Sex differences in behavior and metabolism

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To my family

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ABSTRACT

Men and women exhibit distinct illness patterns and disparate responses to pharmacotherapies. However, there is a scarcity of preclinical studies that systematically compare the sexes. The overarching aim of this thesis was to identify sex differences, and their underlying mechanisms, in metabolic and behavioral control within rodent models of obesity and anxiety. Specifically, we explored novel brain targets and mechanisms for the control of appetite, energy expenditure, and emotionality. In **Paper I**, we found that mice and rats subjected to diet-induced obesity responded with sexually divergent eating behavior and adaptations in energy expenditure. In **Paper II**, we showed that obesity reduced the expression of interleukin-6 (IL-6) in the brain of males only, and that the role of IL-6 in the parabrachial nucleus (PBN) is sexually dimorphic, such that it is only necessary for normal brown adipose tissue thermogenesis in males. In **Paper III**, we discovered that the locus coeruleus (LC) is a novel site for the behavioral effects of the hunger hormone ghrelin. We demonstrated that males have higher ghrelin receptor levels in the LC, and that there is a sex difference in response to ghrelin receptor activation and blockade, in regards to food motivation and anxiety-like behavior. Finally, in **Paper IV**, we investigated whether brain-produced estrogen plays a role in body weight regulation. We found a sexually dimorphic role of aromatase in the amygdala, such that it is only necessary for normal energy homeostasis and food motivation in females. Collectively, the work presented in this thesis underscores the significance of considering biological sex in the context of energy balance regulation and associated behaviors. These findings contribute to a broader conversation on addressing sex differences in human disease with the ultimate goal of enhancing the success of drug development.

Keywords: sex differences, eating, brown adipose tissue, motivation, anxiety-like behavior
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Sammanfattning på svenska

Trots att sjukdomsförekomst och läkemedelsreaktioner skiljer sig mellan kvinnor och män baseras merparten av vår kunskap om medicin på forskning som har utförts på män och handjur. Denna snedvridning har gett upphov till kunskapsluckor som bland annat har resulterat i att kvinnor drabbas av läkemedelsbiverkningar i större utsträckning än män. Konsekvenserna sträcker sig bortom biverkningar – om grundläggande forskning inte undersöker eventuella könsbetingade skillnader kan vi gå miste om behandlingsmöjligheter som hade varit gynnsamma för ett av könen.

Denna avhandling utforskar könsskillnader i beteende och metabolism hos råttor och möss. Vi har fokuserat på två vanliga hälsoproblem hos människor: fetma och ångest. Fetma är en global hälsoutmaning kopplad till psykiatriska sjukdomar och nedsatt immunförsvar. Vår forskning visade att hanar och honor reagerar olika på fetma och övervikt. I vår första studie erbjöd vi möss och råttor av båda könen en diet med högt fett- och sockerinnehåll. Honor åt färre totala kalorier än hanar men föredrog den ohälsosamma dieten före sin vanliga diet. Detta tyder på att deras matintag kan vara mer driven av njutning. Vi fann också att hanar och honor förbränner energi på olika sätt. Honråttor uppvisade större energiförbränning, vilket troligen bidrog till större motståndskraftighet mot viktuppgång. Å andra sidan var hanråttor bättre på att kompensera för det höga fettinnehållet i den ohälsosamma maten genom att öka aktiviteten i sin bruna fettvävnad. Trots att den här kompensationen inte var tillräcklig för att förhindra viktuppgång så bidrog den troligen till att bibehålla normala blodsockernivåer.

I vår andra studie tittade vi på ämnet interleukin-6 (IL-6) som är känt för sin roll i inflammation. Vid fetma är nivåerna av IL-6 förhöjda i blodomloppet men reducerade i den vätska som omger hjärnan, cerebrospinalvätskan. Vi observerade att hanråttor och möss med fetma hade reducerade nivåer IL-6 i en del av hjärnan som kallas parabrachialkärnan (PBN), medan detta inte gällde för honor. Genom att manipulera IL-6 i PBN kunde vi kontrollera kroppstemperatur och vikt hos endast hanråttor. Intressant nog återställdes de reducerade nivåerna av IL-6 när de feta hanarna utsattes för kyla. Detta ledde också till en ökning av den bruna fettvävnadens förbränningsförmåga. Dessa resultat tyder på att utvecklingen av

fetmaläkemedel som riktar sig mot signalvägar relaterade till IL-6 bör beakta potentiella könsskillnader.

I vår tredje studie undersökte vi hormonet grelin som frisätts från magen och spelar en avgörande roll för att överföra hungersignaler till hjärnan och påverka humöret. Vi upptäckte ett nytt hjärnområde där grelin verkar. Detta hjärnområde, känt som locus coeruleus (LC), är viktigt för kontrollen av motivation och emotionella tillstånd. Våra resultat visade att hanar och honor reagerar olika på grelinsignaler i LC. Grelin som administrerades till LC ökade matintaget och motivationen för socker hos båda könen, men minskade ångestliknande beteende endast hos honor. Att blockera grelinsignaler i samma hjärnområde minskade matintaget och ökade ångesten hos båda könen, men påverkade motivation endast hos honor.

Avslutningsvis, i den fjärde studien, undersökte vi effekterna av aromatas, enzymet som är ansvarigt för syntesen av östrogen. Även om majoriteten av östrogen produceras av könsorgan och fettvävnad, finns aromatas även i hjärnan hos både män och kvinnor. Trots det vet vi fortfarande lite om detta östrogen som produceras i hjärnan, särskilt med avseende på energibalans. När vi hämmade hjärnans aromatas, vilket minskade produktionen av östrogen, ökade endast honor i vikt. Detta berodde på nedsatt energiförbrukning och ökat intag av specifikt fettrik mat. Vi fann också att minskningen av aromatas i amygdala, en hjärnregion som är involverad i emotionella processer, spelade en avgörande roll i dessa effekter. Eftersom aromatashämmare redan används i klinisk praxis, främst för behandling av bröstcancer, är det avgörande att förstå deras fulla påverkan på beteende och ämnesomsättning.

Sammanfattningsvis betonar denna avhandling vikten av att inkludera både hanar och honor i prekliniska studier. Dessa resultat bidrar till en mer omfattande diskussion om att systematiskt analysera eventuella könsskillnader i grundläggande forskning, med syftet att i slutändan förbättra framgången av läkemedelsutveckling.

Научно популарни сажетак

Упркос разликама у учесталости обољења и реакцијама на лекове између мушкараца и жена, већина нашег медицинског знања заснива се на истраживањима изведеним превасходно на мушкарцима и мужјацима. Ова полна пристрасност у истраживању довела је до непотпуног разумевања начина на који терапија утиче на жене, што често доводи до испољавања више нуспојава лекова код жена у односу на мушкарце. Међутим, полна пристрасност у истраживању има, поред нуспојава лекова, и друге последице. Ако не узимамо у обзир оба пола и поређење између њих, можемо пропустити терапеутике који би били врло ефикасни код једног пола али никад не би прошли клиничка испитивања због недостатка статистичке анализе у односу на пол.

У овој докторској дисертацији смо истраживали разлике у метаболизму и понашању између мужјака и женки пацова и мишева. Усредсредили смо се на два честа обољења код људи: гојазност и анксиозност. Гојазност је глобални здравствени изазов који је повезан са психијатријским и имунским поремећајима. Наше истраживање открило је да мужјаци и женке различито одговарају на гојазност и прекомерно стицање телесне масе. У првом раду из ове дисертације, понудили смо мишевима и пацовима оба пола избор између стандардне и дијете богате мастима и шећерима. Женке су уносиле мање калорија свеукупно у односу на мужјаке али су бирале нездраву храну у односу на нормалну. Ово указује да су навике у исхрани женки можда више подстакнуте задовољством. Такође смо показали да мужјаци и женке различито сагоревају енергију. У почетним условима, женке пацова су показале већу локомоторну активност и активност мрког масног ткива, што је вероватно допринело почетном одолевању у добијању телесне масе. Са друге стране, мужјаци пацова су боље балансирали дијету богату мастима тако што су појачавали метаболизам путем активације мрког масног ткива које помаже у сагоревању калорија и регулацији шећера у крви.

У другом раду из дисертације концентрисали смо се на супстанцу под именом интерлеукин-6 (ИЛ-6) која је позната по својој улози у запаљенским процесима. Док је гојазност повезана са високим нивоом ИЛ-6 у крви, нивои ИЛ-6 у течности која окружује мозак, цереброспиналној течности, се смањују са гојазношћу. Приметили смо да гојазни мужјаци мишева и пацова имају смањени ниво ИЛ-6 у делу мозга који се зове парабрахијално једро (ПБЈ), што није био случај са гојазним женкама. Подешавањем нивоа ИЛ-6

специфично у ПБЈ, успели смо да контролишемо телесну температуру и добитак телесне масе мужјака пацова. Занимљиво, излагање животиња хладноћи успоставило је нормалне нивое ИЛ-6 и повећало активност мрког масног ткива у сагоревању калорија. Ови резултати указују на то да будући третмани гојазности морају узети у обзир полне разлике.

У трећем раду из ове дисертације испитивали смо хормон грелин који се ослобађа из црева и игра кључну улогу у преносу сигнала за глад ка мозгу, такође утичући на расположење. Открили смо ново место испољавања дејства грелина. Тај регион мозга, познатији као ромбоидна јама (лоцус цоерулеус , ЛЦ), укључен је у контролу мотивације и емотивних стања. Наши резултати су показали да мужјаци и женке испољавају другачији одговор на стимулацију грелином у ЛЦ. Примена грелина у ЛЦ повећала је унос хране и жељу за шећером у оба пола, али је смањила анксиозности-слично понашање само код женки. Блокада сигнала грелина у истом региону мозга смањила је унос хране и повећала анксиозност код оба пола. Међутим, блокада сигнала грелина је утицала на искуство задовољства храном само код женки.

Коначно, четврти рад из ове дисертације бави се ароматазом - ензимом одговорним за настанак естрогена. Иако се већина естрогена производи у репродуктивним органима и масном ткиву, ароматаза је такође присутна и у мозгу оба пола. Међутим, о овом естрогену који настаје у мозгу се и даље врло мало зна, нарочито у односу на енергетски баланс. Када смо спречили дејство ароматазе у мозгу, само су женке добиле на тежини. Овај добитак телесне масе је био последица смањеног трошења енергије и повећаног уноса специфично калоричне хране. Такође смо открили да је смањење ароматазе у амигдали, региону мозга укљученом у емотивне процесе, одиграло кључну улогу на поменуте процесе. Имајући у виду да су инхибитори ароматазе класа лекова који су већ у клиничкој употреби, углавном за третман канцера дојке, изузетно је важно разумети њихов свеукупан утицај на понашање и метаболизам.

Укратко, наши резултати истичу важност укључивања оба пола у пре клиничка истраживања. Ови резултати доприносе ширем дијалогу о аспекту полних разлика у болестима људи са крајњим циљем повећавања успешности развоја нових лекова.

List of papers

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

I. **Sex and species differences in the development of diet-induced obesity and metabolic disturbances in rodents.**
Maric I, Krieger JP, van der Velden P, Börchers S, Asker M, Vujicic M, Wernstedt Asterholm I, Skibicka KP.
Frontiers in Nutrition, 2022; 9: 828522.

II. **Parabrachial interleukin-6 reduces body weight and food intake and increases thermogenesis to regulate energy metabolism.**
Mishra D, Richard JE, Maric I, Porteiro B, Häring M, Kooijman S, Musovic S, Eerola K, López-Ferreras L, Peris E, Grycel K, Shevchouk OT, Micallef P, Olofsson CS, Wernstedt Asterholm I, Grill HJ, Nogueiras R, Skibicka KP.
Cell reports, 2019; 26(11): 3011-3026.e5.

III. **From the stomach to locus coeruleus: new neural substrate for ghrelin's effects on ingestive, motivated and anxiety-like behaviors.**
Maric I, López-Ferreras L, Bhat Y, Asker M, Börchers S, Bellfy L, Byun S, Kwapis J, Skibicka KP.
Frontiers in Pharmacology, 2023; 14: 1286805.

IV. **Sex-specific effects of amygdala aromatase in the control of energy balance and food reward.**
Maric I, Richard JE, Taing L, López-Ferreras L, Byun S, Bhat Y, Skibicka KP.
Manuscript

Scientific contributions beyond this thesis

Peripherally restricted oxytocin is sufficient to reduce food intake and motivation, while CNS entry is required for locomotor and taste avoidance effects.
Asker M, Krieger JP, Liles A, Tinsley IC, Borner T, Maric I, Doebley S, Furst CD, Börchers S, Longo F, Bhat YR, De Jonghe BC, Hayes MR, Doyle RP, Skibicka KP.
Diabetes, Obesity and Metabolism, 2023; 25(3): 856-877.

Neural pathway for gut feelings: vagal interoceptive feedback from the gastrointestinal tract is a critical modulator of anxiety-like behavior.
Krieger JP, Asker M, van der Velden P, Börchers S, Richard JE, Maric I, Longo F, Singh A, de Lartigue G, Skibicka KP.
Biological Psychiatry, 2022; 92(9): 709-721.

From an empty stomach to anxiolysis: molecular and behavioral assessment of sex differences in the ghrelin axis of rats.
Börchers S, Krieger JP, Maric I, Carl J, Abraham M, Longo F, Asker M, Richard JE, Skibicka KP.
Frontiers in Endocrinology, 2022; 13: 901669.

Commonly-used rodent tests of anxiety-like behavior lack predictive validity for human sex differences.
Börchers S, Krieger JP, Asker M, Maric I, Skibicka KP.
Psychoneuroendocrinology, 2022; 141: 105733.

CONTENT

Abbreviations

AAV	adeno-associated virus
AgRP	agouti-gene-related protein
AR	androgen receptor
Arc	arcuate nucleus of the hypothalamus
ASR	acoustic startle response
BAT	brown adipose tissue
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CNS	central nervous system
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
ER	estrogen receptor
FISH	fluorescent in situ hybridization
GHSR	growth hormone secretagogue receptor, ghrelin receptor
GLP-1	glucagon-like peptide 1
HPA	hypothalamic-pituitary-adrenal
IHC	immunohistochemistry
IL-6	interleukin-6
LC	locus coeruleus
LEAP2	liver-enriched antimicrobial peptide-2

LH	lateral hypothalamus
MCH	melanin-concentrating hormone
mRNA	messenger RNA
NAc	nucleus accumbens
NPY	neuropeptide Y
NTS	nucleus tractus solitarus
OF	open field
ORX	orexin
OT	oxytocin
PBN	parabrachial nucleus
PFC	prefrontal cortex
POMC	proopiomelanocortin
PR	progestin receptor
PVH	paraventricular nucleus
PYY	peptide YY
qPCR	quantitative polymerase chain reaction
siRNA	small interfering RNA
TH	tyrosine hydroxylase
UCP-1	uncoupling protein-1
VMH	ventromedial hypothalamic area
VTA	ventral tegmental area
WAT	white adipose tissue

Definitions in short

Sex differences are defined as biological differences determined from the gonadal and chromosomal composition. While this thesis categorizes sex as male or female in the context of experimental rodents, it is important to recognize that in animals (including humans) variations to this binarity do occur.

Gender differences are relevant only for research with humans and encompass gender identity, social structures and culturally acquired or attributed characteristics.

INTRODUCTION

Sex differences in prevalence, manifestation, and drug response have become evident in numerous medical conditions (Mauvais-Jarvis et al., 2020). While these disparities are increasingly recognized, the foundation of our knowledge about health and disease is predominantly derived from research conducted on male cells, male rodents, and men (Beery & Zucker, 2011; Woitowich et al., 2020). This bias holds significant implications, no less in the context of one of the most pressing global health challenges of our time – the striking surge in overweight and obesity rates. Women are disproportionately affected by eating disorders and morbid obesity compared to men (Flegal et al., 2016). Differences between genders can arise from the sociocultural factors imposed on humans, however, animal studies have revealed that there is an effect of biological sex in many aspects of energy balance regulation (Benz et al., 2012; Morselli et al., 2016). Some of these, albeit not all, can be attributed sex hormones and their fluctuations throughout life (Wang & Xu, 2019).

Weight gain is a result of a positive energy balance, where energy intake exceeds energy expenditure. Appetite is complex and modulated by the body's internal state and the perception and processing of environmental stimuli. In the control of eating, the brain integrates an array of peripheral signals communicating energy reserves, along with intricate signals encompassing sensory pleasures, past experiences, emotional states, circadian rhythms, immune responses, and more. The role of food in modern Western society has transcended its fundamental purpose of providing nutrition for survival. The abundant availability of tasty, energy-dense food, promotes overeating and leads to an excess accumulation of body fat. In our battle against obesity, gaining insights into the fundamental mechanisms driving this disrupted eating behavior is of utmost importance. From an energy

balance point of view, it may not be possible to solve the problem of excessive weight gain by focusing on eating behavior alone. Energy expenditure is divided in three main components: basal metabolism, adaptive thermogenesis and physical activity. On this side of the energy balance equation, brown fat stands out as an intriguing therapeutic target due to its inherent ability to burn calories (Nedergaard & Cannon, 2010). Nonetheless, our understanding of the tissue's role in the development and prevention of obesity remains limited.

The consequences of excessive weight gain extend far beyond metabolic concerns; it intertwines with psychiatric disorders and impaired immune function (Bapat et al., 2022; Milaneschi et al., 2019). To combat the rise of obesity and comorbidities effectively, a comprehensive understanding of the neural circuits governing energy intake and expenditure is imperative. Despite the increasing recognition of the interaction between sex and body weight regulation, further explorations about how the underlying mechanisms differ between males and females are necessary. This thesis aims to encourage a deeper appreciation for sex as a modifier of behavior and metabolism, as part of a crucial step toward research that will bring advantages to men and women alike.

Origins of sex differences

Biological sex differences result from the interplay of genetic and endocrine mechanisms that evolved due to evolutionary forces. Some are intrinsic and driven by direct effects of sex chromosome genes, the organizational effects of hormones during early development, and epigenetic chromatic modifications. The initiation of biological sex differences is controlled by genes on the X and Y sex chromosomes, with females typically possessing the XX karyotype, and males the XY karyotype. During development, male and female embryos are exposed to distinct surges of androgens and estrogens respectively (Arnold &

Breedlove, 1985). This leads to 'organizational effects', which have permanent impact on shaping anatomy and physiology. For instance, male rodents and humans respond to weight loss by compensatory overeating, while females compensate by reduced energy expenditure (Shi et al., 2007; Valle et al., 2005; Zandian et al., 2011). This outcome was suggested to be due to the masculinization of the developing brain, resulting in sex-specific morphological differences within the hypothalamic melanocortin system in mice (Nohara et al., 2011). Beyond the organizational effects, a big portion of observed sex differences are due to 'activational effects' that are mediated through the acute effects of sex steroids (Frye et al., 2008; Santollo & Eckel, 2008; Walf & Frye, 2010).

Sex steroids encompass estrogens, androgens and progestins. These hormones interact with their specific receptors, namely progestin receptors (PR), androgen receptors (AR) and estrogen receptors (ER), distributed throughout the body and the brain of both males and females (MacLusky & McEwen, 1980; Shughrue et al., 1997; Simerly et al., 1990). Progestins are synthesized from cholesterol, and can be further converted to androgens (Miller & Auchus, 2011). Subsequently, estrogens are synthesized from androgens by the enzyme aromatase (encoded by the CYP19A1 gene) (Simpson et al., 1994). Both males and females produce testosterone and estrogen. In men, testes are producing majority of circulating androgens, while the same holds true for ovaries and estrogen in premenopausal women. In contrast, in men and postmenopausal women estrogen synthesis primarily takes place in other tissues than the gonads. This is possible due to the presence of aromatase in organs beyond the gonads, including the adipose tissue and brain (Callard et al., 1978; McTernan et al., 2000; Roselli et al., 1998).

Women, as well as female rodents, have fluctuating plasma levels of sex hormones produced by the ovaries. The human menstrual cycle

spans slightly shorter than a month on average, while the rodent estrous cycle is approximately four days long. Nonetheless, the hormonal fluctuations are very similar between species and affect, and shape, brain physiology (Rocks et al., 2022). In the past, the exclusion of female animals in pre-clinical research was often justified with the misconception that females exhibited more variable behavior than males due to these hormonal fluctuations. However, recent research has dispelled this myth, as it has been shown that male rodents are equally variable and also undergo hormonal changes, albeit not in the predictable cyclical pattern observed in females (Becker et al., 2016; Prendergast et al., 2014; Smarr & Kriegsfeld, 2022).

The effects of ovarian estrogens have been extensively studied in the context of eating behavior. During the ovarian cycle, variations in food intake occur in both women and rodents. Lowest food intake is observed following periods of high estradiol levels, namely the periovulatory phase in women and estrus in rats (Danker-Hopfe et al., 1995; Roney & Simmons, 2017). The anorexic effects of estradiol are further confirmed with ovariectomy (Asarian & Geary, 2002). In rats, the surgical removal of ovaries results in hyperphagia (overeating), increased food reward, and body weight gain. Central administration of estradiol is enough to reverse these effects, and ERs in the brain are necessary for estradiols anorexigenic effects (Palmer & Gray, 1986; Rivera & Eckel, 2010). The influence of androgens on eating behavior in males have received less attention, possibly because androgen levels remain relatively stable throughout reproductive life and are not subject to abrupt changes as the ones associated with pregnancy or menopause (Kaufman & Vermeulen, 2005). Hypogonadism in men is associated with obesity, but castration leads to weight loss which is partially reversed with testosterone administration (Fernandez et al., 2019; Gentry & Wade, 1976; Kim et al., 2021). Notably, the interpretations of testosterones effects can be complicated by

aromatase converting it into estrogen, that holds the same anorexic effect in males as it does in females.

Aromatase, the sole enzyme responsible for estrogen production, is present in the brains of both women and men (Biegon, 2016). This signifies that estrogen's actions in the brain are not solely reliant on peripheral sources but also involve local production. The specific functions of locally produced brain estrogen are still a subject of ongoing research. Interestingly, human imaging studies have hinted that aromatase activity in various brain regions could be associated with vital factors ranging from personality traits to body weight (Biegon et al., 2020; Takahashi et al., 2018). Surprisingly, despite these intriguing leads, there has been an absence of studies investigating the impact of local estrogen synthesis in the brain on appetite and energy balance. Aromatase inhibitors, a commonly used class of drugs for breast cancer treatment, can penetrate the brain, but we know little about potential long term side effects of their action in the brain (Curtaz et al., 2022). Hence, studying the significance of local brain estrogen synthesis is essential not only for a fundamental understanding of physiology but also for gaining insights into potential side effects associated with this drug treatment.

Peripheral signals in energy balance regulation

Energy balance is regulated by a complex neuroendocrine network that connects peripheral organs with the central nervous system (CNS). A continuous communication between the gastrointestinal tract, brain, and adipose tissue affects appetite, food intake and energy expenditure. This regulation involves various circulating hormones that can be classified as anabolic or catabolic.

Adipose tissue is an endocrine organ that signals to the brain, relaying information about available energy stores. A major long-term feedback

factor released from the adipose tissue is leptin. Plasma leptin correlates to fat mass, and acts in the brain to promote catabolic effects by reducing appetite and increasing energy expenditure when fat stores are sufficient (Davis et al., 2011; Hayes et al., 2010; Rosenbaum et al., 1996). This ideally creates a homeostatic mechanism where rising fat mass leads to elevated leptin levels, subsequently curbing further weight gain. Generally, individuals suffering from obesity have elevated levels of leptin due to increased fat mass, but their brains become resistant to its anorexic signals (Considine et al., 1996; Matheny et al., 2011; Seeley et al., 1996). Hence, leptin therapies have not yielded success in promoting weight loss in cases of obesity. Leptin levels are greatly influenced by sex hormones, with opposite response to estradiol and testosterone. Females are more sensitive to the anorexic effects of leptin and central estradiol administration improves leptin sensitivity in ovariectomized females and males (Clegg et al., 2006).

The gastrointestinal tract releases peptides to communicate ongoing information about meals. When food reaches the duodenum, cholecystokinin (CCK) is immediately released to signal satiety and inhibit eating (Kissileff et al., 1981). Close by, in the jejunum, production of glucoregulatory glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) takes place during and after meals to signal satiety (Woods et al., 2004). Moreover, the pancreas produces the hormones insulin, glucagon, somatostatin and amylin, which are not reviewed in this thesis.

Altogether, these signaling mechanisms aid the CNS in generating appropriate responses for acute meal control, and long-term control of energy intake and expenditure to preserve homeostasis.

Ghrelin

Ghrelin, often called the hunger hormone, is the only gut peptide that promotes appetite by signaling energy deficiency and modulating food reward (Kojima et al., 1999). Intriguingly, ghrelin's influence extends beyond energy regulation, also encompassing mood and cognition (Chuang & Zigman, 2010). Ghrelin is primarily synthetized by the empty stomach in response to fasting, and reaches the brain where it acts on the growth hormone secretagogue receptor (ghrelin receptor, GHSR) (Cummings et al., 2001). The ghrelin receptor can be found in brain regions important for energy homeostasis, reward processing, and emotionality (Zigman et al., 2006). Exogenous ghrelin administration into discrete brain sites expressing GHSR, for instance the arcuate nucleus of the hypothalamus (Arc), lateral hypothalamus (LH) ventral tegmental area (VTA), nucleus tractus solitarus (NTS), hippocampus and amygdala, potently promotes appetitive behavior (Abizaid & Horvath, 2012; Alvarez-Crespo et al., 2012; Faulconbridge et al., 2003; Le May et al., 2019; López-Ferreras et al., 2017). Lack of ghrelin signaling disrupts the rewarding properties of natural rewards such as food and mating, but also artificial rewards such as nicotine, cocaine and alcohol (Jerlhag et al., 2010; Jerlhag et al., 2009; Jerlhag & Engel, 2011). Liver-enriched antimicrobial peptide-2 (LEAP2) was recently identified as an endogenous peptide that acts as a competitive antagonist and inverse agonist of GHSR (Ge et al., 2018; M'Kadmi et al., 2019). LEAP2 works by blocking the effects of ghrelin binding, as well as the constitutive activity of the receptor, essentially opposing its hunger-stimulating actions. Emerging reports have demonstrated that endogenous ghrelin access into the brain is limited (Perello et al., 2019). The Arc, and specifically orexigenic neurons synthesizing agouti-gene-related protein and neuropeptide Y (NPY/AgRP) (Chen et al., 2004), are crucial mediators for the effects of peripheral ghrelin. Moreover, higher levels of peripheral ghrelin are hypothesized to access the paraventricular nucleus (PVH) through CSF, and modulate the

hypothalamic-pituitary-adrenal (HPA) stress axis (Cabral et al., 2015; Cabral et al., 2016). Previous reports have indicated some inconsistency in the effects of ghrelin on anxiety. Rodent studies have shown that ghrelin can have both anxiety-inducing (anxiogenic) and anxiety-reducing (anxiolytic) effects, and these effects appear to depend on various factors, including prior exposure to stress (Carlini et al., 2004; Currie et al., 2012; Lutter et al., 2008; Patterson et al., 2013). Notably, our research group has demonstrated that ghrelin's ability to reduce anxiety-like behavior relies on the absence of food prior to anxiety testing (Alvarez-Crespo et al., 2012).

Similarly to the leptin resistance that develops in obesity, there are indications of diet-induced obesity leading to ghrelin resistance in the hypothalamus of rodents (Lockie et al., 2015). Individuals with obesity exhibit reduced circulating ghrelin at baseline, and do not undergo the typical post-meal drop in plasma levels (Cummings, 2006). Some human studies have reported that following fasting, women have higher ghrelin levels than men (Barkan et al., 2003; Espelund et al., 2005). Moreover, our group has shown that the ghrelin axis is sexually dimorphic in rats, such that females exhibit higher plasma ghrelin levels and lower hepatic expression of LEAP2 (Börchers et al., 2022).

Interleukin-6

Cytokines, including interleukin-6 (IL-6), are released from immune cells and are predominantly associated with inflammatory mechanisms. IL-6 plays a critical role in immune system modulation and the promotion of inflammatory reactions. However, it has also been implicated in obesity and the control of energy balance (Benrick et al., 2009; V. Wallenius et al., 2002). Adipose tissue serves as a significant origin of IL-6, and, like circulating leptin levels, the secretion of IL-6 is positively correlated with fat mass (Khaodhiar et al., 2004; Mohamed-Ali et al., 1997).

Chronic low-grade inflammation associated with excessive adipose tissue is a key driver of metabolic syndrome (Monteiro & Azevedo, 2010). Interestingly, central injection of IL-6 increases energy expenditure and decreases fat mass (Timper et al., 2017; K. Wallenius et al., 2002). It's possible that the primary source of centrally acting IL-6 comes from local production in the brain. This is suggested by the observation that IL-6 levels in the cerebrospinal fluid (CSF) are higher than the concentrations found peripherally. Indeed, brain regions crucial for energy regulation, like the LH, produce this cytokine (López-Ferreras et al., 2021; Miyahara et al., 2000). In contrast to the positive correlation of obesity and serum IL-6, the opposite stands true for IL-6 levels in the CSF of obese men (Stenlöf et al., 2003). It is worth noting that the limited research on central IL-6 and its role in obesity has almost exclusively concentrated on male subjects.

Central nervous system integration

Even though we know better, we often eat too much. Food intake is driven not only by the necessity to restore energy balance but also by the allure of palatable foods, which can continue to stimulate consumption even when a sense of fullness is present. Indeed, in the case of obesity, negative feedback from metabolic satiety signals like leptin proves ineffective in maintaining energy balance. Feeding behavior control is orchestrated by an extensive neural network that spans from basal forebrain to the caudal brainstem. The scientific literature often refers to two types of behavioral control, that are closely intertwined: homeostatic mechanisms that responds to variations in metabolic need, and hedonic mechanisms that rely on cognition and the pleasure derived from food.

The hypothalamus

The hypothalamus was early recognized to be a crucial center for energy homeostasis. Lesioning studies of the ventromedial hypothalamic area (VMH) demonstrated animals gaining weight due to hyperphagia and reduced energy expenditure, whereas lesions of the LH produced the opposite effects (Anand & Brobeck, 1951; Cox & Sims, 1988; Hetherington & Ranson, 1940). Receptors for the adiposity signal, leptin, were found to be most abundant in the Arc (Elmquist et al., 1999). Furthermore, two important neuronal populations, sensitive to leptin and other peripheral signals such as ghrelin, were identified in the Arc: anorexigenic POMC/CART neurons and orexigenic NPY/AgRP neurons (Mercer et al., 1996; Seeley et al., 1997). Supported by the initial lesion studies of the LH, which resulted in weight loss, it is evident that the LH primarily functions as an anabolic region that also drives reward (Margules & Olds, 1962). Within the LH the orexigenic peptides melanin-concentrating hormone (MCH) and orexins (ORX) are expressed (Qu et al., 1996; Toshinai et al., 2003). On the other hand, neurons in the PVH express primarily anorexigenic neuroactive substances, including corticotropin-releasing hormone (CRH) and oxytocin (OT) (Hill, 2012). The hypothalamic brain regions are strongly interconnected, and receive and send projections among one another but also to the forebrain and hindbrain.

The hindbrain

The brainstem receives sensory information from the periphery and coordinates autonomic functions such as heart rate, digestion, and metabolism. It is shown to serve a crucial role in the regulation of meal size and thermogenesis. Research conducted by Grill and colleagues using the chronic decerebrate (CD) rat model, where the caudal brainstem is surgically isolated from the forebrain, highlighted the

significant role of the brainstem in regulating feeding behavior (Grill, 1980; Kaplan et al., 1993). The CD rat does not initiate meals on its own and does not engage in compensatory food intake following fasting. However, the CD rats were able to adjust meal size according to energy density and reduce intake following leptin administration. Taken together, this suggests that the caudal brainstem is sufficient for the sensory feedback related to taste and ingestion, as well as some aspects of nutrient sensing. However, without communication with the forebrain there is a lack of adaptive responses to changing energy needs. The NTS is the brainstem region most thoroughly investigated in relation to eating behavior. It is the afferent center of the vagus nerve, and receives direct sensory inputs related to food intake, such as stomach distention (Huo et al., 2007). Furthermore, the NTS integrates various signals from the periphery, it reacts to changes in blood glucose levels and peripheral feeding peptides (Rinaman, 2010). In addition to being produced in the intestines, the anorexic hormone GLP-1 is synthesized within neurons in the NTS. This central production of GLP-1 is believed to be the primary source acting in the brain, as GLP-1 released by the intestines is likely metabolized before it can reach the brain (Holt et al., 2019).

PARABRACHIAL NUCLEUS

The parabrachial nucleus (PBN) is an important hub for integrating interoceptive and exteroceptive inputs. It responds to taste, thermal sensation, visceral malaise, arousal and energy status. Moreover, PBN receives meal-related satiety signals from the NTS and integrates them with various neurochemical signals, including leptin (Alhadeff et al., 2014). One important aspect of feeding control imposed by PBN is aversion-induced anorexia, such that neurons in this area terminate feeding in response to pain, heat and stress (Chen et al., 2018; Jen-Hui et al., 2023).

LOCUS COERULEUS

Adjacent with PBN is the major source of noradrenergic innervation in the brain, the locus coeruleus (LC). It is crucial for arousal, and thus implicated in pathologies related with hyperarousal such as anxiety disorders (Morris et al., 2020). Interestingly, anxiety disorders are more prevalent in women, and the LC is well-investigated in regards to its sexual dimorphism (Bangasser et al., 2016). Stress alters LC activity, an effect mediated by CRF, and activates downstream targets to cause vigilance (Koob, 1999). Studies have shown that the female LC displays increased sensitivity to the effects of CRF, and this heightened sensitivity is not associated with activational hormonal levels but rather with organizational differences (Bangasser et al., 2010; Bangasser et al., 2013; Curtis et al., 2006). Recently, the LC has started receiving attention in the field of feeding. It was reported that LC activity is necessary for fear-induced feeding suppression through projections to PBN (Yang et al., 2021). Moreover, Sciolino and colleagues demonstrated that satiety can modulate LC activity, and identified that activation of noradrenergic projections to the LH suppressed feeding and triggered anxiety-like behavior (Sciolino et al., 2022).

The reward system

The mesolimbic system, often referred to as the reward system, comprises neural networks that play a key role in regulating the physiological and cognitive aspects of reward processing. These neural substrates are activated both prior to and following the receipt of a reward, establishing connections between stimuli and pleasure, ultimately prompting behaviors that fuel the pursuit of the rewarding stimuli in question. Eating behavior, like drinking, sleeping and mating, represents natural reinforcements essential for the preservation of a species. Artificial rewards, like substances of abuse, have the potential to hijack the reward system that originally evolved to regulate motivation for the natural rewards necessary for survival (Koob, 1992).

The mesolimbic dopamine pathway is centered around the VTA (Wise, 2004). Dopaminergic neurons originating from the VTA project to brain regions associated with executive, emotional, and motivational functions (Wise & Bozarth, 1984). Projections from the VTA to the nucleus accumbens (NAc) are well recognized for goal-directed motivated behaviors (Bardo et al., 1996). Moreover, bidirectional communication takes place between the VTA and the amygdala, hippocampus, prefrontal cortex (PFC), and hypothalamus (Alonso-Alonso et al., 2015). Hence, the regulation of food intake and energy expenditure is under the combined control of energy status, emotional processes, memory, cognition, and reward responses.

Metabolic signals involved in the homeostatic regulation of energy balance discussed earlier, are also implicated in the rewarding aspect of eating. Leptin, insulin, ghrelin, and GLP-1 all possess receptors within the mesolimbic pathway and act there to modulate food seeking and motivation to work for food (Alhadeff et al., 2012; Dossat et al., 2013; Fulton et al., 2006; Palmiter, 2007; Skibicka et al., 2011). Remarkably, GLP-1R and GHSR have been found to exert influence beyond regulating food rewards and have gained attention in their ability to modulate the effects of addictive substances as well (Davis et al., 2007; Graham et al., 2013; Jerlhag et al., 2010; Klausen et al., 2022).

Previous reports, suggest that females may have a more hedonically-driven feeding behavior (Buczek et al., 2020). For instance female rats show higher activation of reward-related brain regions and a stronger shift for palatable food in a conditioned place preference (Sinclair et al., 2017). Ovarian estradiol modulates reward derived from food, and estradiol administration selectively targeting the VTA of female rats reduces operant behavior for sucrose in the progressive ratio (Richard et al., 2017).

The sexual dimorphism of adiposity

Appetite, once essential for preventing starvation, can become maladaptive in our modern, food-abundant society and result in overeating and, ultimately, obesity. The incidence of overweight and obesity has increased dramatically in recent years, and the World Health Organization (WHO) estimates that approximately 1.9 billion adults worldwide are overweight. This escalating concern is not to be taken lightly, given the strong correlations between obesity and cardiovascular diseases, diabetes, some cancers, and autoimmune and neurodegenerative disorders. Moreover, epidemiological studies indicate a bidirectional relationship between excessive body weight and psychiatric disorders, alluding to the presence of shared mechanisms underpinning these seemingly distinct conditions (Milaneschi et al., 2019). While there is a myriad of potential contributors to obesity, the heightened availability and consumption of food is frequently identified as a prime suspect for the current epidemic. In fact, studies show that consumption of a high-fat high-sugar diet can lead to metabolic consequences independent of obesity development, suggesting that unhealthy food *per se* can have adverse effects on physiology (la Fleur et al., 2011). Nonetheless, due to its palatability, the Western diet promotes overeating in susceptible individuals and results in a state of positive energy balance and adiposity, despite the many feedback mechanisms to maintain homeostasis discussed above.

Excess energy is primarily stored as triglycerides in white adipose tissue (WAT), but the location of this fat storage matters significantly for health outcomes. Visceral fat, which accumulates in the abdominal region, is strongly linked to metabolic disorders, while subcutaneous fat, found just beneath the skin, appears to offer some protection against these conditions (Frank et al., 2019). Interestingly, there is a clear sexual dimorphism in fat distribution (Palmer & Clegg, 2015). Men, despite generally having lower overall body fat, are prone to

storing fat in the visceral depot. When storage becomes excessive, is associated with an increased inflammatory state and endocrine release of pro-inflammatory metabolites, which can ultimately cause complications such as insulin resistance (Foster & Pagliassotti, 2012). On the other hand, premenopausal women tend to favor subcutaneous fat depots, which possess better adaptability for growth and, thus, a metabolically healthier phenotype. However, with the onset of menopause, there is a shift in fat storage that aligns postmenopausal women more closely with men in terms of metabolic risk. The timing of these changes implies involvement of ovarian hormones, and the fact that estrogen replacement therapy can mitigate these effects emphasizes the pivotal role of this hormone in particular (Gambacciani et al., 1997).

One could speculate that the evolutionary basis to the sexually dimorphic white adipose tissue deposition could be due to the differences in lipolytic activity between the depots and the presumed roles of our prehistorical ancestors. In men, the evolutionary forces driving the accumulation of visceral fat may be rooted in its rapid mobilization capability, useful for shorter-term energetic challenges such as perilous hunting scenarios. Conversely, women had to survive pregnancy and lactation in a very challenging environment. There is a close connection between reproduction and adiposity, women suffering from anorexia nervosa stop ovulating due to a decline in leptin signaling insufficient fat depots to support a pregnancy (Frisch, 1990). On the other hand, pregnancy leads to growing subcutaneous adipose tissue depots that can be utilized during the energy demanding period of lactation.

While WAT is an endocrine organ that functions as a storage reservoir for excess energy, brown adipose tissue (BAT) can expend energy via nonshivering thermogenesis during cold exposure (Cannon & Nedergaard, 2004; van Marken Lichtenbelt et al., 2009). Brown

adipocytes contain high amounts of mitochondrial uncoupling protein 1 (UCP-1), that following sympathetic stimulation allows for heat generation utilizing fatty acids and glucose (Chondronikola et al., 2016; Hanssen et al., 2015). This unique property is essential to maintain body temperature, and enable survival in cold environments. Notably, cold-exposure and the concurrent BAT activation has been proven advantageous for glucose homeostasis and insulin sensitivity in both healthy individuals and individuals with type 2 diabetes (Chondronikola et al., 2014). Therefore, it is rather unsurprising that the activity and prevalence of BAT is found to be lower in older and obese populations (Pfannenberg et al., 2010). BAT research in humans has experienced a revival in recent years, reigniting interest for using this tissue as a potential therapeutic target for boosting energy expenditure and promoting weight loss. Intriguingly, women appear to have higher BAT mass and thermogenic activity (Pfannenberg et al., 2010). Further human studies are needed to elucidate the molecular basis of these differences, but rodent studies indicate that female BAT may contain higher mitochondrial density, UCP-1 expression and is more sensitive to β-adrenergic stimulation (Rodriguez-Cuenca et al., 2002)

The sex bias in biomedical research

Out of the ten prescription drugs pulled from the U.S. market between 1997 and 2001, eight were withdrawn due to health risks affecting women (U.S. Government Accountability Office, 2001). It is astonishing that, despite the rigorous and expensive drug development process, we fail to detect severe side effects in half of the population until the drug is already on the market. Throughout history, women have been neglected in biological research (Liu & Mager, 2016). It wasn't until the 1990s that the inclusion of female participants in federally supported phase III clinical trials became obligatory, as mandated by the US National Institute of Health Revitalization Act of

1993. Evidence shows that excluding women from trials has led to an unrepresentative assessment of drug efficacy and side effects (Correa-De-Araujo, 2006).

The underrepresentation of females in basic scientific studies and animal disease models is remarkable and remains an ongoing issue. A bibliometric analysis from 2011 reported that a selective focus on male rodents was observed in a striking 80% of published neuroscience studies (Beery & Zucker, 2011). Subsequently, the US National Institutes of Health introduced a policy requiring funding recipients to consider sex as a biological variable (Clayton & Collins, 2014). Nevertheless, despite improvements in the inclusion of females, significant gaps persist in the reporting and analysis of data by sex (Woitowich et al., 2020).

Considering that pre-clinical research marks the initial stage in the development of a new treatment, this bias creates a scenario where drugs are developed based on male biology primarily. Without considering the role of sex, there is a risk of assuming a general effect when it only applies to one sex. Moreover, lack of sex analysis can lead to mistakenly disregarding treatments when there are offsetting effects in the two sexes. An example that illustrates this issue is the pain medication MorphiDex that failed late-stage clinical trials (Galer et al., 2005; Institute of Medicine Forum on & Nervous System, 2011). Although women were enrolled, the data was not analyzed by sex. None of animal studies conducted prior to the clinical trials had involved females (Mogil, 2020), and it was not until after the unsuccessful clinical trials that it became evident that the effect of the active components could not be demonstrated in female rodents (Grisel et al., 2005). Therefore, it is quite likely that an effect in men may have been cancelled out when combined with the data of women. This is likely one of many cases where a drug with the potential to benefit one sex was disregarded in clinical trials.

AIM

The overarching aim of this thesis was to identify sex differences in metabolic and behavioral control within rodent models of obesity and anxiety.

The specific aims were:

Paper I
To determine whether characteristics of diet-induced obesity differ between both sexes of rats and mice, and to investigate the potential energy balance disturbances underlying the observed divergence.

Paper II
To explore if diet-induced obesity induces changes in the expression of interleukin-6 within specific brain regions, in both male and female rodents. Additionally, we sought to determine whether interleukin-6 within the parabrachial nucleus is necessary for maintaining normal energy balance.

Paper III
To examine if ghrelin signaling in the locus coeruleus mediates ingestive, reward and anxiety-like behavior, and whether these effects vary between male and female rats.

Paper IV
To investigate the influence of local brain estrogen synthesis on eating behavior in both male and female rats, with a specific focus on the role of aromatase in the amygdala.

METHODOLOGICAL CONSIDERATIONS

This section provides a general overview and rationale of the methods used in this thesis. For specific procedural details, please refer to the "Materials and Methods" sections in each respective paper.

Experimental model

The work of this thesis is based on studies carried out using male and female Sprague Dawley rats, as well as C57BL/6N mice (**Paper I** and **II**). The rodent neural circuits and neurotransmitter systems involved in eating behavior and anxiety are similar to those in humans. For example, monogenetic mutations that induce obesity in humans do so in rodents as well (Krude et al., 1998; Montague et al., 1997; Yaswen et al., 1999). As evidenced by the findings in **Paper I**, variations do exist in diet-induced obesity between mice and rats, and their potential for translational relevance may differ across various disease aspects. There are already established differences between mice and rats in terms of ingestive behavior, for instance mice tend to eat smaller, more frequent, meals than rats. Hence, to enhance confidence in the translatability of novel findings and drug effects to humans, it is advisable to confirm them in both species. Furthermore, certain practical considerations in the experimental setup may raise questions about translational relevance. For instance, the need to individually animals after some surgeries (**Paper II**, **III** and **IV**), which affects their emotional state, is notable given the social nature of rats. Additionally, conducting all experiments during the light cycle, despite rodents being nocturnal animals, might not mimic natural conditions very well. Although rodents are more active during the dark cycle and typically consume the majority of their daily food intake then, it is important to note that rats do not have consolidated sleep patterns throughout the

light cycle and still consume approximately 25% of their daily intake during this period (Sidlo et al., 1995). Moreover, we tried to avoid individual housing in experiments not requiring it, and made sure that our study design aligned with the 3R principle in mind (replace, reduce, refine), to minimize animal suffering.

All studies conformed to and received approval by the local Ethics Committee for animal care at the Institute of Experimental Biomedicine at the University of Gothenburg in Sweden. Additionally, a big part of the experiments in **Paper III** and **IV** were performed at Pennsylvania State University and received approval by the local Institutional Animal Care and Use Committee. Ethical permit numbers are specified in each paper.

Obesogenic diets

While animal models with single-gene mutations are useful for studying the specific contribution of certain genes to energy metabolism, they fall short in mimicking the complexity of the current obesity epidemic. Instead, the diet-induced obesity paradigm provides a model that better replicates interactions between polygenetic predispositions and weight gain in an obesogenic environment. In laboratory settings, a variety of palatable diets are utilized to induce obesity in rodents, all sharing common characteristics of being high in energy content and abundant in sugars and fats. "The 'cafeteria diet,' first introduced by Sclafani and Springer in 1976, comprises an unstandardized combination of unhealthy food items people commonly consume, such as cookies, candy, and chips (Sclafani & Springer, 1976). Nowadays, commercially produced energy-dense pellets are used more regularly. The most common pellets contain either 45% or 60% fat by energy, and are therefore classified as 'high-fat diets'. Nevertheless, it is worth noting that while their carbohydrate content (20%) is lower than their fat content, these diets do contain

simple sugars (sucrose) as opposed to regular chow pellets, making them more than just high-fat diets. Another vital element in inducing overeating and emulating the obesogenic conditions in humans is the emphasis on variety, often referred to as the 'buffet effect'. With this concept in mind, Susanne la Fleur's laboratory introduced a free-choice high-fat high-sugar diet, offering lard and a 30% sucrose solution with standard chow. This approach demonstrated a more sustained hyperphagia than the inclusion of just one unhealthy component (la Fleur et al., 2014).

In **Paper I,** a combination of commercial 60% HFD-pellets and chow were used to induce obesity in mice and rats. In **Paper II**, mice were fed a 60% HFD while rats were offered a free-choice high-fat high-sugar diet. In **Paper III**, the animals were fed a chow-based diet during the entire study, except for the initial experiment in which they were provided with a free-choice paradigm to test food preference following a ghrelin injection. In **Paper IV** we employed Susanne la Fleur's free-choice paradigm in the initial experiments, and 60% HFD-pellets in the gonadectomized cohort.

Surgeries

In **Paper IV**, rats were gonadectomized to investigate the effects of reduced estrogen synthesis in amygdala without the compensation of circulating sex hormones sourced from the gonads. Bilateral ovariectomy is the best characterized model for mimicking the human ovarian hormone loss after menopause. In **Paper II**, telemetric E-mitter transponders were implanted in the abdominal cavity of rats. In **Paper II**, **III** and **IV** stereotaxic surgeries were performed to implant cannulas for drug delivery. Lastly, stereotaxic surgeries were utilized for delivery of viral vectors in **Paper II** and **IV**.

All surgeries were performed under ketamine/xylazine anesthesia administered intraperitonially. It is common practice in our lab to administer a dosage that is up to 15% lower, per kilogram, to female rats. However, there is no published data that confirms our observed sex difference in anesthesia sensitivity. A rat brain atlas (Paxinos & Watson) was used for decision of appropriate coordinates to target the lateral parabrachial nucleus and paraventricular nucleus (**Paper II**), the locus coeruleus (**Paper III**) and the central amygdala (**Paper IV**). Notably, all available rat atlases are based on mapping of the male brain. Evidence suggests variations in the size of some brain regions, such as the medial preoptic area and dorsal locus coeruleus (Babstock et al., 1997; Hofman & Swaab, 1989), between male and female rats. It is tempting to question what additional morphological differences could be identified, and whether the development of a female rat brain atlas could enhance the precision of surgical procedures and collection of micropunches from female brains.

Viral vectors

Viral vectors, such as adeno-associated viruses (AAVs), are essential tools for gene delivery in scientific research. In **Paper II** and **IV**, AAVs were utilized to knock down the IL-6 gene and CYP19a1 gene, respectively. In both cases this was done with AAVs carrying small interfering RNA (siRNA) designed to target the messenger RNAs (mRNA) of interest, induce their degradation and reduce the expression of the corresponding gene. This tool is of great value, as it enables precise gene targeting in specific regions. It is particularly useful for investigating the physiological significance of a substrate, such as determining whether the expression of an endogenous peptide, e.g., aromatase, is necessary for normal feeding behavior. Throughout history, transgenic knockout models have played a pivotal role in unraveling gene function, and knockout mice remain integral to metabolic research (Yazdi et al., 2015). While this approach has been

invaluable for elucidating the fundamental roles of genes, the newer knockdown techniques that we choose to use offer distinct advantages. In transgenic animals with global null mutations of specific genes, organisms' risk to develop compensatory mechanisms, complicating the physiological interpretation of the gene. In contrast, AAV-mediated knockdowns in adult animals enable a more direct assessment of the gene's influence on physiology. We also utilized AAVs for retrograde neural tract tracing. In **Paper II** the viral vector AAV2(Retro)-eSyn-EGFP was administered into the PVH, to be taken up by neurons and transported along their axons to the cell bodies. This technique, together with RNAscope, was used to confirm the hypothesis that IL-6 expressing neurons from the lPBN innervate the PVH.

Drugs

In **Paper II**, IL-6 and leptin were administered together into the lPBN at subthreshold doses. This approach serves as a method for assessing synergy, and the finding that combining two substances at doses that are individually ineffective can produce an effect indicates potentially shared downstream signaling pathways. Gaining insights into the common downstream signaling pathways can provide a more profound comprehension of the mechanisms driving the observed effect, offering valuable insights into disease processes and intervention development. Additionally, employing lower doses of each substance to attain the desired effect holds notable pharmacological significance and paves the way for investigating novel drug combinations capable of delivering improved therapeutic advantages.

To investigate the effects of ghrelin signaling in the LC (**Paper III**) we administered rat ghrelin and LEAP2 centrally. The exogenous ghrelin used in this study was the acylated form, often regarded the

physiologically 'active' form and extensively studied for its impact on energy balance. The antagonist employed, LEAP2, represents a recently identified endogenous antagonist of the GHSR (M'Kadmi et al., 2019). Given its novelty, there is currently a scarcity of published studies concerning the effects of exogenously administered LEAP2 into discrete brain regions. Notably, as LEAP2 functions as an inverse antagonist, diverging from many previously used synthetic GHSR antagonists, it might unveil fresh insights in the field of ghrelin research as its utilization becomes more widespread.

To ensure optimal compatibility with the respective injection sites, peptides were dissolved in artificial cerebrospinal fluid (aCSF) for central administration, and saline solution for peripheral administration.

Temperature and locomotor activity measurements

Locomotor activity and adaptive thermogenesis are two major components of energy expenditure. Several experimental assays monitor an animal's movements as secondary measure, such as the open field test or operant conditioning (as described in **Paper I** and **IV**). While these tests can provide insights into the impact of a manipulation on locomotor activity, caution is necessary when interpreting the results due to the limited duration of these tests and confounding factors related to the novelty of the testing environment. To obtain the most accurate measurements of how chronic or acute treatments affect energy expenditure, we employ E-mitter telemetry (**Paper II**). This approach relies on implants that continuously monitor locomotor activity and temperature in rats within their home cage. Additionally, we utilize infrared imaging technology (FLIR) to assess temperature in a non-invasive way (**Paper I**, **II** and **IV**). The nature of this technique enables the acquisition of spatial temperature data, providing an assessment that goes beyond core temperature alone.

However, it's important to note the limitations with this technique, such as potential insulating effects of fur and fat, making it more of a relative measure rather than a precise detection of absolute temperatures.

Operant conditioning

Motivation, 'wanting', is an important component of reward, together with hedonic 'liking'. Food motivation is closely linked to the pleasure and sensory experience associated with eating, and the specific incentive value of the food item plays an important role. Nonetheless, in order for food reward to influence eating behavior, pleasure needs to be transformed into motivation Adding to the complexity, while 'liking' and 'wanting' are frequently intertwined, lessons drawn from substance abuse disorders have shown that motivated reward-seeking behavior can exist in the absence of pleasure. Physiological states like hunger strongly influence the motivation for food, with an increase in motivation when hungry and a decrease as satiety is achieved. The brain's mesolimbic pathway, particularly the release of dopamine, have a significant impact on food motivation (Alonso-Alonso et al., 2015). Food cravings are a common manifestation of food motivation, driven by sensory cues and emotional states. Taken together, it is clear that food motivation is crucial to understand in the study of disordered eating.

Operant conditioning, as studied through the Skinner box paradigm, is a key concept in behavioral neuroscience. In our assay, rats are trained to press a lever to obtain a sucrose (**Paper III)** or fat pellet (**Paper IV**), with the aim to measure the motivation to work for a food reward. The progressive ratio (PR) schedule is designed to make the task progressively more challenging as the rat works for each successive reward. To summarize, this means that the number of lever presses required to obtain each additional sucrose or fat pellet increases. At the

end of the test, the number of lever presses represent how motivated the animal was to obtain the reward.

Anxiety-like behavior

The brain regions and circuits that control food intake, reward processing, and motivation are intricately interconnected with those responsible for emotional regulation. Therefore, it is highly relevant to include anxiety testing in the research of eating behavior. The assessment of anxiety-like behavior in rodent models encompasses a range of assays designed to uncover responses that can resemble the intricate human condition of anxiety.

The open field (**Paper II** and **III**) test assesses the animal's exploration of an unfamiliar environment and measures the balance between the desire to explore a novel space and the anxiety of an open space. In the open field (OF) test, locomotion can be an important confounding factor, particularly when studying both male and female rats. This is because female rats tend to exhibit higher levels of spontaneous locomotion compared to males. When studying anxiety-like behavior in the OF, and other locomotor-based tests such as the elevated plus maze, the differences in locomotion between sexes or in response to a treatment can complicate the interpretation of results. To mitigate the risk of misinterpretation, we employ an analysis of covariance (ANCOVA) to assess whether the observed effects on anxiety-like behavior remain significant after accounting for differences in locomotor activity. In addition, we started incorporating alternative tests to more accurately evaluate anxiety-related behaviors in our animal models. In **Paper III**, anxiety-like behavior is measured with the acoustic startle response (ASR), a test where the rat is placed in a confined chamber and exposed to sudden noise bursts. The startle response involves the rapid and involuntary motor response, typically a whole-body flinch or jump. A noise burst elicits a startle response

both in humans and laboratory animals, making this a highly translatable assay (Davis & Whalen, 2001). This response can be quantified by measuring the magnitude of the motor reaction – a greater startle response is an indicator of higher vigilance, which is a fundamental characteristic of anxiety disorders.

Biochemical procedures

For the study of gene expression, frozen brains were sectioned using a cryostat and micropunches of the PBN (**Paper II**), LC (**Paper III**) and amygdala (**Paper IV**) were obtained. In **Paper I** and **II**, BAT samples were collected and flash frozen during the terminal experiment. The fat samples and brain micropunches were processed using a Qiagen lipid kit for mRNA extraction, followed by a reverse transcription step to synthesize cDNA. Gene expression in the samples was assessed by Quantitative Polymerase Chain Reaction **(**qPCR) using TaqMan® gene expression assays for the target genes. During the qPCR process the template is amplified exponentially over several cycles, and fluorescent signals from the Taqman probe is measured. A highly expressed gene results in higher fluorescent signal. The number of cycles it takes for the fluorescent signal to reach a threshold, will define the cycle threshold (CT) value for a sample. Analysis of the resulting data was then performed using the 2-ΔΔCT method, with beta actin (a highly expressed stable gene) as endogenous control gene (Livak & Schmittgen, 2001). This method was used to measure the expression of genes relevant for BAT thermogenesis in **Paper I**, to test if they were altered in response to diet-induced obesity. In **Paper II** and **IV**, qPCR was utilized to confirm the reduced expression of the genes targeted for viral knockdown. Lastly, in **Paper III**, we analyzed how GHSR expression in the LC differed between males and females.

While qPCR excels at quantitative mRNA analysis, fluorescent in situ hybridization (FISH) techniques like RNAscope adds important nuances to the gene expression data. RNAscope is a technique

employed to visualize mRNA within brain sections affixed to glass slides. This method involves the application of target probes and signal amplifiers, which ultimately generates fluorescent markers for the specific target mRNA that can be imaged. This visual confirmation is vital for in pinpointing in what cell type the target gene is expressed. More importantly, it provides the ability to label for several targets, enabling the detection of co-expression. In **Paper II**, RNAscope was employed to detect if IL6 is co-expressed with labels for neurons, microglia and astrocytes in lPBN, to investigate what cell type in this brain region is responsible for the production of this cytokine. It was also utilized in the retrograde tracing experiment discussed in the section about viral vectors. In **Paper III,** GHSR was visualized within the locus coeruleus and confirmed to be co-expressed with TH. Lastly, in **Paper IV**, CYP19A1 was visualized throughout neurons in the amygdala, providing support for the capacity to locally produce estrogen.

Finally, in **Paper II**, Western blot was utilized to measure UCP-1 protein levels in BAT and immunohistochemistry (IHC) was used to detect TH in BAT. The Western blot process begins by separating proteins in a sample based on size using gel electrophoresis, to then transfer them to a membrane that is treated with a primary antibody targeting the protein of interest, and a secondary antibody that is conjugated with a dye allowing quantification. Immunohistochemistry is primarily used for spatial distribution and localization of proteins within sections of tissues. Similarly, to the Western blot technique, it relies on primary and secondary antibodies, designed to target the protein of interest and create a signal that can be imaged. In addition to visualizing the protein in the tissue, the signals can be quantified using imaging processing tools.

To summarize, qPCR and FISH experiments primarily provide information about gene expression at the mRNA level. IHC and

Western blot serve as crucial tools for confirming the presence and quantifying the protein products of the studied genes. Integrating these techniques is highly beneficial as it provides a cross-validation mechanism, confirming that alterations at the mRNA level are genuinely reflected in protein expression changes, which is not always the case. However, limited antibody availability and specificity is an issue for some proteins like aromatase, causing us to rely primarily on gene expression quantification.

Statistical analysis

In the studies presented here, data from males and females are consistently examined separately and are never combined. Throughout majority of the studies, data of both males and females are analyzed using a two-factor ANOVA, with sex as one of the variables. A two-factor ANOVA assesses the influence of two independent variables, here 'sex' and 'treatment/diet' on a dependent variable. This allowed us to not only assess main effects of the independent variables but to also explore potential interactions.

RESULTS

Paper I

In this work we sought to explore the effect of sex and species in the development of diet-induced obesity. Both male and female rats and mice were offered a free choice of a 60% HFD and standard chow. Regardless of the species, males demonstrated greater caloric intake and weight gain when exposed to the HFD. Females displayed a lower level of overeating than males, but exhibited a higher preference for the unhealthy diet. While rats preferred the HFD over chow, the preference displayed by mice was dramatic, with nearly all of their total intake coming from the palatable diet. Mice showed an inferior ability to compensate for the higher energy density of the HFD, and they consumed a pellet mass similar to controls. Despite male mice gaining more weight, it was the females who experienced a remarkable accumulation of metabolically unhealthy, visceral fat. Furthermore, the severe level of overeating in mice coincided with compromised energy expenditure, namely reduced levels of locomotor activity and BAT thermogenesis. Collectively, mice of both sexes exhibited a more pronounced obesity phenotype, further evidenced by their disrupted glucose homeostasis. In contrast, obese rats maintained normal glucose tolerance when subjected to an oral glucose challenge. In both rats and mice, females exhibited a higher BAT thermogenesis at baseline compared to males of the same species. However, only female rats displayed a higher locomotor activity than males, potentially contributing to their blunted weight gain. Intriguingly, we found that only male rats increased BAT temperature and expression of genes relevant for thermogenesis in response to the obesogenic diet.

Paper II

Based on prior discoveries of reduced IL-6 levels in the CSF of obese human patients (Stenlöf et al., 2003), this study opted to examine IL-6 gene expression in brain regions key for energy balance in obese male and female rodents. Among the various brain regions analyzed, the PBN emerged as the singular area demonstrating a significant decline in IL-6 gene expression in animals subjected to diet-induced obesity. Intriguingly, this was a sex-specific effect only observed in male mice and rats. Due to this lack of response of IL-6 in the PBN of females to any metabolic challenges applied, majority of remaining experiments aiming to reveal the mechanism of metabolic effects of IL-6 were performed in male rats only.

Exogenous administration of IL-6 targeting lateral lPBN reduced food intake and produced hyperthermia by significantly increasing BAT temperature. To elucidate the physiological function of IL-6 in the lPBN of male rats, we applied an AAV-mediated knockdown specifically within this brain region, effectively reducing IL-6 expression. Reduction of endogenous IL-6 in the lPBN resulted in body weight gain, that was driven by reduced BAT temperature. Two molecular mechanisms were identified as potential explanations for the attenuated thermogenesis: impaired sympathetic input to BAT and impaired hypothalamus-pituitary-thyroid (HPT) axis. This was characterized by reduced levels of tyrosine hydroxylase (TH) in the BAT of knockdown animals, and a decline in plasma thyroid hormones. Given the regulatory role of the PVH in the HPT axis, we hypothesized that the dysregulation caused by loss of PBN IL-6 may be linked to disrupted signaling to the PVH. To test this hypothesis, we targeted the PVH with a retrograde AAV. The neural tract tracing revealed the presence of retrogradely labeled cell bodies specifically in the lPBN, and more importantly co-expression of IL-6 mRNA on

nearly all retrogradely labeled cells - indicating that lPBN neurons are a source of IL-6 in the PVH.

Furthermore, the knockdown animals showed indications of increased anxiety-like behavior when tested in the OF. To investigate if stress, similarly to obesity, could be a source of dysregulated IL-6 in the PBN, we exposed male rats to acute restraint stress. However, this did not reveal any alterations in IL-6 gene expression. On the other hand, cold exposure potently increased IL-6 expression in the PBN. Crucially, this elevation was evident not only in males consuming standard chow, but also in obese males - providing evidence that cold exposure can, to some extent, reinstate IL-6 levels in the lPBN that are diminished in obesity.

Paper III

Here, we identified the LC as a novel target for the behavioral effects of ghrelin. First, we established that ghrelin receptors are indeed present in the LC of both male and female rats. Notably, gene expression analysis unveiled remarkable disparities in receptor expression between the sexes, with males exhibiting significantly higher levels than females. This finding raised intriguing questions about potential sex-specific responses to ghrelin signaling in the LC. Exogenous administration of ghrelin targeting the LC stimulated chow intake in both males and females. While the acute response was similar, a sex-specific delayed effect was notable, with only females consuming more chow after 24 hours. Furthermore, intra-LC ghrelin elevated motivated behavior for a sucrose reward in a progressive ratio operant test, with no sex divergence. To explore the impact of blocking ghrelin signaling at the level of LC in fasted animals, we utilized the endogenous GHSR antagonist LEAP2. Intra-LC administration of LEAP2 reduced food intake in both sexes when offered within one hour of injection. However, it decreased chow intake in females only when administered two hours post-injection. Moreover, in females, intra-LC microinjection of LEAP2 decreased the motivation for sucrose. Although it didn't significantly impact food-seeking behavior, locomotor activity was reduced during specific time intervals. In contrast, males displayed no significant changes in food motivated behavior following LC GHSR blockade. Lastly, we wanted to explore if ghrelin's effects in the LC extends to anxiety-like behavior. Intra-LC ghrelin exhibited anxiolytic effects in females in the ASR test, with significantly lower startle amplitudes at the highest sound intensity. Remarkably, these effects did not manifest in males, as ghrelin treatment failed to alter their startle responses in the ASR test. Conversely, acute pharmacological blockade of LC-GHSR with LEAP2, increased startle response in both male and female rats.

Paper IV

In this study, our primary objective was to investigate the necessity of estrogen synthesis in the brain for the regulation of energy balance. To achieve this, we conducted experiments with gonadally intact, and gonadectomized, males and females. Osmotic pumps were surgically implanted targeting the lateral ventricle, and we proceeded to continuously infuse the aromatase inhibitor Letrozole over a 26-day period. Simultaneously, we closely monitored changes in body weight and the consumption of a free choice high-fat high-sugar diet, including chow, lard, and sucrose. The results yielded intriguing sex differences. Males subjected to Letrozole treatment did not exhibit any significant effects on body weight, and consumed less energy than control males. In contrast, females both displayed weight gain in response to the treatment regardless of hormonal state. Notably, ovariectomized females experienced a more pronounced increase in body weight, coupled with a heightened energy intake. In gonadally intact females, the weight gain observed was not attributed to increased food consumption, hinting at changes in energy expenditure as the underlying cause. A recent imaging study revealed a negative correlation between amygdala aromatase availability and human body mass index, which prompted us to explore if aromatase in the amygdala of rats is necessary for energy balance. We utilized a virogenetically-mediated knockdown to investigate the involvement of aromatase in the rat amygdala on energy balance and food motivated behavior. Amygdala-specific aromatase knockdown did not yield significant alterations in body weight in neither intact nor gonadectomized animals when fed a chow-only diet. It was in an obesogenic environment that females with amygdala aromatase knockdown displayed heightened body weight gain and calorie intake. In ovariectomized females, this was accompanied by an enhanced food motivation, indicating that the overeating was hedonically driven. Surprisingly, amygdala aromatase knockdown led to a reduction in

BAT thermogenesis in intact females, while the opposite was true in ovariectomized females. Ultimately, intact and orchiectomized males displayed no notable effects from the knockdown in any of the measured parameters.

DISCUSSION

The findings presented in **Paper I** offer valuable insights into the complex interplay between sex, species and the development of diet-induced obesity. It is evident that both sex and species play critical roles in shaping the obesity phenotype and its associated metabolic outcomes. One of the key takeaways is the divergence between species of animal models. The striking difference in dietary preference between rats and mice, with mice almost exclusively consuming the palatable diet, underscores the complexity of species-specific responses to obesogenic diets. This discrepancy calls for caution regarding the generalizability of findings from one species to another and emphasizes the importance of selecting appropriate animal models based on the specific research objectives. It also raises questions about what underlying mechanisms may be causing this divergent response. Considering that HFD mice consumed a pellet mass equivalent to those on a standard diet, it is possible that mice to a greater extent rely more on signals related to stomach distention to terminate consumption of palatable food. The female preference for HFD is intriguing in the light of a recent brain imaging study of obese men and women. They found that women with obesity had alterations in brain signatures associated with emotion-related eating and reward driven eating (Bhatt et al., 2023).

The observed compromised energy expenditure in mice, characterized by reduced locomotor activity and diminished BAT thermogenesis, is an intriguing finding of this study. This compromised energy expenditure undoubtedly contributes to the more pronounced obesity phenotype observed in mice. However, our data does not provide conclusive evidence regarding the causal relationship between weight gain and impaired energy expenditure. It remains uncertain whether this dysregulation precedes the onset of morbid obesity, or if it is the result of the extensive fat accumulation causing compromised mobility

and increased insulating adipose tissue. However, thermal imaging has some limitations and the molecular profile of BAT in obese mice is not fully conclusive. Quantification of proteins involved in thermogenesis, as well as measurement of circulating thyroid hormones, would aid us in confirming the biological basis of the suspected phenotype. Lower prevalence of BAT has been documented in obese individuals, but it appears that it does not necessarily mean its activity is diminished (Kulterer et al., 2022). However, also in humans the causal relationship in this context remains elusive. In the light of results from **Paper II**, it is plausible that the mice in **Paper I** developed a more advanced obesity, and suffered diminished BAT activity, for instance due to the reduction of IL-6 in PBN. Diet-induced thermogenesis has been reported in mice, but with shorter HFD exposure (Essen et al., 2017). In male rats, the extent of overeating reached such staggering proportions that energy expenditure appeared inadequate, even when augmented. However, it is noteworthy that while this sex-specific diet-induced thermogenesis did not rescue male rats from weight gain, it appeared to play a role in maintaining relatively healthy glucose tolerance despite an accumulation of unhealthy fat mass (Chondronikola et al., 2014). It remains a question whether the BAT activity in rats would subside similarly to what is seen in mice, after longer exposure to the obesogenic diet.

Surprisingly, there is a notable discrepancy in weight gained by males and females in **Paper I** and **Paper II**, such that males in Paper II gained less than their chow-fed counterparts compared to females. The rationale behind this discrepancy remains speculative, given the differing study designs. In **Paper II**, the animals were individually housed and introduced to the obesogenic diet at an earlier age (5 weeks rather than 10 weeks), the diet regimen extended for a longer duration (14 weeks as opposed to 10 weeks), the dietary composition differed (free access to chow, lard, and sucrose water instead of chow and HFD pellets). Therefore, further investigations are warranted to elucidate

which specific environmental factor may have contributed to the less pronounced overeating observed in the males of Paper II. Nevertheless, it is imperative to consider this aspect, as it underscores the notion that varying environmental conditions may yield different patterns of sex differences.

In **Paper II**, we conducted an analysis of various brain regions critical for the regulation of energy in mice and rats exposed to obesity, with a particular focus on changes in IL-6 expression. Here, the PBN stood out as the singular area where IL-6 gene expression was significantly altered in response to diet-induced obesity. However, this reduction was observed exclusively in male mice and rats, constituting a clear sexual dimorphism. The study was motivated by prior observations of reduced IL-6 levels in the cerebrospinal fluid (CSF) of obese human patients (Stenlöf et al., 2003). However, it is crucial to emphasize that the human study exclusively featured obese men and the question of whether obese women exhibit reduced IL-6 levels in CSF remains unanswered. Nonetheless, the findings of Paper II suggest that obesity might selectively impair brain IL-6 in males. Although the study successfully demonstrated the necessity of IL-6 within the PBN for normal thermoregulation, it became evident that this was not the case for females as PBN IL-6 levels remained unaltered following metabolic challenges. Should IL-6 or related downstream signaling be pursued as a target for obesity treatment, clinical trials must recognize the potential sex-related disparities. Failure to do so may lead to diluted effects in a clinical trial including both men and women, without a thorough analysis of the sex-specific responses. A recent paper from our laboratory found that IL-6 in the LH mediates food intake in males, but only food motivation in females (López-Ferreras et al., 2021). We did detect IL-6 expression in female PBN, but did not pinpoint its role. It calls for investigation if the same neural projections to the PVH are present in females and whether they serve alternative functions. It is conceivable that IL-6 in the female PBN exclusively

plays a role in regulating food-motivated behavior as is the case in the LH. Nevertheless, our study did not examine this aspect, nor did we investigate the impact of knockdown in females. It is also plausible that PBN IL-6 in females might be implicated in stress regulation, despite the minimal relevance for the measured parameters in males. Prior research has revealed a sex-specific influence of IL-6 on stress responses. In this context, female mice show an elevated corticosterone response to restraint stress, and this distinction diminishes in the absence of IL-6. While IL-6 knockout female mice have a reduced HPA activation in response to stress, the response in IL-6 KO males remain similar to wild type (Bethin et al., 2000).

Prevalence in stress associated pathologies, such as anxiety disorders, are overrepresented in women (Altemus et al., 2014). The findings presented in **Paper III** further extend our understanding of ghrelin's impact on the interplay between feeding behavior and emotionality. While a prior study suggested the locus coeruleus (LC) as a potential location for ghrelin binding, the impact of ghrelin signaling at this site has remained unexplored until now (Cabral et al., 2013). The divergent outcomes observed between sexes invite two essential questions: is the sensitivity to dysregulation, for instance by stress, in this system sexually divergent, and could this system be more effectively targeted for treating anxiety in one sex over the other? Exploring the cellular profile of GHSR expressing cells in the LC merits attention. Furthermore, discerning the involvement of norepinephrine as a downstream mediator of ghrelin will provide valuable insights into the regulatory mechanisms at play. GHSR KO mice have repeatedly been shown to exhibit increased anxiety-like behavior, and one previous study suggested an obligatory role for GHSR expressed specifically in catecholaminergic neurons in mediating stress-induced overeating, antidepressant-like behavior and food reward (Chuang et al., 2011). The paper did not elucidate what catecholaminergic sites that could be responsible for these effects, but LC is a plausible candidate. There are

other sites than LC with GHSR-TH co-expression, such as the NTS and VTA, and these have been shown to mediate ghrelin's orexigenic effects, however here we show a GHSR-TH population that also alters anxiety-like behavior. Furthermore, Chuang and colleagues' research, along with the majority of other studies examining the impact of ghrelin on anxiety-like behavior, predominantly involve male animals. Considering the sexually divergent response of LC to stress, it surely calls for investigation to see how ghrelin can mitigate effects following stress-exposure also in females.

In **Paper IV**, we demonstrate that inhibiting estrogen synthesis specifically in the brain is sufficient to induce sex-specific changes in feeding behavior. We further establish that estrogen production within the amygdala is crucial for maintaining normal thermoregulation and feeding behavior in an obesogenic environment, particularly in ovariectomized females. Notably, a recent human study has shown a negative correlation between the amount of amygdala aromatase and body weight, bolstering the translational relevance of our findings and suggesting a potential causal link between the two factors (Biegon et al., 2020). While reduced estrogen levels are the hypothesized cause for the effects following loss of aromatase, we must also consider the potential change in substrate, specifically testosterone, as a contributing element. Moreover, aromatase activity in the amygdala does not necessarily mean a local release of estrogen. Hence, we aim to investigate where these cells project and what their downstream targets may be. Intriguingly, the presence of aromatase in this brain region in males raises questions about its function at this site. Unlike our findings, the human imaging study did not identify sex differences in the correlation between BMI and amygdala aromatase activity.

The implications of the findings in **Paper IV** emphasize the importance of considering not only peripheral estrogen but also the brain-derived sources of estradiol in future investigations related to the

effects of sex hormones on energy balance. Regarding IL-6, we've gained insights into how peripheral and central sources can yield distinct effects. Similarly, when examining aromatase, it is essential to investigate how the brain-specific pool is modified in pathophysiological contexts.

Importantly, our work may contribute to insights for the clinical use of aromatase inhibitors. This drug class is associated with side effects like weight gain and hot flashes (Rand et al., 2011). Our results propose that these side effects may stem from central actions of aromatase inhibition, rather than peripheral actions. It is tempting to theorize that these side effects could be prevented by limiting the drug's entry into the brain, thereby ensuring it primarily targets peripheral sites. This approach aligns with ongoing research involving compounds like oxytocin and GLP-1 analogs, where drug molecules are linked to more complex organic structures to prevent central side effects (Asker et al., 2023; Borner et al., 2020). Finally, our results hold translational significance, as unpublished findings suggest that aromatase inhibitors contribute to weight gain in patients and reduce the effectiveness of weight loss medications among breast cancer survivors (Endocrine Society, 2023).

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