsing, sections were incubated for 1 hour with fluorescent secondary antibodies diluted in the blocking solution. Nuclear visualization was achieved through DAPI staining for 3-4 minutes. Sections were then carefully rinsed and mounted using Prolong Diamond anti-fade mounting medium (P36965; ThermoScientific).
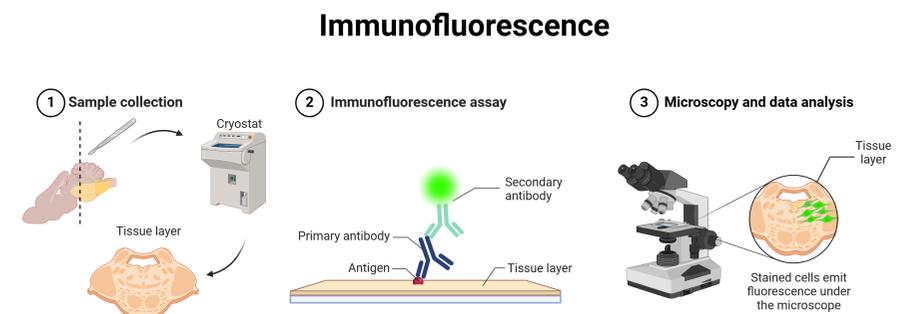
Immunofluorescence

Figure 4: Illustration of immunofluorescence workflow

3.6.1.3 DAB IMMUNOSTAINING
For 3'diaminobenzidine (DAB) visualization, the protocol followed identical initial steps through primary antibody incubation. Subsequently, instead of fluorescent secondary antibodies, sections were incubated for 1 hour with biotinylated secondary antibodies. Following thorough rinsing, sections underwent 30-minute incubation with avidin-biotin complex (ThermoScientific, Waltham, MA, USA). The final visualization was achieved through development with DAB solution containing 0.3% nickel sulfate for enhanced contrast. To validate antibody specificity, control sections were processed with mismatched primary and secondary antibodies, consistently yielding negative staining results and confirming absence of non-specific cross-reactivity.

Table 1: list of antibodies used in IHC

Antibody	Dilution	Catalog Number	Source and RRID
Primary Antibody			
Sheep anti-DBH	1:1000	ab19353	Abcam, Cambridge, UK. RRID: AB_731851
Rabbit anti-TH	1:1000	ab112	Abcam, Cambridge, UK. RRID: AB_297840
Mouse anti-FosB	1:200	ab11959	Abcam, Cambridge, UK. RRID: AB_298732
Rabbit anti-Cfos	1:2000	14,609	Cell Signaling Technology, Danvers, MA, USA. RRID: AB_2798537
Rabbit anti-NPY	1:1000	Ab221145	Abcam, Cambridge, UK. RRID: AB_2894872
Secondary Antibody			
Goat anti-rabbit Alexa fluor 488	1:1000	A31627	Thermo Fisher Scientific, Waltham, MA, USA
Donkey anti-sheep Alexa fluor 488	1:1000	A-11015	Thermo Fisher Scientific, Waltham, MA, USA
Goat anti-mouse Alexa fluor 594	1:1000	A-11005	Thermo Fisher Scientific, Waltham, MA, USA
Goat anti-mouse IgG (biotinylated)	1:1000	ab6788	Abcam, Cambridge, UK.RRID:AB_954885
Nuclear stain			
DAPI	1:5000	D1306	Thermo Fisher Scientific, Waltham, MA, USA

3.6.2 RNASCOPE

RNAscope analysis was implemented as a high-sensitivity technique for detecting specific messenger RNA transcripts in the desired tissue. Perfused tissue was sectioned at 12 μm thickness and stored in 10% formalin. On the day before RNAscope assay, sections were fixated on the SuperFrost Plus glass slides (VWR, Randor, PA, USA) air dried and left overnight in the oven (ACD HybEz II hybridization system) at 60°C. Next day, mounted slides were quickly rinsed (2x) in Phosphate Buffer Solution, and subsequently dehydrated through graded ethanol series (50%, 70%, 2×100%). Following dehydration, hydrophobic barrier was drawn around the tissue using an ImmEdge hydrophobic barrier pen and sections were treated with protease solution (pretreatment IV) for 30 minutes at a 40°C in the HybEz oven. Protease was quickly rinsed (2x) with Wash Buffer provided by RNAscope ACD kit (Newark, CA, USA). Target probe hybridization was performed under stringent conditions at 40°C in the HybEz oven for 120 minutes, followed by a series of pre-amplification and amplification steps (AMP1, 40°C for 30 min; AMP2, 40°C for 15 min; AMP3, 40°C for 30 min) that enabled single-molecule detection sensitivity. Multiple target genes were simultaneously visualized using different fluorescent tyramides, with sections counterstained with DAPI and mounted using ProLong Diamond anti-fade medium (P36965; ThermoScientific,

Waltham, MA, USA). This method proved particularly valuable for validating protein-level findings while maintaining precise spatial information about gene expression within intact tissue sections. Detailed descriptions of probes used are provided in a Table 2:

Table 2: Probes used for RNAscope

Probe/Reagent	Dilution	Catalog Number
FosB C1	1:1	539,721
TH C2	1:50	317,621-C2
DBH C2	1:50	464,621-C2
Slc17a6 C2	1:50	319,171-C2
Gad1 C3	1:50	511,931-C3
Fluorescent tyramide:		
Opal 520	1:750	FP1487001KT
Opal 570	1:750	FP1488001KT
Opal 620	1:750	FP1495001KT

All RNAscope probes were obtained from Advanced Cell Diagnostics Inc. (Newark, CA, USA) and Opal fluorophores from Akoya Biosciences (Marlborough, MA, USA).

3.6.3 CONFOCAL MICROSCOPY

High-resolution imaging was conducted using a Zeiss LSM 700 laser scanning confocal microscope system. This microscope utilizes point-focused laser illumination and pinhole detection to reject out-of focus light, allowing detailed visualization of cellular and subcellular structures. Images of tissues processed with IHC and RNAscope were captured using a Plan APO 40×/1.40 oil lens for high-magnification imaging and a Plan Fluor 20×/0.75 lens for anatomical overviews. The system's advanced optical sectioning capabilities enabled generation of three-dimensional data through Z-stack acquisition protocols provided by Center for Cellular Imaging at the University of Gothenburg. Image capture utilized specific laser lines and optimally calibrated filter sets optimized for each fluorophore's spectral properties. Comprehensive analysis of NTS was achieved through systematic tile scan imaging using a 3×3 grid pattern obtained via Zen Black software from Zeiss. Image processing and analysis followed standardized protocols using FIJI software (ImageJ v1.53f51), including z-stack processing into maximum intensity projections and uniform adjustment of brightness and contrast across comparative images and tile-stitching using the Grid/Collection stitching plugin with default parameters.

3.6.4 CELL QUANTIFICATION AND IMAGING

Quantification of immunoreactive cells was performed using a standardized counting protocol. For regional analysis, cell counts were conducted unilaterally on anatomically matched sections, with the investigator blinded to experimental conditions. The average number of labeled cells per section was calculated for each animal by dividing the total count by the number of sections analyzed. Treatment group averages were then derived by combining individual animal means and dividing by the group size. Anatomical boundaries for specific regions were precisely defined using anatomical landmarks from mouse atlas (Allen Brain Atlas).

For verification of noradrenergic neuron ablation, tyrosine hydroxylase (TH)-positive cells in the medial NTS were quantified bilaterally under blinded conditions. Strict exclusion criteria were implemented, whereby saporin-treated animals retaining more than 30% of TH-immunoreactive neurons were classified as incomplete lesions and excluded from further analysis.

Digital image processing was performed using FIJI software platform. Standardized adjustments for brightness and contrast were applied to optimize visualization of immunolabeled cells. For comprehensive regional analysis, high-resolution overview images were generated by assembling multiple fields using the FIJI tile scan stitching plugin.

3.6.5 STATISTICAL ANALYSIS

Statistical analyses were performed using a combination of parametric tests to evaluate treatment effects and group differences. Two-tailed, unpaired Student's t-tests with equal variance assumption were employed to assess differences between experimental groups for measurements including sodium and water evaluations, cell counting data, cumulative food intake, and specific time point comparisons of body weight changes. For longitudinal data analysis, particularly body weight and food intake changes over time, repeated measures Analysis of Variance (ANOVA) was utilized. When Mauchly's test of sphericity showed significance ($p \leq 0.05$) for treatment × time interactions, corresponding values were reported; otherwise, Greenhouse-Geisser corrections were applied. Data normality was evaluated using Shapiro-Wilk test and Q-Q plot visual inspection. Results were expressed as mean ± standard error of the mean (SEM), with statistical significance set at $p < 0.05$. For comprehensive analysis, both treatment effects alone and treatment × time interactions were assessed. Statistical computations were performed using IBM SPSS Statistics

(version 29.0.0.0) and Microsoft Excel, with graphical representations generated using GraphPad Prism (version 9.4.1). This statistical approach enabled robust analysis of both acute and longitudinal treatment effects while accounting for temporal variations in physiological responses.

3.7 METHODOLOGICAL STRENGTHS AND LIMITATIONS

The experimental approaches employed in these studies offered several notable strengths. The use of multiple complementary techniques (immunohistochemistry and RNAScope, detected with confocal microscopy) provided robust validation of findings through different methodological angles. The immunohistochemical analysis enabled precise protein localization while maintaining tissue architecture, and RNAScope technology offered single-molecule detection sensitivity at the RNA level. High-resolution confocal microscopy with standardized protocols ensured reliable quantification and detailed visualization of cellular structures. A particular methodological strength was the development of a novel, less traumatic loading method that allowed for separation of surgical effects from loading effects by introducing a two-week recovery period between capsule implantation and loading. The careful attention to controls, including secondary antibody controls and vehicle treatments, enhanced the reliability of the findings. However, several limitations should be noted. The studies primarily relied on male rodents, potentially limiting generalizability across sexes. The analysis of neuronal activation patterns, particularly in spinal cord segments, showed some discrepancies between intraperitoneal and subcutaneous loading methods, making it challenging to fully differentiate between direct loading effects and secondary mechanical responses due to the placement of the capsule. Additionally, while the capsaicin ablation experiments provided valuable insights, the lack of control experiments for neuronal activation following capsaicin injections left some mechanistic questions unanswered.

3.8 ETHICAL CONSIDERATIONS

All experimental procedures were conducted in accordance with rigorous ethical standards and received explicit approval from the local ethics committee for animal care at the University of Gothenburg (approval numbers 3308/20, 1874/18, and 5944/23). The studies adhered to relevant guidelines and regulations and were reported following ARRIVE (Animals in Research: Reporting

In Vivo Experiments) guidelines. Animal welfare was prioritized throughout the experiments, with careful attention to minimizing suffering through appropriate anesthesia (isoflurane or Sedastart) and pre-operative analgesia (Rimadyl, 5 mg/mL, dose: 1 mL/kg). Animals were housed under standardized conditions with appropriate temperature, humidity, and light/dark cycles, with free access to food and water. Exclusion criteria were clearly defined and implemented when animals showed signs of complications or distress, such as wound infections, deviating energy intake, or lethargy. While complete investigator blinding was not always feasible due to the nature of the capsule-loading procedures, cell counting was performed blindly to minimize bias. The number of animals used was justified through sample size calculations based on previous similar studies, adhering to the principle of using the minimum number of animals necessary to achieve statistically significant results.

3.9 INNOVATIVE METHODOLOGICAL CON-
TRIBUTIONS

The studies present several notable methodological innovations that were not previously investigated. A key development is the introduction of a novel, less traumatic loading method that enabled clear separation between surgical effects and loading effects through a two-week recovery period between capsule implantation and loading. The design of fillable capsules with external access through a threaded tube represents another technical advancement, allowing for controlled in vivo manipulation of loading conditions without additional surgical interventions. The studies also demonstrate innovative combinations of complementary techniques, integrating immunohistochemistry, RNAScope, and high-resolution confocal microscopy to validate findings through multiple methodological angles. Particularly noteworthy was the systematic approach to investigate spinal cord activation patterns across different segments, providing detailed mapping of neuronal responses to loading conditions. The use of intrathecal capsaicin injection for selective ablation of sensory nerve populations represented a sophisticated approach to investigate neural pathways. Furthermore, the careful implementation of metabolic cage trials with standardized measurement protocols enabled precise quantification of physiological responses to loading interventions, while maintaining rigorous control over potential confounding variables.

4 RESULTS

4.1 PAPER 1

Reduction of body weight by increased loading is associated with activation of norepinephrine neurones in the medial nucleus of the solitary tract

Our initial investigations revealed that increased load on weight-bearing extremities activated specific neuronal populations in the medial nucleus of the solitary tract (mNTS). Through complementary approaches of immunohistochemistry and RNAscope analysis, we demonstrated that both intraperitoneal (IP) and subcutaneous (SC) load implantation resulted in significant increases in FosB immunoreactivity in the mNTS. Notably, in load mice approximately 55% of these FosB-positive neurons co-expressed tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DbH), key enzymes for norepinephrine synthesis. This co-expression pattern was significantly higher as compared with control animals (~15% of activated neurons co-expressed TH), which indicated a specific activation of norepinephrinergic neurons. The functional significance of these neurons was confirmed through selective ablation using anti-DbH conjugated saporin, which attenuated the load-induced reduction of body weight and food intake.
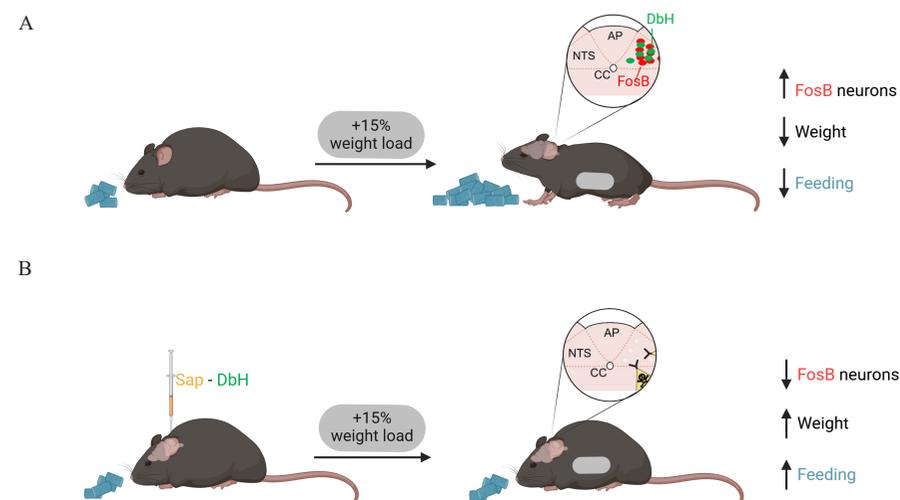

Figure 5: Illustration of results from Paper I. (A) Load application in mice fed with HFD leads to reduction in body weight and food intake while increasing in FosB expression in DbH producing neurons in the NTS.. (B) Ablation of DbH-positive neurons using Sap-DbH attenuates load-induced reductions in body weight, food intake, and diminishes load-induced increase in FosB expression in the NTS.

4.2 PAPER 2

Increased weight-bearing load reduces biological body weight while sodium and water balances are mainly unaffected

Using a novel, innovative two-step loading methodology which allowed more specific investigation of acute physiological responses to load, we demonstrated that increased weight-bearing load reduced body weight by 4.2% compared to controls, accompanied by decreased food intake. Through detailed metabolic cage studies, we observed no alterations in water or sodium balance, either acutely (days 1-2) or after sustained loading (days 8-9). These findings strengthened our previous observations that load-induced weight reduction primarily affects fat mass rather than fluid balance, suggesting a specific metabolic response rather than changes in body fluid homeostasis.
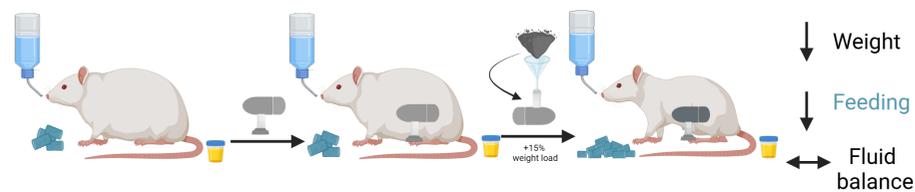

Figure 5: Illustration of results from Paper II. Rats fed with HFD reduce their weight and feeding upon receiving weight-bearing load, while their fluid balance remains unaffected.

4.3 PAPER 3

Weight reduction by artificial load requires sensory signaling in the dorsal horn of the lumbar spine

Our most recent findings revealed a crucial role for sensory signaling in the dorsal horn of the lumbar spine in the homeostatic regulation of body weight. We observed distinct patterns of neuronal activation in response to different loading methods. IP load activated neurons in segments T13-L6, while SC load showed more focused activation in segments L3-L5, which receive sensory input from the hindlimbs.

Through targeted ablation studies using intrathecal capsaicin administration, we demonstrated that these spinal sensory pathways are essential for the weight reducing effects of increased load. This discovery provides compelling evidence for a sensory pathway through which information from weight-bearing bones is transmitted from the hindlimbs to the central nervous system, advancing our understanding of the Gravitostat mechanism.
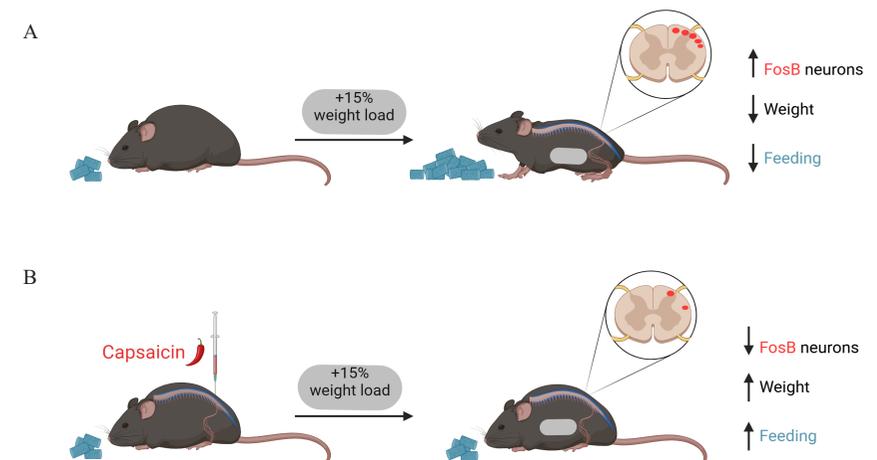

Figure 5: Illustration of results from Paper III. (A) Load application in mice fed with HFD leads to reduction in body weight and food intake while increasing the expression of FosB neurons within DH of the lumbar spine. (B) Ablation of sensory neurons using Capsaicin attenuates load-induced reductions in body weight and food intake, and diminishes load-induced increase in FosB expression in the DH of the lumbar spine.

These findings collectively establish a comprehensive framework for understanding load-induced weight regulation, from initial sensory detection through spinal processing to central integration in the brainstem, while excluding alternative mechanisms such as fluid balance alterations.

5 DISCUSSION

5.1 HISTORICAL CONTEXT AND PREVIOUS GRAVITOSTAT FINDINGS

The discovery of the Gravitostat mechanism in 2018 represented a paradigm shift in our understanding of homeostatic body weight regulation [158]. This homeostatic system, responding to loading of weight-bearing bones, demonstrated that gravitational force could influence metabolic regulation - a concept previously underexplored. Our early studies established that increased load reduced body weight and food intake in rodents independently of leptin, suggesting a novel pathway distinct from known metabolic regulatory systems [156]. Additionally, several studies done by our group in humans have explored the effect of increased load in overweight and obese people. We have shown that wearing weighted wests leads to a decrease in body weight and fat mass [159]. However, the mechanisms of the observed effects of weight load in both rodents and humans were not yet explored.

As noted above, load-induced regulation of body weight and food intake functions independently of leptin. While leptin's appetite-suppressing effects are most pronounced in lean subjects, the Gravitostat's anti-obesity properties are most pronounced in obese rodents [160]. This suggests a complementary regulatory system: leptin acts as a protective factor up until the obesity threshold, after which the Gravitostat "kicks in" [157]. Additionally, leptin's role may be primarily anti-anorectic, as maintaining appropriate levels of adipose tissue is vital for multiple physiological functions beyond energy storage and thermoregulation, such as endocrine health via adiponectin production and estrogen regulation [161,162]. Thus, as the leptin level in serum is the primary indicator of the body's adiposity level to the brain, a reduced leptin level may likely serve as a critical signal to ensure thermoprotection and preservation of reproductive health when fat mass drops below a healthy threshold.

The Gravitostat may then be the specific anti-obesity mechanism that becomes relevant specifically in individuals who have exceeded healthy weight thresholds.

5.1.1 EVOLUTIONARY CONSIDERATIONS

It is well known that our bodies have developed multiple survival strategies protecting us from threats, such as predatory-protection reflexes. These mechanisms operate along a threat imminence continuum, from prediction and prevention to rapid "fight or flight" behavior responses associated with modulation of the sympathetic nervous system. This in turn allows for an immediate reaction to threats, for instance through the periaqueductal gray and hypothalamic circuits [163].

Similarly, from an evolutionary perspective, our bodies have developed sophisticated mechanisms balancing energy preservation with survival capability. While storing energy as fat tissue offers crucial advantages for survival during food scarcity and provides thermal insulation [161], excessive adiposity can severely compromise mobility and metabolic health - factors critical for survival. Although leptin may have evolved as a primary signal for maintaining adequate energy stores and for supporting reproductive function through adipose tissue regulation, its effectiveness diminishes with obesity development ("leptin resistance") [164][165]. This suggests the necessity for additional regulatory mechanisms, potentially explaining the evolutionary role of the Gravitostat. As a body weight regulator, the Gravitostat appears to represent an evolutionary safeguard that becomes activated specifically when excess weight begins to compromise mobility.

Since body weight regulation is a central homeostatic system of importance for various mechanisms of survival, it is likely that many such systems function in parallel to ensure the survival of the organism. The proposed evolutionary framework supports the presence of multiple weight-regulatory systems and explains why mechanical load-sensing would be particularly important for mobile organisms. Moreover, it provides a context for understanding why such mechanisms might be impaired in our modern sedentary society, with its high prevalence of obesity and related chronic diseases [11,166–168].

5.2 THE GRAVITOSTAT IN THE BRAIN

Noradrenergic neurons in mNTS are needed for Gravitostat signaling

The observation that weight loading decreased food intake implicated the Gravitostat in the behavioral regulation and brain function. A major aim of this study was to identify the brain areas and brain functions that mediate the Gravitostat effect. The discovery of a specific neuronal activation pattern in rodents with increased load, demonstrated by FosB immunostaining in the medial Nucleus of the Solitary Tract (mNTS), marked a pivotal shift in our understanding of Gravitostat mechanisms. NTS is well known as a key integrating center for regulation of energy homeostasis [136].

Our initial hypotheses focused on endocrine pathways due to osteocyte involvement [156], and such may play a role in the Gravitostat signal. However, the Gravitostat might primarily operate through neuronal mechanisms, including afferent cranial nerves like the vagus, afferent spinal sensory nerves, or via the spinosolitary tract [169]. The latter pathway would transmit the signal from the spine to the NTS in the brainstem.

A crucial breakthrough was the identification of a predominance of noradrenergic cells among the load-activated neurons in the medial part of Nucleus of the Solitary Tract (mNTS) in the brain. Approximately 55% of FosB+ neurons in the mNTS co-expressed tyrosine hydroxylase (TH) and dopamine-beta hydroxylase (DbH); key enzymes in norepinephrine (NE) synthesis from dopamine (DOPA) [170,171]. This finding aligns with previous research on norepinephrine's role in appetite suppression, particularly in pharmacological contexts such as amphetamine-based therapies [172,173].

Methodologically, we validated our findings through both intraperitoneal (IP) and subcutaneous (SC) load models. While the IP model provided initial insights, the SC model confirmed that load-induced effects were more likely to be independent of potential confounding factors such as vagal stimulation or gastrointestinal compression [174–176]. Both models demonstrated consistent patterns of neuronal activation in the mNTS, reinforcing the specificity of the load response.

The remaining 45% of FosB+ neurons that don't express noradrenergic markers suggest additional cell populations involved in load sensing. While some activation might reflect non-specific effects of capsule implantation, the consistent FosB+ expression patterns in both IP and SC models may indicate a more specific load-induced response in these cells.

In summary, this study demonstrated that effects of increased load on body weight and food intake require specific neurons in the mNTS. Ablation of these activated cells showed that noradrenergic cells are needed for this regulation. As the weight regulation and neuronal activation happen regardless of implantation method, this provides robust evidence of an independent physiological mechanism caused by the increased load itself.

5.3 GRAVITOSTAT IN THE PERIPHERY

Gravitostat's weight-reducing effects are independent of fluid and sodium dynamics

The development of a novel loading methodology represented a significant advancement in Gravitostat research, enabling isolation of increased loading effects from confounders due to surgical trauma. By implementing a two-stage approach - initial capsule implantation followed by in vivo loading after a recovery period - we effectively minimized potential surgical artifacts that could influence physiological responses to increased load. This methodological refinement provided a more precise platform for examining acute physiological adaptations to increased load.

Given the acute effects of load on body weight, we aimed to determine if this effect could be attributed to a direct loss of fluids or, indirectly, via changes in sodium dynamics [177,178]. This investigation was particularly relevant as the brain is involved in fluid and sodium homeostasis through a complex circuitry involving the NTS, the same area where we observed load-induced neuronal activation [179,180]. Through comprehensive metabolic cage studies, we monitored sodium and water balance at two strategic time points: immediately post-loading and during an extended acute phase. This dual-timepoint analysis allowed differentiation between immediate responses and sustained effects while maintaining focus on acute physiological adaptations.

The results demonstrated that increased load, using our novel method, reduced biological body weight by 4.2% compared to controls, accompanied by a significant reduction in food intake. Notably, neither immediate nor extended acute measurements revealed alterations in water intake patterns, sodium balance, or overall fluid homeostasis. The absence of changes in fluid dynamics at both time points provided robust evidence that load-induced weight reduction operates independently of water and sodium regulation.

These findings substantially strengthen the previously established findings that Gravitostat-mediated weight regulation primarily influences energy balance and adiposity, rather than fluid balance [156]. The preservation of normal fluid homeostasis despite significant weight reduction suggests that the Gravitostat operates through distinct pathways focused on energy balance regulation. This understanding advances our mechanistic knowledge of body weight regulation and provides crucial insights for developing targeted therapeutic approaches for obesity management.

This study's methodological strength lies in its ability to isolate load effects through minimized impact of the initial surgical trauma, provide precise temporal resolution of physiological responses, and quantitatively assess multiple components of fluid homeostasis. These advances in experimental design have enhanced our understanding of the Gravitostat's specificity in regulating body weight through energy balance rather than fluid dynamics.

Sensory innervation in lumbar spine is needed for Gravitostat signalling

Identifying neuronal activation patterns in the dorsal horn of the lumbar spine represented a crucial step in understanding the Gravitostat's peripheral pathway. If the Gravitostat signal from the osteocytes in weight-bearing hindlimbs involves neurons, it is likely transmitted by the sciatic nerve, via the dorsal root ganglion to the dorsal horn of the spine. Using both IP and SC loading models, we demonstrated distinct patterns of FosB-positive, activated neurons in the dorsal horn (DH) of mice. IP load activated cells in segments T13-L6, while SC load showed a more focused activation of cells in segments L3-L5. This differential activation pattern aligns with our current understanding of the dorsal horn as a sophisticated integration center for multiple independent neural circuits [181].

Moreover, a broader activation pattern observed in IP-loaded mice (T13-L6) likely reflects the complex nature of visceral sensory processing, as these segments are known to receive diverse afferent inputs from abdominal organs through distinct pathways. This system is much more widely studied in the rat than the mouse, but is likely to not differ too much [182]. More specifically, thoracolumbar and lumbosacral regions represent two discrete populations of DRG mediated transmission that independently innervates visceral organs, whereas T13-L1 innervate distal colon and rectal area, and L5-S1 receive input from rectal area [183]. In contrast, the more focused activation pattern observed in SC-load mice (L3-L5) suggests a more specific sensory pathway activation, reflecting direct innervation from the sciatic nerve which innervates hindlimbs [184].

A key finding was that capsaicin-mediated ablation of sensory neurons in the lumbar spine attenuated both the weight-reducing effects of load and the neuronal activation patterns in the IP mouse model. Thus, intact sensory signaling in the dorsal horn of lumbar segments is necessary for load-induced weight regulation. Additionally, the localization of load-responsive neurons to laminae I-III, which primarily receive sensory input from C- and Aδ fibers from deep tissues including bone [185], suggests a specific pathway for mechanical load sensing.

Altogether, these findings establish that sensory innervation in the lumbar spine serves as a crucial component in the Gravitostat pathway, providing a neural circuit through which mechanical loading information can be transmitted to metabolism-regulating centers in the brain. This may be accomplished either via direct projection neurons, or via further modulation of a signal that is then transmitted to deeper laminae and from there onwards to the brain. Further studies are needed to determine this step of the Gravitostat pathway. This work bridges the gap between peripheral load sensing and central regulation of body weight, offering new insights into the physiological mechanisms of body weight homeostasis.

6 CONCLUSION

Increased load in obese rodents leads to a reduction in body weight and food intake. The observed effects require intact noradrenergic neurons in the mNTS in the brainstem and TrpV1-containing sensory nerves in the lumbar spine. Additionally, as this process is independent of fluid and sodium dynamics, acute reduction of body weight by load seems to happen primarily due to fat loss, rather than fluid loss.

6.1 CLINICAL IMPLICATIONS

Taken together with evidence from human studies, pre-clinical research on the Gravitostat has provided us with novel insights about weight regulating systems in our body which could lead to the development of preventative strategies as well as pharmacological targeting of neurons involved. Non-pharmacological interventions grounded in our findings help society by encouraging more movement to activate the anti-obesity properties of sensed weight-bearing due to excess adiposity. Additionally, albeit speculation, people with obesity may activate this mechanism by wearing heavy backpacks.

7 FUTURE PERSPECTIVES

1. Further cellular investigation of the identity of mNTS load-activated neurons is required. Even though a large population of these neurons express NE, it is unclear if this substance is the key mediator of the Gravitostat. One such promising substance that has been implicated in regulation of body weight and is known to be co-expressed with NE is prolactin releasing hormone [140]. Further studies using targeted inhibition of NE signaling, rather than ablation of the whole NE-producing neuron, are needed to elucidate NE's role in load-induced reduction in body weight and food intake. Additionally, further studies are needed for determining expression of neuropeptides within load-activated mNTS neurons. This would allow the use of specific functional studies which would give us the possibility of manipulating these neurons by using novel methods such as chemogenetics for mimicking long-term neuronal activity and optogenetics for investigation of acute responses.

2. Identification of downstream brain pathways from mNTS. So far, we haven't been able to determine if the Gravitostat signal ends in mNTS or projects to other energy homeostasis regulating regions of the brain. While some hypothalamic areas have been explored (such as ARC showing increased activation in NPY neurons), they haven't given us a clear answer, as we believe this activation represents a failed compensatory mechanism. Therefore, given the complexity of signaling within other areas in hypothalamus, such as PVN (described in Introduction), it would be worth investigating distinct neuronal populations involved in appetite reduction, such as oxytocin and MC4R. The goal is to find downstream signals from the mNTS and therefore it would be best to use subcutaneous load model or two-step intraperitoneal load model. The use of neuroanatomical tracers, such as Cholera Toxin subunit Beta (CTB), injected into mNTS and combined with immunostaining markers for neuronal activation (FosB) and the aforementioned neuropeptides would allow quantification of active markers and allow mapping of mNTS projections involved in load-induced appetite reduction. Furthermore, other nuclei known to be of importance for body weight regulation, such as the lateral PBN, could be similarly investigated.

3. As in the brain, additional investigations of spinal responses would include identifying specific substance released in response to load. While we have observed the requirement of TRPV1 channels (required for a response to capsaicin), cellular mechanisms including release of neuropeptides would give us

answers about specificity and therefore allow the employment of manipulation approaches. It has been shown that cell populations within the dorsal horn with functions either as inhibitory or excitatory interneurons, as well as direct projection neurons, contain non-overlapping neuropeptides. Additionally, direct connection of spine with brain as well as with osteocytes in relation to load warrants further investigation.

4. Translation of rodent studies to human studies. As described above, our research group was able to establish connections with human results, i. e. loss of body weight and fat loss in humans wearing weighted wests. However, more studies are needed to determine if the same pattern of neuronal activation described in this thesis happens in humans.

5. While the main focus of our studies was on the mechanism of the Gravitostat, our research has opened the door for other applications of load, such as in strength training. Therefore, more research is needed to investigate specific cellular mechanisms within muscle tissue. Since we have found indications that load reduces appetite via nervous connections between mNTS and DH of the lumbar spine, it would be interesting to see if these same neurons are activated in response to weight-lifting. This may be of special importance if a future drug based on the principles of the Gravitostat turns out to have similar muscle-wasting side effects as current GLP-1 analogs.

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