The role of relaxin-3/RXFP3 system in hyperphagia

Thèse

Camila De Avila Dal'Bo

Doctorat en neurosciences
Philosophiæ doctor (Ph. D.)

Québec, Canada

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Sous la direction de :
Vincent Joseph, directeur de recherche
Carlo Cifani, codirecteur de recherche

Résumé

Les troubles alimentaires sont souvent déclenchés par le stress et sont plus fréquents chez les femmes que chez les hommes. Les premiers symptômes apparaissent au début de l'adolescence, mais le fondement biologique des différences selon le sexe est inconnu. Parmi les neuropeptides orexigènes étudiés jusqu'à présent, la relaxine-3 (RLN3) est le seul à être déclenché après un épisode de stress. La RLN3 a été découverte en 2001 et appartient à la superfamille de l'insuline. Il est produit dans le noyau incertus (NI) du cerveau et se lie avec une forte affinité à son récepteur spécifique, RXFP3. Des études pharmacologiques ont démontré que les injections exogènes de RLN3 dans le cerveau de rats augmentaient la prise alimentaire et affectaient l'activité des axes hypothalamo-hypophyso-surrénalien (HPA) et gonadique (HPG), ainsi que les niveaux du comportement anxieux. Ces effets étaient différents chez les femelles et chez les mâles. De plus, la déplétion des neurones RLN3 positifs dans le NI des rats mâles n'a pas affecté la prise alimentaire ou l'axe HPA, alors que les effets chez les femelles étaient encore inconnus et sont traités dans la présente étude par une déplétion induite par un miRNA. La présente étude a montré une diminution du poids corporel, un déséquilibre dans la prise alimentaire, ainsi que des changements dans les niveaux du comportement anxieux. De plus, la déplétion de la RLN3 dans le NI a perturbé la régulation de la corticostérone et augmenté les taux de c-*fos* d'ARNm d'ocytocine (OT) et de vasopressine arginine (AVP) dans les régions du cerveau impliquées dans la régulation du stress et de la prise alimentaire. De plus, des études in vitro ont révélé que la RLN3 se lie également à RXFP1, le récepteur spécifique de la relaxine, mais avec une affinité plus faible. La deuxième étude vise à identifier les effets de la liaison de RLN3 à RXFP3 et RXFP1. Des rats mâles ont reçu des injections intracérébroventriculaires de RLN3 et de l'agoniste sélectif de RXFP3, A2. Les résultats ont montré que la prise alimentaire était augmenté à la fois par RLN3 et RXFP3-A2, mais que les effets orexigènes de RXFP3-A2 étaient significativement plus forts que ceux de la RLN3. La prise d'eau et l'activité des axes HPA et HPG ont été significativement augmentées par RLN3, mais pas par RXFP3-A2. Inversement, seule l'activation du

RXFP3-A2 a permis de réduire les concentrations plasmatiques d’OT. Sur la base de ces résultats, nous concluons que la RXFP1 stimulé par la RLN3 est impliqué dans la prise d'eau et dans l'activation des axes HPA et HPG. La réduction de la stimulation de la prise alimentaire par RLN3 par rapport à RXFP3-A2 peut être liée à l'activation des circuits orexigènes et anorexigènes par RLN3. Ces résultats sont cohérents avec le rôle putatif de la RLN3 dans l'ajustement fin des réponses au stress et la régulation de la prise alimentaire.

Abstract

Eating disorders are frequently triggered by stress and are more prevalent in women than men. First symptoms appear during early adolescence, but the biological basis for sex-specific differences is unknown. Among the orexigenic neuropeptides studied thus far, relaxin-3 (RLN3) is the only one to be triggered after a stress episode. Relaxin-3 was discovered in 2001 and belongs to the insulin superfamily. It is produced in the nucleus incertus (NI) of the brain and binds with high affinity to its cognate receptor, relaxin-family peptide receptor 3 (RXFP3). Pharmacological studies showed that exogenous RLN3 injections into the brains of rats increased food intake and affected activity of the hypothalamic-pituitary-adrenal (HPA) and gonadal (HPG) axes, as well as levels of anxiety-like behavior. These effects were different in females and males. In addition, the depletion of the RLN3-positive neurons in the NI of male rats did not affect food intake or the HPA axis, while the effects in females were still unknown and are addressed in the present study using a miRNA-induced depletion. The present study found a resulting decrease in body weight, an imbalance in food intake, and changes in levels of anxiety-like behavior. Furthermore, the depletion of NI RLN3 disrupted corticosterone regulation and increased levels of *c-fos*, oxytocin *(*OT), and arginine vasopressin (AVP) mRNA in brain regions involved in stress and food intake regulation. In addition, in vitro studies revealed that RLN3 also binds to RXFP1, the cognate receptor for relaxin, with lower affinity. The second study aims to identify the effects of RLN3 binding to RXFP3 and RXFP1. Male rats received intracerebroventricular (icv) injections of RLN3 and a selective RXFP3 agonist, A2. Results showed that food intake was increased by both RLN3 and RXFP3-A2, but that the orexigenic effects of RXFP3-A2 were significantly stronger than those of RLN3. Water intake and activity of the HPA and HPG axes were significantly increased by RLN3, but not by RXFP3-A2. Conversely, only RXFP3-A2 activation decreased OT plasma levels. Based on this evidence, we conclude that RXFP1 is involved in the RLN3 stimulation of water intake and activation of the HPA and HPG axes. The reduced food intake stimulation by RLN3 compared to RXFP3-A2 may be related to activation of both orexigenic and anorexigenic circuits by RLN3.

These results are consistent with the putative role of RLN3 in fine-tuning stress responses and food intake regulation.

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Chapter 1

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List of abbreviations

ACTH – adrenocorticotrophin
AVP – arginine vasopressine
aCSF – artificial cerebrospinal fluid
BE – binge eating
BED – binge eating disorder
BMI – body mass index
BNST– bed nucleus of the stria terminalis
BNSTv – ventral part of bed nucleus of the stria terminalis
BNSTd – dorsal part of bed nucleus of the stria terminalis
BW – body weight
cAMP – cyclic adenosine monophosphate
CCK – chlolecystokinin
CeA – central amygdala
CBG – corticoid binding globulin
CRF – corticotropin-releasing factor
DRD2 – dopamine receptor D2 gene
ED – eating disorder
EPM – elevated plus maze
ER – estradiol receptor
ERK - extracellular signal-regulated kinases
FSH – follicular-stimulating hormone
FTO – fat mass and obesity-associated gene
GLP-1 – glucagon-like peptide-1
GnRH – gonadotropin-releasing hormone
GPCR - G protein-coupled receptor
HPA – hypothalamic-pituitary-adrenal
HPG – hypothalamic-pituitary-gonadal
5-HTT – serotonin transporter gene
icv – intracerebroventricular
IHC – immunohistochemical
ISH – *in situ* hybridization
L/D – light/dark box
LH – luteinizing hormone
LHA – lateral hypothalamic area
LHAl – lateral part of the lateral hypothalamic area
LHApf – perifornical part of the lateral hypothalamic area
LOF – large open field
MAPK – mitogen-activated protein kinases
MCH – melanin-concentrating hormone
MC4R – melanocortin 4 receptor
MPAv – ventral part of the medial preoptic area
NAc – nucleus accumbens
NI – nucleus incertus
NIc – nucleus incertus pars compacta

NId – nucleus incertus pars dissipata
OD – optical density
OT– oxytocin
OVLT – organum vasculosum of the lamina terminalis
PVNm – magnocellular part of the paraventricular hypothalamic nucleus
PVNp – parvocellular part of the paraventricular hypothalamic nucleus
RLN3 – relaxin-3
RXFP1 – relaxin-family peptide receptor 1
RXFP3 – relaxin-family peptide receptor 3
RXFP3-A2 – RXFP3-selective agonist relaxin-3 analogue 2
SON – supraoptic hypothalamic nucleus
VTA – ventral tegmental area

Dedication

I dedicate this thesis to Dr Elena Timofeeva, my lovely supervisor who passed away in September of 2017. Elena was an inspiring/sweet/strong woman who gave me the chance to discover science. I will especially never forget a lab meeting we had prior to our institute's annual meeting that year. She was clearly not satisfied with the narrative of the poster I would present at the venue. She criticized it and recommended changes, but I defended my hypothesis and said that I was comfortable defending it as it was. At the end of the meeting, she smiled to me and said, "I'm challenging you because I know the potential you have!"

Thank you, Elena.

Acknowledgement

For all of you who have literally or figuratively held my hand and encouraged me, jumped into my crazy scientific ideas, journeys, projects, scholarships, fellowships, prizes, presentations, experiments, travels, and even the chocolate cravings and some crying: thank you from the bottom of my heart. You are the best and I could never have made it without you!

I thought about naming every single person, but there are simply too many to list and it would be frustrating to omit a single one.

Also, special thanks to everyone who gave me a “no” as an answer and made me develop alternative routes for survival in science. Thankfully I got a decent amount of “no,” adapted, and survived!

Finally, I considered promising to temper my sometimes overly ambitious pursuits from now on, but I feel everyone who knows me would see this for the lie that it is, and I might lose my credibility before even starting my introduction. Instead, I will promise to keep my passion for science and, more than anything else, for all the amazing experiences that science brings me.

“No one is big enough to be independent of others” - *Dr. William Worrall Mayo*

Foreword

Chapter 1 and 2 are submitted and published manuscripts, respectively.

Chapter 1: Effects of silencing relaxin-3 production in *nucleus incertus* neurons on food intake, body weight, anxiety-like behaviour and limbic brain activity in female rats*. *Submitted.*

Camila de Ávila[1-3], Sandrine Chometton[1], Sherie Ma[2]§, Lola Torz Pedersen[3], Elena Timofeeva[1]**†, Carlo Cifani[1,4]** and A.L. Gundlach[2]**

1 *CRIUCPQ, Faculty of Medicine, Department of Psychiatry and Neuroscience, Université Laval, Québec (Québec), Canada*
2 *The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria, Australia*
3 *NNF CBMR, Nutrient and Metabolite Sensing, Faculty of Health and Medical Science, University of Copenhagen, Copenhagen, Denmark*
4 *Pharmacology Unit, School of Pharmacy, University of Camerino, Camerino, Italy*

* Dedicated to Elena Timofeeva, a fine scientist, colleague, supervisor and mentor
** ET, CC and ALG jointly supervised this research
† Deceased 28/09/2017
§ Current Address: Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia

In this paper, CA was responsible for animal procedures. CA and SM performed animal surgeries. CA generated data for figures 1-4., and SC for figures 5-7. LT generated figures 1-7. CA, SC, and ALG wrote the paper with corrections of LT and CC.

Chapter 2: Differential effects of relaxin-3 and a selective relaxin-3 receptor agonist on food and water intake and hypothalamic neuronal activity in rats. Brain Behavioral Research 336 (2017) 135-144

Camila de Ávila*[a], Sandrine Chometton*[a], Christophe Lenglos[a], Juliane Calvez[a], Andrew L. Gundlach[b], Elena Timofeeva[a].

*These authors contributed equally to this research.

[a] Faculté de Médecine, Département de Psychiatrie et de Neurosciences, Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec, Université Laval, Québec (QC), G1V 0A6, Canada.

[b] The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria 3052, Australia; and Florey Department of Neuroscience and Mental Health, The University of Melbourne, Melbourne, Victoria 3010, Australia.

In this paper, CA and SC were responsible for animal procedures and generation of data. CL and JC performed surgeries and injections. CA, SC, ALG and ET wrote the paper with corrections of CL and JC.

In addition to this work done exclusively as part of my doctoral project, I have also had the opportunity to participate in other articles listed below.

- Aliou B. Gueye, Leandro F. Vendruscolo, **Camila de Ávila,** Catherine Le Moine, Muriel Darnaudéry, Martine Cador (2018) Unlimited sucrose consumption during adolescence generates a depressive-like phenotype in adulthood. Neuropsychopharmacology 43:2627–2635.

- Alan Kania, Anna Czerw, Agnieszka Grabowiecka, **Camila de Ávila**, Tomasz Blasiak, Zenon Rajfur, Marian H Lewandowski, Grzegorz Hess, Elena Timofeeva, Andrew L Gundlach, and Anna Blasiak (2017) Inhibition of oxytocin and vasopressin neuron activity in rat hypothalamic paraventricular

nucleus by relaxin-3/RXFP3 signalling. The Journal of Physiology 595: 3425-3447.

- Juliane Calvez, **Camila de Ávila**, Geneviève Guèvremont, Elena Timofeeva (2016) Sex-specific effects of chronic administration of relaxin-3 on food intake, body weight and hypothalamo-pituitary-gonadal axis in rats. Journal of Neuroendocrinology 28:12.

- Juliane Calvez, **Camila de Ávila**, Louis-Olivier Matte, Geneviève Guèvremont, Andrew Gundlach, Elena Timofeeva (2016) Role of relaxin-3/RXFP3 system in stress induced binge-like eating depends on activity of relaxin-3 system in female rats. Neuropharmacology 102:207-215.

- Juliane Calvez; **Camila de Ávila,** Elena Timofeeva (2016) Sex-specific effects of Relaxin-3 on food intake and body weight gain. British Journal of Pharmacology 174:1049-1060.

- Juliane Calvez, **Camila de Ávila,** Geneviève Guèvremont, Elena Timofeeva (2015) Stress differentially regulates brain expression of corticotropinreleasing factor in binge-like eating prone and resistant female rats. Appetite 107:585-595.

- Juliane Calvez, Christophe Lenglos, **Camila de Ávila,** Geneviève Guèvremont, Elena Timofeeva (2015) Differential effects of central administration of relaxin-3 on food intake and hypothalamic neuropeptides in male and female rats. Genes, Brain and Behavior *14:550-563.*

Introduction

Eating disorders (EDs) have shown discrepancies in incident rates, wherein prevalence is particularly high for women (Hoek and Van Hoeken, 2003, Hudson et al., 2007, Preti et al., 2009). Stress appears to be one the most important environmental factors which affect eating behavior (Lo et al., 2007). It is crucial to better understand the neuronal basis of these sex-specific effects of stress in order to develop strategies for treating EDs in women.

It is important to emphasize that EDs contain psychological and sociological diagnostic criteria, and that episodes can be triggered by factors other than stress, such as cultural background, social and ideological influences, and past traumas. The concern for individuals presenting these pathologies extends beyond physical health concerns and includes issues of self-image (Andersen, 2017). Because of this consideration, incidences in humans might include co-occurring compensatory mechanisms such as food restriction, purging, and excessive exercising (American Psychiatric Association, 2013). The focus of this thesis is to investigate the involvement of the relaxin-3/RXFP3 system in stress-induced hyperphagia, using a rat model. Understanding the physiological mechanisms underlying eating disorders in an animal model is essential in order to elucidate new therapeutic approaches for patients.

The following introduction is comprised of an overview of EDs, mechanisms involved in stress-induced food intake, and their sex-specific differences. Finally, a promising therapeutic target for stress-induced eating disorders will be described, the relaxin-3/RXFP3 system.

Eating disorders

Eating disorders definition

According to the American Psychological Association (APA), EDs are "illnesses in which the people experience severe disturbances in their eating behaviors and related thoughts and emotions. People with eating disorders typically become pre-occupied with food and their body weight" (American Psychiatric Association, 2013).

Types of eating disorders

The spectrum of EDs includes anorexia nervosa, bulimia nervosa, and binge eating disorders (BED). According to the current Diagnostic & Statistical Manual of mental disorders (DSM-5), individuals must fulfill the following criteria to have a positive diagnosis.

Anorexia nervosa

- Persistent restriction of energy intake leading to significantly low body weight (in percentiles relative to age, sex, and developmental trajectory)
- Either an intense fear of gaining weight or of becoming fat, or persistent behavior that interferes with weight gain
- Distorted perception of one's body weight or physique and/or undue influence of body shape and weight on self-image

Subtypes of anorexia:

A. Restricting type

This is the most commonly known type of anorexia nervosa, whereby a person severely restricts their food intake. Restriction may take many forms and may follow obsessive and rigid rules (e.g. only eating food of one color).

B. Binge eating or purging type

This subtype refers to persons restricting their energy intake as above, but also includes bouts of either binge eating or purging behavior (e.g. self-induced vomiting, over-exercise, misuse of laxatives, diuretics or enemas). These are cases of anorexia which present some symptoms of bulimia without sufficiently fulfilling the diagnostic criteria of a bulimia nervosa diagnosis.

Bulimia nervosa

- Recurrent episodes of binge eating (BE). An episode of BE is characterized by both of the following:
    - Eating, in a discrete period of time (e.g. within any 2-hour period), an amount of food that is significantly larger than most people would eat during a similar period of time and under similar circumstances
    - A sense of lack of control over eating during the episode (e.g. a feeling that one cannot control what or how much one is eating)
- Recurrent excessively compensatory behavior in order to prevent weight gain, such as self-induced vomiting and misuse of laxatives, diuretics, or other medications
- The BE and compensatory behaviors both occur, on average, at least once a week for three months
- Self-image is disproportionally influenced by body shape and weight

Binge eating disorder

- Recurrent episodes of BE. An episode of BE is characterized by both of the following:
    - Eating, in a discrete period of time (e.g. within any 2-hour period), an amount of food that is significantly larger than most people would eat during a similar period of time and under similar circumstances

    - A sense of lack of control over eating during the episode (e.g. a feeling that one cannot control what or how much one is eating)
- The BE episodes are associated with three or more of the following:
    - Significantly more rapid consumption rate than normal, relative to that individual
    - Eating until feeling uncomfortably full
    - Eating large amounts of food when not feeling physically hungry
    - Eating alone because of feeling embarrassed by how much one is eating
    - Feeling disgusted with oneself, depressed, or very guilty afterward
- Evident distress regarding BE is present
- BE occurs, on average, at least once a week for three months
- BE episodes are not associated with the recurrent use of inappropriate compensatory behaviors (as in bulimia nervosa), and does not occur exclusively during the course of bulimia nervosa or anorexia nervosa

A spectrum of physical characteristics is seen among individuals suffering from eating disorders. Body mass index is only one of such characteristics, and is reflected in the spectrum shown in Figure 1.

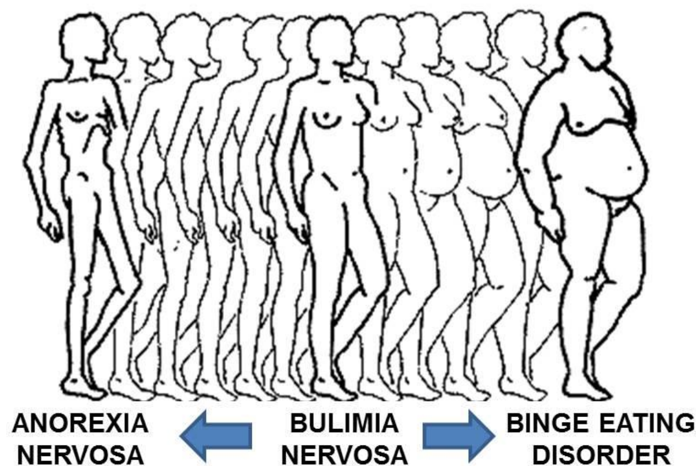

Figure 1. Spectrum of body mass index (BMI) in individuals with eating disorders

Anorexia nervosa: a persistent restriction of energy intake leads to significantly low body weight (relative to age, sex, height, and developmental trajectory). Bulimia nervosa: BE episodes followed by recurrent inappropriate compensatory behavior in order to prevent weight gain. Approximately 65 percent of individuals with bulimia nervosa have BMI's which fall within a normal/healthy range of 18.5 and 29.9 (Hudson et al., 2007). Binge eating disorders (BED): recurrent episodes of BE not associated with the recurrent use of inappropriate compensatory behaviors, thusly contributing to a higher BMI (American Psychiatric Association, 2013).
Adapted from http://staff.washington.edu/jrees/ch1edado.html

Risk factors

The greater the number of risk factors present, the more intense these factors are, and the more vulnerable the period of development is, the higher the likelihood of developing an eating disorder. Risk factors are therefore probabilistic, not deterministic. They are culture-bound, occurring primarily in industrialized cultures that value thinness and have sufficient availability of food so that being overweight is a realistic possibility. Individual idealization of underweight BMIs in societies experiencing widespread starvation is rare, except within the governing or wealthy classes of developing countries that have adopted westernized body images (Andersen, 2017).

Genetic predisposal

Although no large-scale studies of gene association with eating disorders have yet been conducted (Yilmaz et al., 2015), the probability of familial predispositions to BED has been shown to be significant (Bulik et al., 2003, Javaras et al., 2008). In addition, studies show that polymorphisms of certain genes, such as those encoding the serotonin transporter (5-HTT), the dopamine type 2 receptor (DRD2), the melanocortin pathway type 4 receptor (MC4R), and the fat mass and obesity

associated (FTO) gene, are associated with hyperphagia and obesity (Branson and Potoczna, 2003, Monteleone et al., 2006, Frayling et al., 2007, Cecil et al., 2008, Davis et al., 2012). However, for these genetic predispositions to induce hyperphagia, environmental factors are usually necessary. These factors include, but are not limited to, stressful stimuli and food availability. Stressful stimuli are factors that can induce a multi-systemic response in the body to face a challenge that exceeds specific mechanisms of the homeostatic response (Day, 2005).

Prevalence

Studies report higher prevalence rates of eating disorders in females (Table 1), leading researchers to conclude that they are biologically more prone to developing them. More specifically, the prevalence of anorexia nervosa and bulimia nervosa is significantly higher in females (Woodside et al., 2001, Hoek and Van Hoeken, 2003, Hudson et al., 2007), though this sex-specific discrepancy lessens in prevalence rates for bulimia (Hudson et al., 2007, Striegel-Moore et al., 2009, Forrester-Knauss and Zemp Stutz, 2012) (Table 2). However, even in this case, bulimia displays significantly different characteristics in the two sexes. For example, in many countries, women with bulimic hyperphagia more often have a desire to appear slimmer in alignment with socially permeated ideals, while men rarely seek slim figures, which may be culturally synonymous with weakness and vulnerability (Andersen, 1999, Striegel-Moore and Bulik, 2007, Forrester-Knauss and Zemp Stutz, 2012, Strother et al., 2012). In addition, women report more frequent loss of control than men during binge eating episodes and are more likely to use compensatory behaviors characteristic of bulimia (e.g., vomiting, laxative use), while men tend to compensate for it through exercise (Weltzin et al., 2005).

One additional factor that can contribute to binge eating episodes and excess weight gain is dietary restriction (Gluck, 2006). For example, women restrict their overall food consumption more often than men, and this restriction is positively correlated with stress-induced over-consumption of palatable food (Weinstein et al., 1997, Zellner et al., 2006, Habhab et al., 2009). Indeed, dietary

restriction is thought to be linked to an increase in blood cortisol levels in women, while a link between cortisol levels and restriction has not been established in men (Rutters et al., 2009). These high cortisol levels in women may stimulate food intake (Epel et al., 2001).

The lifetime prevalence of anorexia nervosa and bulimia nervosa is three times higher in women than men (Hoek and Van Hoeken, 2003, Hudson et al., 2007, Preti et al., 2009), as shown in the table below.

	Male		Female		Total	
	%	(SE)	%	(SE)	%	(SE)
I. Lifetime prevalence						
Anorexia nervosa	.3*	(.1)	.9*	(.3)	.6	(.2)
Bulimia nervosa	.5*	(.3)	1.5*	(.3)	1.0	(.2)
Binge eating disorder	2.0*	(.5)	3.5*	(.5)	2.8	(.4)
Subthreshold binge eating disorder	1.9*	(.5)	.6*	(.1)	1.2	(.2)
Any binge eating	4.0	(.7)	4.9	(.6)	4.5	(.4)
II. Twelve-month prevalence†						
Bulimia nervosa	.1*	(.1)	.5*	(.2)	.3	(.1)
Binge eating disorder	.8*	(.3)	1.6*	(.2)	1.2	(.2)
Subthreshold binge eating disorder	.8	(.3)	.4	(.1)	.6	(.2)
Any binge eating	1.7	(.4)	2.5	(.3)	2.1	(.2)
(n)	(1220)		(1760)		(2980)	

Abbreviations: SE, standard error.
*Significant sex difference based on a .05 level, 2-sided test.
†None of the respondents met criteria for 12-month anorexia nervosa.

Table 1. Lifetime and 12-month prevalence estimates of eating disorders

The lifetime prevalence of anorexia nervosa and bulimia nervosa were three times higher for women compared to men. The ratios of male to female prevalence of individual EDs, as well as binge eating in general, shows relative consistency in both 12-month and lifetime rates.

Adapted from (Hudson et al., 2007)

Age of onset of eating disorders

The first signs of eating disorders normally appear at adolescence (Bulik, 2002). Figure 2 shows the cumulative lifetime prevalence of anorexia nervosa, bulimia nervosa, BED, subthreshold BED, and any BE. Median age of onset of anorexia nervosa, bulimia nervosa, BED, subthreshold binge eating disorder, and any BE ranged from 18–21 years. Survival analysis based on retrospective age-of-onset reports suggests that the risk of bulimia nervosa and BED increased with successive birth cohorts (Hudson et al., 2007).

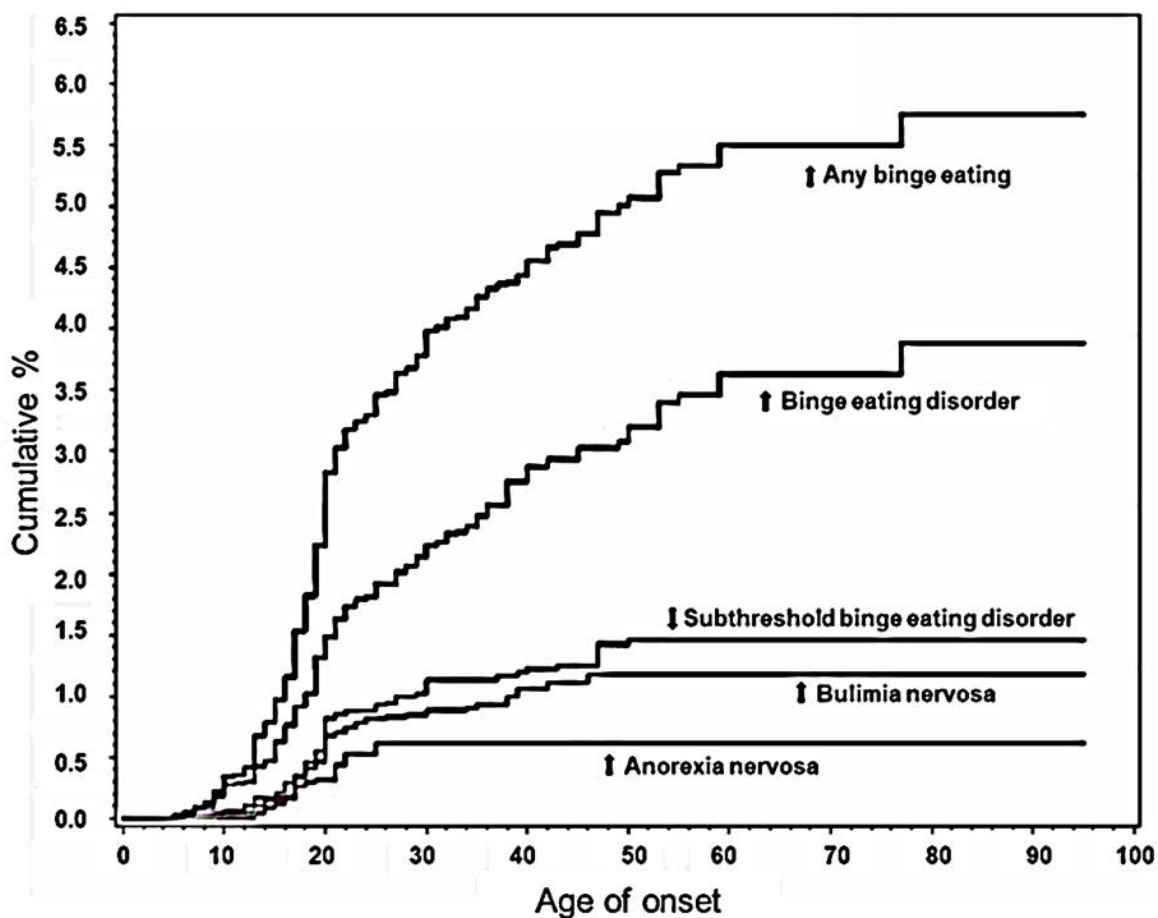

Figure 2. Cumulative lifetime eating disorders

Median age of onset of the five disorders ranged from 18–21 years. The period of onset risk was shorter for anorexia nervosa than for the other disorders, with the

earliest cases of the other disorders beginning about 5 years earlier than those of anorexia nervosa (ages 10 vs. 15), and no cases of anorexia nervosa beginning after the mid-20s, whereas some cases of the other disorders began at a much older age.

Adapted from (Hudson et al., 2007)

Environmental factors

Acute psychological stressors, such as visualization of images causing social rejection, have been identified as factors which may trigger BE episodes (Cattanach et al., 1988, Tuschen-Caffier and Vögele, 1999). In individuals with bulimia, physical acute stressors, such as exposure to cold, can also increase the desire to eat (Gluck et al., 2004). Chronic stress has also been shown to contribute to BEDs. Indeed, most individuals with bulimia report chronic stress (Stunkard and Allison, 2003, Freeman and Gil, 2004, Smyth et al., 2007).

Food availability has also been shown to play an important role in bulimia. The abundance of energy-rich and highly palatable food promotes the likelihood of BE episodes (Allison and Timmerman, 2007). Conversely, dietary restriction, whether imposed by oneself or others, is positively correlated with the development of bulimia (Polivy, 1996). Finally, food insecurity, which particularly affects the poorest populations, is reported to be a contributing factor for episodes of BE; wherein individuals experiencing food deprivation due to poverty tend to consume excessive amounts of high energy density foods when presented with opportunities of unlimited access to food (Townsend et al., 2001).

Additional risk factors

Geography, personality, and family history, among others, are important risk factors that increase the probability of developing an ED. The following table summarizes these factors.

Risk factor	Comments
Geography	Highest incidence in westernized societies and upper socioeconomic classes in developing countries.
Personality (typical, though not required, features)	Anorexia nervosa: sensitive, perfectionist, persevering, self-critical features of temperament, persevering. Bulimia nervosa: impulsivity, emotional intensity, mood lability, dramatic features. Binge-eating disorder: high harm avoidance, low self-directedness, conflict avoidant.
Family history	Increased in families with history of depressive spectrum disorder, obesity.
Interest groups	Students in strict dance schools, bodybuilders, models, wrestlers, participants in sports with high performance and appearance demands (girls gymnastics, figure skating), cross-country runners, media professionals, actors.
Racial and ethnic group	Largely independent of racial and ethnic designation if westernized body ideals for thinness are adopted; increased in African American families of higher socioeconomic status.
Predisposing medical diseases	Type 1 diabetes mellitus, cystic fibrosis, medical prescription of corticosteroids or atypical antipsychotics.
Predisposing psychiatric disorders	Depressive disorders, anxiety disorders, attention deficit hyperactivity disorder, posttraumatic stress disorder, obsessive compulsive disorder.
Location	More common in urban areas than in rural.
Influence of media	Decreased self-esteem, more self-critical body image, after viewing impossible-to-achieve body size/shape ideals in media; onset of eating disorders with introduction of TV and other media, e.g., in South Sea Islands.

Table 2. Additional risk factors

Geography, personality, and family history, among others, are important risk factors that increase the probability of developing an ED.

Adapted from (Andersen, 2017)

Therapeutic best practices

Current therapies for EDs focus on psychological approaches. These approaches can be performed individually or in groups. Besides the patient, family and caregivers may also be involved in the therapy, as their relationship is an important element in the treatment model. Medication can be prescribed without psychological support as a secondary option, carrying specific risk factors to be monitored (Andersen, 2017). The table below summarizes best practices in current therapeutic treatments of EDs.

Cognitive behavioral therapy (CBT)	Cognitive behavioral therapy (CBT) is the most effective evidence-based form of psychotherapy for eating disorders. CBT principles teach patients to challenge, through evidence gathering, their overvalued beliefs about the benefits of slimness/shape change. Interpersonal therapy (IPT) is slower but may be alternative. Dialectical behavioral therapy (DBT) is also slower but may be an alternative for bulimia nervosa comorbid with borderline personality disorder. CBT is often effective in groups.
CBT and Medication-assisted treatment (MAT)	If no experienced cognitive behavioral therapist is available, Selective Serotonin Reuptake Inhibitors (SSRIs), such as fluoxetine, sertraline, and citalopram, often decrease binge-purge symptoms by 50%. However, patients may have relapse if medications are discontinued or may not respond at all. SSRIs are best used if CBT is not by itself fully effective. Medications to increase appetite such as atypical antipsychotics, and cannabinoids, should be avoided.
Individualized psychotherapy	Individualized psychotherapy can help the patient understand that the core underlying issues in treatment are not weight loss or food but strategies to manage emotional distress, identity formation, and self-esteem, for which the eating disorder is a pseudosolution.
Family-based outpatient treatment	Family-based outpatient treatment for adolescents with mild or moderate anorexia nervosa can be effective using the Lock and Le Grange method, in which the family is empowered to act as the treatment team. Inpatient hospitalization may be avoided.
Therapy for the caregivers	Parents and significant others should be provided with psychoeducation about the nature of the eating disorder and companion disorders. They need empathy and coping strategies for preventing "burnout" while caring for individuals with chronic illness and for maintaining healthy family relationships without being monitors of "food police." Resolutions of family functioning issues can be identified in family therapy.

Table 3. Current therapies for eating disorders

Adapted from (Andersen, 2017)

Stress: an important trigger for binge eating

Definition of stress

In 1936, Selye defined stress as “a syndrome produced by nocuous agents,” and since then, stress has received much debate. Today, it is widely accepted that “stress” needs to be defined in terms of the response and that which causes the response (McEwen, 2004). An actual or perceived threat to an organism is referred to as the “stressor” (Schneiderman et al., 2005). Additionally, a stress response could be initiated by both external and internal stressors (Ginty and Conklin, 2011). For example, tissue damage from an accident or foot shock in rodents is externally inflicted stress; whereas public speaking is an internally perceived stress. Stressors shift the physiological set-points such as heart rate, respiratory rate, or extent of vigilance to a resource-hungry unstable state in order to trigger an appropriate response for survival (Wray et al., 2002). Hence, any threat to systems that are essential for life (homeostasis) or situation where the feeling of well-being is challenged triggers an adaptive and beneficial stress response (McEwen, 2004).

Stress activation of the hypothalamic-pituitary-adrenal axis

Stressors activate the hypothalamic-pituitary-adrenal (‘HPA’) axis (Fig. 3), which is composed of the paraventricular nucleus of the hypothalamus (PVN) and the anterior pituitary and adrenal glands. Neurons with cell bodies located within the parvocellular region of the PVN and terminals located in the median eminence release corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), which are transmitted through the portal vasculature. These peptides stimulate the secretion of adenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH is released into the general blood circulation and eventually triggers the secretion of glucocorticoids from the adrenal cortex, with the predominant steroid being cortisol in humans and corticosterone in rodents (Herman and Cullinan, 1997). Glucocorticoids then travel via the general circulatory system to act on nearly every tissue within the body. Given their widespread actions, glucocorticoids trigger negative feedback at the adrenal, anterior pituitary, PVN, and other sites of the

central nervous system (CNS) (Herman, James. P. 2011). Central Nervous System Regulation of the Hypothalamic-pituitary-adrenal axis Stress Response. In Conrad, Cheryl D (Ed), *The Handbook of Stress: Neuropsychological Effects on the Brain* (pp. 30). Chichester, WS: Wiley-Blackwell).

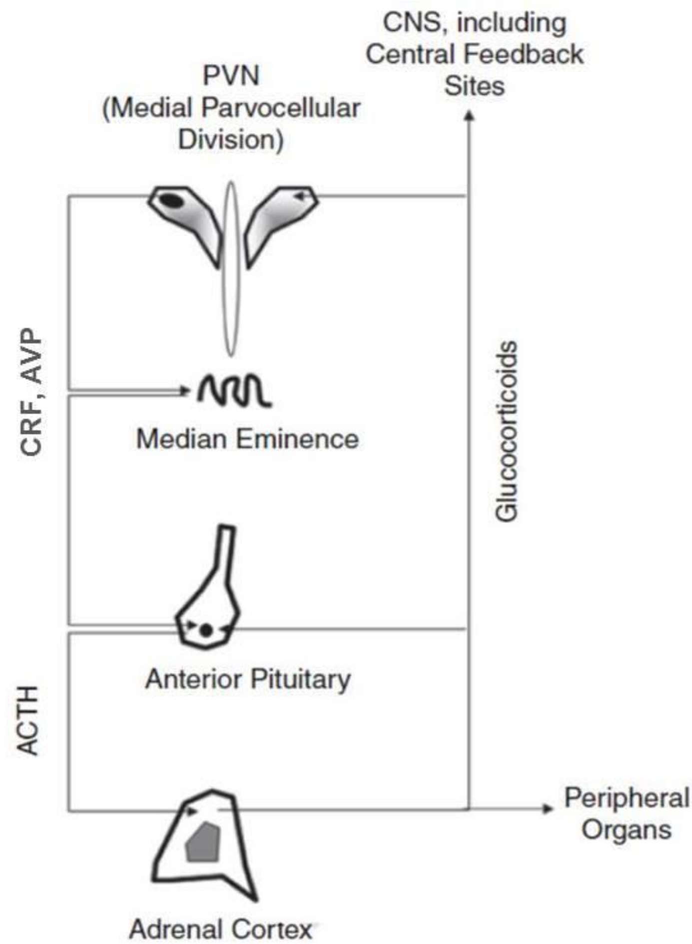

Figure 3. Schematic representation of the hypothalamic-pituitary-adrenal axis: central and peripheral structures and their mediators

Adapted from (Herman, James. P. 2011). Central Nervous System Regulation of the Hypothalamic-pituitary-adrenal axis Stress Response. *Adapted from* (In Conrad, Cheryl D (Ed), *The Handbook of Stress: Neuropsychological Effects on the Brain* (pp. 30). Chichester, WS: Wiley-Blackwell).

Bimodal stress modulation of food intake

The stress response has immediate effects that involve redistributing resources to mechanisms that are critical for survival and away from those that are unnecessary in the immediate term. For example, blood flow is enhanced in the skeletal muscles to assist with immediate movement, while being reduced in the digestive system. Consequently, one of these reductions from the immediate stress response includes reduced hunger or anorexigenic effects. As part of the HPA axis and as a neuromodulator, CRF is a putative anorexigenic peptide involved in the stress-induced inhibition of food intake (Krahn et al., 1986, Smagin et al., 1999). Indeed, studies have shown that in the PVN, CRF injection has an anorexigenic effect (Krahn et al., 1988) and that the anorexigenic effect of stress is related to an increase in CRF (Smagin et al., 1999) or agonists to the CRF type 2 receptor, such as urocortins (Heinrichs and Richard, 1999, Kuperman and Chen, 2008). However, this anorexigenic effect of the CRF would only occur in certain regions, such as the ventromedial hypothalamus (Richard et al., 2002), since injection of CRF into other regions of the hypothalamus, such as the lateral area (Krahn et al., 1988) and medial preoptic area (Dagnault and Richard, 1997), does not induce a significant anorexigenic effect.

Unlike CRF, glucocorticoids stimulate food intake to help restore energy resources depleted during the stress response (Dallman et al., 2004). For example, glucocorticoids exert negative feedback on CRF secretion and can stimulate orexigenic neurons in the arcuate nucleus, leading to increased food intake (Dallman et al., 2004, Takeda et al., 2004) . As a result, logic dictates that during fasting, corticosterone levels are increased while CRF levels are relatively low, thus promoting food intake (Dallman et al., 1999). Considering these notions, a stress response involves immediate cessation of feeding through fast neural actions via CRF, while a slower response to onset of feeding occurs when glucocorticoids are at their peak, about 15 to 30 minutes after the introduction of the stressor.

In today's world, people experience different types of daily stressors: home and work overload, family demands and arguments, transportation and financial problems, among others (Almeida and Kessler, 1998). These numerous daily events can lead to chronic stress and an imbalance in the hypothalamic-pituitary-adrenal axis (Timofeeva and Calvez, 2014).

Imbalance of the HPA axis induces binge eating

Chronic stress or traumatic episodes leading to an imbalance in the activity of the HPA axis can greatly disrupt feeding behavior (Timofeeva and Calvez, 2014). An imbalance in the central response to stress can indeed explain incidence rates of eating disorders being more than three times higher in women than in men (Hoek and Van Hoeken, 2003, Hudson et al., 2007, Preti et al., 2009). In particular, it has been shown that stress or negative emotions were significant causes of BE episodes (American Psychiatric Association, 2013, Timofeeva and Calvez, 2014) and that women were more likely than men to eat due to stress (Laitinen et al., 2002, Zellner et al., 2006).

Sex differences in the activation of the HPA axis

HPA axis and sex hormones

The difference between males and females in the activation of the HPA axis may be partly due to the interaction of steroid hormones released by the gonads. Although overall HPA axis activation is similar in males and females, with an increase in CRF, ACTH, and corticosterone following acute stress, it appears that female rats may experience higher plasma ACTH and corticosterone levels after stress compared to male rats (Babb et al., 2013). However, females have more corticosteroid-binding globulin (CBG) protein, the major transport protein for glucocorticoids, and present similar levels of biologically active glucocorticoids (Tinnikov, 1999, Kudielka and Kirschbaum, 2005).

Following chronic stress, plasma corticosterone levels are also observed to increase in males and females, but reach a higher level in females than in males (Sterrenburg et al., 2011). However, in males, studies have shown a significant increase in CRF mRNA expression that is not found in females (Dunčko et al., 2001, Sterrenburg et al., 2011, Sterrenburg et al., 2012). These observed differences between males and females may be due to the activation of a higher number of glucocorticoid receptor-expressing neurons in female rats (Zavala et al., 2011), which would induce greater inhibition of CRF expression following increased corticosterone levels. Given the inhibitory role of corticosterone on CRF, it may be possible that the higher level of corticosterone in females reduces the anorexigenic effect of CRF following stress. This explains observed decreases in body weight gain in males following certain types of stress, such as immobilization stress, rarely seen in females (Dunčko et al., 2001, Faraday, 2002, Faraday et al., 2005).

Indeed, testosterone administration in castrated males reduces ACTH secretion to a level similar to intact males, while estradiol administration in ovariectomized females increases ACTH secretion to a level similar to intact females (Seale et al., 2004).

The HPG axis (Fig. 4) is composed of the medial preoptic area (MPA) of the hypothalamus, the anterior pituitary gland, and the gonads. The gonadotropin-releasing hormone (GnRH) is the key hypothalamic regulator of the HPG axis. GnRH is released at the median eminence by axonal terminals from neurons with somas located within the MPA. GnRH activates gonadotrophic cells of the anterior pituitary and stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the bloodstream. Following release, LH and FSH stimulate the gonads, effectively stimulating the release of estrogen and progesterone in females, and testosterone in males. These gonadal steroids enter the bloodstream and target many tissues, including the brain (Meethal and Atwood, 2005).

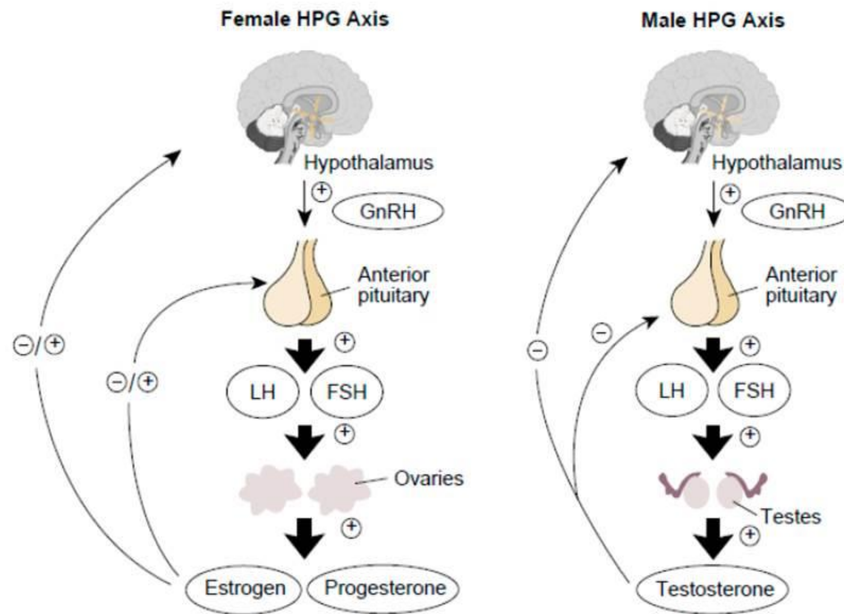

Figure 4. Schematic representation of the hypothalamic-pituitary gonadal (HPG) axis

Adapted from (Kong et al., 2014)

Like glucocorticoids, estrogens and testosterone are fat-soluble hormones and can modulate food intake through their binding to intracellular receptors that act as transcription factors. Testosterone binds to androgen receptors (AR) while estrogens, including the predominate estrogen in rats in humans, 17β-estradiol, bind to estrogen receptors (ER) (Simerly et al., 1990, Shughrue et al., 1997). Furthermore, estradiol binds to GPER1 or GPCR30, a G protein-coupled receptor (GPCR) evoking rapid effects. These effects may include stimulating Ca2+ mobilization from intracellular stores (directly or via epidermal growth factor receptor transactivation), c-fos expression, adenylyl cyclase (AC) and cyclic adenosine monophosphate (cAMP)-mediated signaling, and ERK-1/2 in a variety of cells (Hazell et al., 2009). These receptors are widely expressed in the brain, particularly in the hypothalamus and the regions of the brain connected to the hypothalamus (Simerly et al., 1990, Shughrue et al., 1997, Hazell et al., 2009). Thus, sex hormones could bind and have a potential effect on many areas of the brain, such as the ventromedial hypothalamus, amygdala, and preoptic area

(Madan and DeFranco, 1993). These areas will be explained in more detail in the next section.

Feeding behavior

Physiology of feeding behavior

Hunger and satiety states are coordinated by a series of signals. These signals come from peripheral organs (e.g. gut and stomach) and are integrated centrally by different brain structures. In the brain, the most studied of these structures is probably the hypothalamus. This structure is very well preserved in all mammals, which allows us to study its anatomy and functioning in rodent models, among others. In addition to peripheral signals, the hypothalamus integrates signals from other brain regions such as the nucleus solitary tract (NTS), amygdala, bed nucleus of the stria terminalis (BNST), and paraventricular thalamus (PVT). The difficulty in elucidating the physiological mechanisms governing feeding behavior comes not only from the complexity of interactions between different regions of the brain, but also the complexity of the interactions within the hypothalamus itself, which can be divided into several regions with distinct functionalities. In this section, we will introduce the hypothalamic regions involved in feeding under normal conditions. Next, we will describe the peripheral signals and their integration at the central level. Finally, we will introduce the concept of hedonism.

History

Lesions found in some regions of the hypothalamus in rats have indicated that this structure has an important role in food intake (Kennedy, 1950). The lesions with the most significant effects on food intake are those of the lateral hypothalamic area (LHA), which induce a decrease in food intake, and those of the VMH, which induce an increase in food intake (Anand and Brobeck, 1951, Pandit et al., 2011). As a result, the LHA was first described as a 'feeding center' (Anand and Brobeck, 1951) while the VMH was considered a 'satiety center' (Kennedy, 1950, Panksepp, 1971).

The use of more precise stereotaxic techniques later determined that strict lesions of VMH are not found to produce hyperphagia, but that more dorsal lesions,

in the paraventricular hypothalamus (PVH), did produce hyperphagia (Gold, 1973). Although this study invalidates both the dual-center hypothesis and the involvement of VMH in satiety, this nucleus still represents an important player in the metabolism of energy (Chun-Xia et al., 2011). Along with the other regions of the hypothalamus, it still corresponds to one of the key components of the energy balance. Subsequent studies have also highlighted the importance of the arcuate nucleus (Arc), paraventricular nucleus (PVN), LHA, ventromedial hypothalamus (VMH) in regulating feeding (Fig. 5).

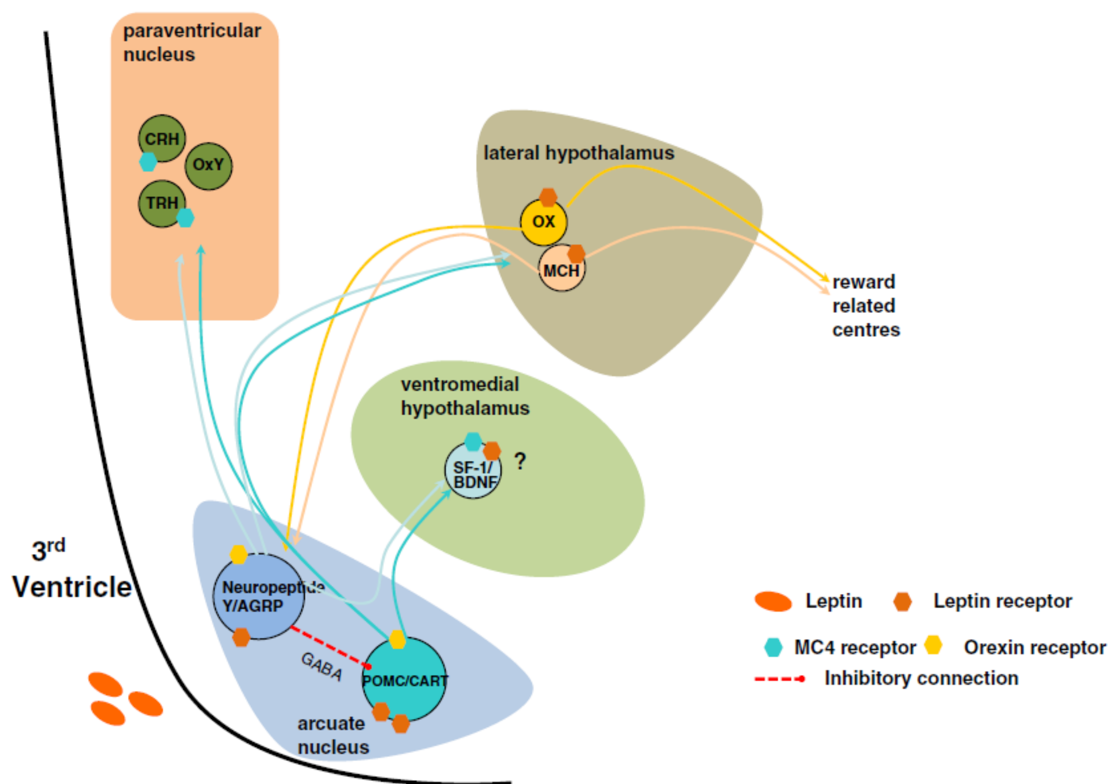

Figure 5. Schematic representation of the principal hypothalamic sub-nuclei involved in feeding behavior

(MCH) melanin-concentrating hormone, (MC4) melanocortin 4, (CRH) corticotropin-releasing hormone (TRH) thyrotropin-releasing hormone, (OxY) Oxytocin, (SF-1) orphan nuclear receptor

Adapted from (Pandit et al., 2011)

Hypothalamic regions

Lateral hypothalamus or lateral hypothalamic area

The LHA is one of the principal sites connecting the homeostatic and hedonic aspects of feeding (Pandit et al., 2011). The former control energy balance by increasing motivation to eat following depletion of energy stores, while the latter increase the desire to specifically consume foods that are highly palatable (high in sugar and/or fat) (Meule and Gearhardt, 2014) during periods of relative energy abundance (Lutter and Nestler, 2009).

As mentioned above, LHA lesions induce a decrease in food intake, while stimulation of this region produces a rapid and sustained food intake (Hoebel and Teitelbaum, 1962, Wise, 1971). However, these lesions and stimulation experiments mask the heterogeneity of the neuronal population of the LHA. Indeed, many studies have shown that the LHA contains distinct neuronal populations which express neurotransmitters with varying roles (Barson et al., 2013).

Orexin is a neuropeptide highly involved in regulation of feeding. Orexin-positive neurons in LHA are inhibited by high concentrations of glucose in extracellular fluid and could therefore act as a direct sensor of the metabolic state of the body (Burdakov et al., 2005). Neurons expressing orexin do not express melanin-concentrating hormone (MCH), and both are orexigenic neuropeptides (Barson et al., 2013).

Blood glucose levels are believed to be an important regulator of LHA's neural activity. Indeed, the neurons expressing orexin are stimulated by a decrease in glucose levels, while neurons expressing MCH are stimulated by increased glucose levels (Burdakov et al., 2005). It has been shown that the absence of orexin reduces insulin sensitivity and can lead to diabetes (Yi et al., 2009).

The neuropeptides expressed in LHA neurons can be regulated by peripheral signals. Indeed, a significant proportion of neurons with orexin and MCH

also express leptin receptors (Hakansson et al., 1999). Leptin down-regulates the expression of the orexin precursor and the orexin type 1 receptor (López et al., 2000). The orexin-positive neurons are also reported to be activated by ghrelin and to induce an increase in food intake independently of the increase in neuropeptide Y (NPY) in the Arc (Toshinai et al., 2003).

The neuronal population of the LHA is also modulated by afferences from many brain regions such as the prefrontal and infralimbic cortex, nucleus accumbens (NAc), BNST, amygdala, and ventral tegmental area (VTA) (Barone et al., 1981, Kita and Oomura, 1982, Hahn and Swanson, 2012). The reciprocity of connections with these extra hypothalamic regions involved in motivated behavior and emotion (Saper et al., 1979, Berk and Finkelstein, 1982) suggests that LHA is also involved in non-homeostatic control of food intake. Distinguished from 'homeostatic feeding,' where food intake is restricted to satisfy one's biological needs, 'non-homeostatic feeding' or 'feeding for pleasure' relates to alterations in dietary patterns with a prevalence of energy-dense fat and sweet foods, food cravings, and compulsive eating (Pandit et al., 2011). Additional details regarding non-homeostatic feeding will be detailed in the section on hedonic feeding.

Ventromedial hypothalamus

The role of the VMH in regulating satiety has been confirmed by studies in which its stimulation inhibited food intake (Hoebel and Teitelbaum, 1962). The VMH contains glucose-sensitive neurons, and the effect of the VMH on food intake would be coordinated with the other regions of the brain which it is connected to. For example, Arc neurons in the melanocortin pathway can stimulate VMH neurons secreting brain-derived neurotrophic factor and thus inhibit food intake (Baoji et al., 2003). In addition, VMH neurons expressing splice factor 1 co-express leptin receptors and contribute to regulating food intake and body weight maintenance (Satoh et al., 1997, Dhillon et al., 2006). A significant portion of the neuronal population of the VMH also express the type 2 CRF receptor, which is involved in the regulation of food intake, particularly in cases of stress-induced anorexia (Pelleymounter et al., 2000).

VMH neurons project to MPOA and BNST (Saper et al., 1979). Despite the absence of direct connections between the VMH and the LHA, interaction between these two regions would be made possible by an indirect connection through the DMH (Luiten and Room, 1980).

Arcuate nucleus

The role of the Arc in food intake is less easily characterized by lesion studies because it includes populations of neurons that inhibit hunger while others stimulate food intake. The neurons that inhibit hunger and stimulate thermogenesis are those that express the cocaine- and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC) which is subsequently cleaved to give rise to the alpha-melanocyte-stimulating hormone (α-MSH). Alpha-MSH induces an anorexigenic effect mainly by activating melanocortin 4 receptor (MC4R) (Miura et al., 2000). Similarly, intracerebroventricular (icv) administration of CART inhibits food intake (Lambert et al., 1998), but the receptors with which it induces this anorexigenic effect remain in need of further investigation (Harrold et al., 2012).

Neurons expressing NPY, agouti-related peptide (AgRP), and GABA stimulate hunger and reduce thermogenesis. Studies using optogenetics have shown that stimulation of neurons expressing NPY is sufficient for inducing food intake in mice, regardless of response from the melanocortin pathway (Kevin and Joel, 2011).

In the brain, the Arc integrates peripheral signals that regulate food intake, such as ghrelin, insulin and leptin (Hewson et al., 2002). Moreover, since the Arc is a region not protected by the blood-brain barrier, these hormones can directly interact with its first-order neuronal population to regulate food intake. The administration of ghrelin in the brain stimulates food intake and is accompanied by activation of the Arc neurons (Hewson and Dickson, 2000). The injection of leptin in the Arc stimulates POMC neurons while inhibiting NPY neurons, both of which express the leptin receptor (Elias et al., 1999, Cowley et al., 2001). Administration of insulin into the third ventricle, in the immediate proximity of the Arc, increases

the expression of POMC, while injection of a melanocortin antagonist prevents the insulin-induced inhibition of food intake (Benoit et al., 2002). The POMC and NPY neurons expressing the insulin receptor may both play a role in the integration of the insulin receptor`s anorexigenic effect (Plum et al., 2005).

Paraventricular nucleus

Similar to the Arc, the PVN is sensitive to food-regulating hormones such as ghrelin, leptin, and insulin. For example, ghrelin injection in this region induces expression of the neuronal activation marker Fos and stimulates food intake (Olszewski et al., 2003), insulin injection induces an increase in thermogenesis and decrease in food intake (Menéndez and Atrens, 1991), and leptin injection increases expression of the anorexigenic neuropeptide CRF in PVN (Huang et al., 1998). Overall, the PVN is more likely to have an inhibitory role on food intake. Indeed, lesions in the PVN stimulate food intake and lead to obesity (Leibowitz et al., 1981).

The PVN can be distinguished in two parts; the first part containing parvocellular neurons (PVNp), and the second part containing magnocellular neurons (PVNm) (Swanson and Kuypers, 1980) . The neurons of PVNm secrete oxytocin (Sabatier et al., 2013) and vasopressin (Pei et al., 2014), with no overlapping of expression (Vandesande and Dierickx, 1975) , while the neurons of PVNp mainly secrete CRF (Sawchenko et al., 1984, Uehara et al., 1998)

The anorexigenic effect of oxytocin, vasopressin, and CRF are exerted through distinct mechanisms. The anorexigenic effect of CRF may be partly explained by an interaction of CRF neurons with POMC and NPY neurons within the Arc. Indeed, the connections between these neurons are reciprocal in nature (Fekete et al., 2000). On the one hand, it has been shown that CRF secretion by PVN neurons can directly inhibit food intake induced by NPY-secreting neurons (Heinrichs et al., 1993, Menzaghi et al., 1993). On the other hand, injection of melanocortin receptor antagonists reduces the inhibition of stress-related food intake, suggesting a contributing role for POMC neurons in CRF-induced anorexia

(Vergoni et al., 1999, Liu et al., 2007). In addition, the CRF can modulate food intake by interacting with other regions that express the CRF type 2 receptor, such as VMH (Richard et al., 2002). Indeed, this receptor is involved in regulating food intake, unlike the CRF type 1 receptor, which is more likely to be involved in other types of stress- and anxiety-related behavior (Pelleymounter et al., 2000, Richard et al., 2002, Timofeeva et al., 2002).

Oxytocin secreted by the PVNp is shown to be particularly important in the delivery of the anorexigenic signal from leptin to brainstem regions such as NTS and area postrema, while oxytocin secreted by the PVNm is reported to be more involved in regulating water homeostasis (Olszewski et al., 2010). As for vasopressin, in addition to its intrinsic anorexigenic effect (Meyer et al., 1989), it could also play a potentiating role in the effect of CRF (Gillies et al., 1982), thus reinforcing its anorexigenic effect. Finally, although these three neurotransmitters are anorexigenic, they have separate and distinct functions. Oxytocin is also involved in sexual reproduction, vasopressin has a significant role in antidiuretic mechanisms (i.e. water retention), and CRF is the initial factor secreted within the HPA axis, causing the body's neuroendocrine stress response.

Supraoptic nucleus

The supraoptic nucleus (SON) has a relatively similar functional anatomy to that of the magnocellular part of the PVN, suggesting a comparable role for these two regions in inhibiting food intake (Swanson and Sawchenko, 1983). It contains mainly magnocellular neurons that secrete oxytocin and vasopressin from their axonal endings located in the posterior pituitary gland (Rhodes et al., 1981).

Medial preoptic area

The medial preoptic area (MPOA) is a sexually dimorphic region in rats (Gorski et al., 1978). Several studies have shown that it can play a role in food intake, as the injection of growth hormone-releasing hormone and galanine-related

peptide into the MPOA increases food intake (Mascarenhas, 1986, Vaccarino and Hayward, 1988, Taylor et al., 2009).

A number of MPOA neurons also produce CRF, though this number is reportedly higher in female rats than in males (McDonald et al., 1994). However, unlike the CRF produced in the PVN, the CRF produced in the MPOA does not induce an anorexigenic effect in rats (Dagnault and Richard, 1997) and is reported to be involved in stress responses with an inhibitive impact on the reproductive system (Rivest et al., 1993). The MPOA also has male and female sex hormone receptors (Madan and DeFranco, 1993). However, the inhibitory effect of estradiol on food intake is not mediated by the MPOA, as an estradiol implant in this region does not reduce food intake in orvariectomized female rats (Hrupka et al., 2002).

Extra-hypothalamic brain structures

Although the hypothalamus has the most studied role in regulating food intake, other brain areas are also involved, particularly areas which are directly connected with the hypothalamus.

Amygdala

The amygdala is a subcortical region of the temporal lobes. The amygdala is part of the limbic system and is involved in memory, decision-making, and emotion (Swanson and Petrovich, 1998). Numerous studies of lesions and stimuli have also highlighted its role in food intake (Anand and Brobeck, 1952, Crovetti et al., 1995, Rollins and King, 2000). However, the observed effects on food intake appear to differ between stimulated and injured amygdala subregions. For example, a lesion in the cortico-medial amygdala inhibits food intake when the rat is placed in a new environment (Sclafani et al., 1970), and larger lesions induce increased food intake and body weight (Rollins and King, 2000). In addition, in rats with intermittent access to palatable food, the increase in CRF in central amygdala (CeA) stimulates excessive palatable food intake and anxiety, while the same CRF in the basolateral amygdala could induce a decrease in average food intake (Cottone et al., 2009).

Finally, the amygdala expresses the MC4R receptor, and the administration of an MC4R antagonist in this nucleus stimulates food intake (Kask and Schiöth, 2000).

Nucleus of the solitary tract

The NTS is located in the brain stem and it is the integration center for many peripheral signals including the vagus nerve (VN) (Berthoud et al., 2006), and it is the first site through which gastro intestinal-afferent information come into the brain (Cui et al., 2011). Afferences of the hypothalamus, amygdala, and BNST originate in the NTS (Norgren, 1978, Ricardo and Koh, 1978), which maintains reciprocal connections with more rostral regions such as the amygdala and hypothalamus (Ricardo and Koh, 1978, Van Der Kooy et al., 1984).

During food intake, activation of the VN via gastric distension and release of other anorexigenic and orexigenic molecules from the gastro intestinal (GI) system leads to activation of NTS (Timofeeva et al., 2005). In addition, many neurons in the medial NTS have leptin receptors that induce activation of this region through interaction with VN signals, inhibiting food intake (Huo et al., 2007).

Bed nucleus of the stria terminalis

A large part of the amygdala nuclei project onto the BNST (Fig. 6) (Weller and Smith, 1982). It has been shown that similar to the amygdala, the BNST also plays a role in stress and anxiety, however the BNST has additional long-term effects (Walker et al., 2003, Walker et al., 2009). The subregions of the BNST present different physiological functions, particularly through the topographical organization of afferences from the amygdala and other limbic structures, such as the infralimbic cortex and the ventral subiculum (Dong et al., 2001).

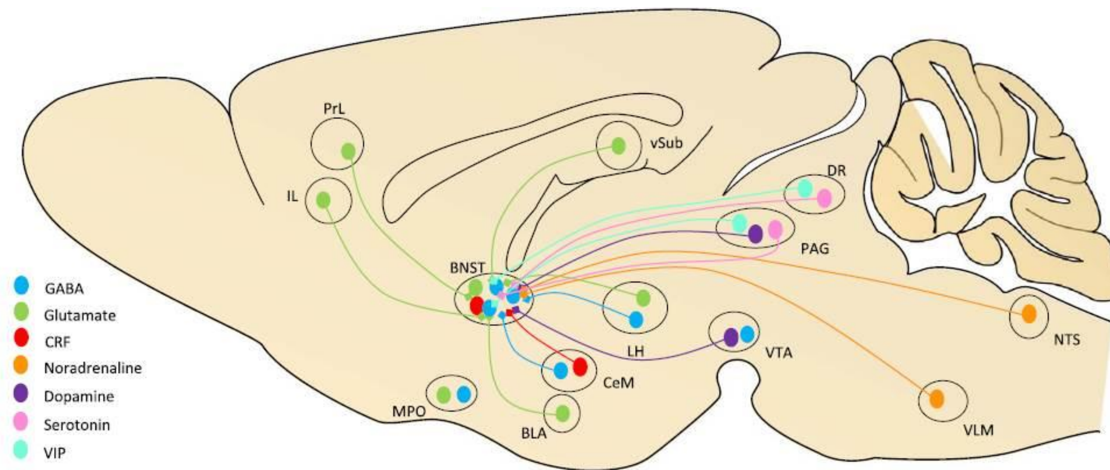

Figure 6. Sagittal schematic of the rat brain depicting main BNST afferents

The BNST receives widespread input from many brain loci involved in processing information related to stress, reward, arousal, social interaction, and viscerosensation. (BLA) basolateral amygdala, (BNST) bed nucleus of the stria terminalis, (CeM) centromedial amygdala, (CRF) corticotropin-releasing factor, (DR) dorsal raphe, (IL) infralimbic cortex, (LH) lateral hypothalamus, (MPO) medial preoptic area, (NTS) nucleus of the solitary tract, (PAG) periaqueductal grey, (PrL) prelimbic cortex, (PVN) paraventricular hypothalamic nucleus, (VIP) vasoactive intestinal polypeptide, (VLM) ventrolateral medulla, (vSub) ventral subiculum, (VTA) ventral tegmental area.

Adapted from (Fu et al., 2018)

In addition to its role in stress and anxiety, the BNST is also involved in drug dependence. Indeed, rats that have experienced drug dependence have an increased level of extracellular CRF in the BNST due to stress. The inhibitive CRF action via antagonists of its specific receptors in the BNST prevents the recovery of drug dependence (Erb and Stewart, 1999). The role of the BNST has also been demonstrated in other motivated behavior such as seeking alcohol, however this incident involves dopaminergic projections of VTA on the BNST (Eiler et al., 2003).

Finally, intra-BNST injections of the nonselective CRF receptor antagonist D-Phe-CRF decreased stress-induced BE in rats with a history of food restriction (Micioni Di Bonaventura et al., 2014).

Paraventricular Thalamus

The PVT is a region of the diencephalon ventrally bordering the dorsal part of the third ventricle in rats (Paxinos and Watson, 2007), and is part of the dorsomedial thalamus (Groenewegen and Witter, 2004). The PVT receives afferences from the BNST, the amygdala (originating in CeA), and different hypothalamic regions such as the lateral and dorsomedial hypothalamus, and is moderately innervated by adrenergic fibers from the NTS (Chen and Su, 1990). In contrast, the PVT densely projects onto the ventral striatum, the prefrontal cortex (Bubser and Deutch, 1998, Otake and Nakamura, 1998) and the amygdala (Moga et al., 1995).

Based on this functional anatomy, it would appear that the PVT sends information from visceral and hypothalamic signals to limbic structures and to the striatum, regulating energy balance, arousal, and the reward system (Kelley et al., 2005, Parsons et al., 2006). The role of the PVT in food intake has also been highlighted in several studies. For example, one hour after food intake, PVN, SON, DMH, CeA, and PVT experience an increase in c-*fos* mRNA, a marker of neuronal activity (Timofeeva et al., 2002). This activation of the PVT following re-feeding may have an inhibitory effect on food intake since inhibition of the PVT, by the injection of GABA agonist, produces an increase in food intake (Stratford and Wirtshafter, 2013). However, since PVT activation can be induced by fasting (Timofeeva and Richard, 2001) and stress (Bubser and Deutch, 1999), it would be unlikely that activation of the PVT under these two other conditions would lead to inhibition of food intake. These divergent effects, observed following the activation of the PVT, could be mediated by the electrophysiological properties of its neuronal population and its many afferences (Kolaj et al., 2014).

Peripheral signals

Many peripheral signals are involved in the controlling of feeding behavior, including those from hormone-producing organs (e.g. gut, intestine, and pancreas). These peripheral signals play a role in diverse feeding behaviors. For instance, the GI system and adipose tissue produce hormones and stimulate the signal transporter, the VN, which modulates food intake. The VN, which is the longest cranial nerve in the body, not only regulates gut physiology, but is also involved in controlling the cardiovascular, respiratory, immune and endocrine systems (Browning et al., 2017). Within the gut, the stimulation of VN, by hormones (e.g.: leptin, insulin, PYY 3-36, and ghrelin) or by gastric distension, induces the transmission of anorexigenic and orexigenic signals through its connection to the brain; more precisely, to the NTS in the brainstem (Fig. 7) (Berthoud, 2008).

Gastro-intestinal system and adipose tissue signals

Insulin

Together with leptin, insulin is one of the principal anorexigenic hormones involved in regulating long-term food intake (Baskin et al., 1999). Insulin is secreted by the pancreatic β-cells in response to an increase in glucose, the preferred energy substrate for most organs, particularly the brain (Mueckler, 1994). Peripherally, insulin stimulates glucose uptake and inhibits glycogenolysis (e.g. glucose production from glycogen in muscles and liver) and neoglucogenesis (i.e. glucose production from non-carbohydrate compounds such as amino acids) to reduce blood glucose levels. Centrally, insulin inhibits food intake (Brüning et al., 2000). Central icv insulin injection inhibits expression of the orexigenic NPY precursor in the hypothalamus (Schwartz et al., 1992).

Glucagon

Glucagon is secreted by the pancreatic α-cells, which are stimulated by a decrease in glucose levels. Similar to insulin, glucagon has an anorexigenic effect. Indeed, it has been shown that endogenous glucagon increases during a meal and

when glucagon is sequestered during this time with intraperitoneal injections of glucagon antibodies, both meal size and duration increase (Heppner et al., 2010). In addition, glucagon injection into the hepatic portal vein has been shown to inhibit food intake in rats with intact VN (Geary and Smith, 1983), and pancreatic secretion is stimulated by vagal afferent firing (Browning et al., 2017). The VN is believed to be an important mediator of the anorexigenic effect of glucagon at the central level, since it projects to brainstem regions involved in food intake such as the NTS and the area postrema (Sawchenko, 1983).

Unlike insulin, glucagon has a hyperglycemic effect. Glucagon binds to its receptor, mainly located on the plasma membrane of liver cells. It stimulates glycogenolysis and neoglucogenesis, thereby increasing glucose levels. Glucagon receptors are also expressed in smaller quantities in other organs such as the brain (Svoboda et al., 1994). Icv injection of glucagon into the brain has a powerful anorexigenic effect and is mediated by the hypothalamus (Inokuchi et al., 1984).

Glucagon-like peptide 1

Glucagon-like peptide 1 (GLP-1) is a peptide secreted by intestinal L-type cells. Its secretion is stimulated by the presence of nutrients in the lumen of the intestine.Unlike glucagon, GLP-1 stimulates and enhances the effects of insulin, leading to a decrease in glucose levels. In addition, GLP-1 inhibits glucagon secretion. The central icv and peripheral administration of GLP-1 or agonist of the GLP-1 receptor, exendin-4, have an anorexigenic effect in rats, while the antagonist exendin 3-39 has an orexigenic effect in satiated rats (Turton et al., 1996, Baraboi et al., 2011). The GLP1R receptor is found in cells that produce insulin and glucagon, but also in certain hypothalamic and extra-hypothalamic regions of the brain (Merchenthaler et al., 1999, Tornehave et al., 2008).

Leptin

Leptin is a protein secreted mainly by fat tissue (Ahima et al., 2000), and its production level varies according to body fat mass (Frederich et al., 1995). It acts

as an anorexigenic hormone in the long term by inhibiting food intake (Jeffrey and Jeffrey, 1998) and increasing energy-releasing mechanisms such as thermogenesis (Gong et al., 1997). Leptin levels are decreased during fasting, potentially caused by a decrease in blood glucose and insulin levels (Boden et al., 1996).

Leptin is also secreted by cells in the stomach (Bado et al., 1998, Sobhani et al., 2000). Unlike leptin which originates in adipose tissue, leptin from the stomach is believed to play a role in modulating food intake in the short term (Sobhani et al., 2000). Indeed, leptin levels in the stomach are rapidly stimulated by food intake (Bado et al., 1998, Cinti et al., 2001). The role of leptin from adipose tissue on long-term food intake is mediated by OB-R receptors in the brain, especially in the hypothalamus (Tartaglia et al., 1995). The role of leptin of gastric origin is believed to be mediated by the VN and cells of the GI system that also express the OB-R receptor (Tartaglia et al., 1995, Wang et al., 1996, Wang et al., 1997).

Peptide YY 3-36

The peptide YY 3-36 (PYY 3-36) is anorexigenic and is mainly produced by intestinal cells. Its plasma concentration is stimulated by food intake and is proportional to the amount of calories consumed (Grandt et al., 1994) (Sawchenko, 1983). Peripheral and central administration of this peptide inhibits food intake (Batterham et al., 2002). PYY 3-36 has been found to produce its anorexigenic effect via the Y2R receptor, which is expressed by NPY neurons of the Arc (Gong et al., 1997, Batterham and Bloom, 2003). In addition, its anorexigenic effect requires an intact VN, suggesting an interaction between the two (Zarbin et al., 1983). However, Tschöp et al (2004) failed to observe inhibition of food intake following the injection of PYY 3-36 at the periphery and central level, leaving some doubt about the anorexigenic effect of this peptide.

Cholecystokinin

Along with PYY 3-36, cholecystokininin (CCK) is one of the main peripheral hormones in short-term food regulation. CCK is a peptide secreted by certain cells of the small intestine, neurons of the enteric system, and the brain, and it has an anorexigenic role (Fink et al., 1998). Its production is stimulated by food intake and its signaling is mainly within the VN and brainstem (Crawley & Corwin, 1994; Zarbin et al., 1983).

Ghrelin

Ghrelin is a hormone mainly produced by X-cells of the stomach and secreted by the GI system. Unlike all the factors mentioned above, ghrelin has an orexigenic effect (Kojima et al., 1999, Ariyasu et al., 2001). However, the knock down of ghrelin, its enzyme, or its receptor in mice produced no alteration in food intake and body weight (Sun and Tank, 2003). The administration of ghrelin in the periphery or icv induces an increase in food intake (Wren et al., 2000) and decreases the use of lipids, leading to an increase in body fat (Tschöp et al., 2000). Its activity is mediated by the activation of growth hormone secretagogue receptors (GHS-R), also called ghrelin receptors. This receptor is found in the periphery and in the brain. In the brain, ghrelin specifically activates the NPY neurons of the Arc (Wang et al., 2002).

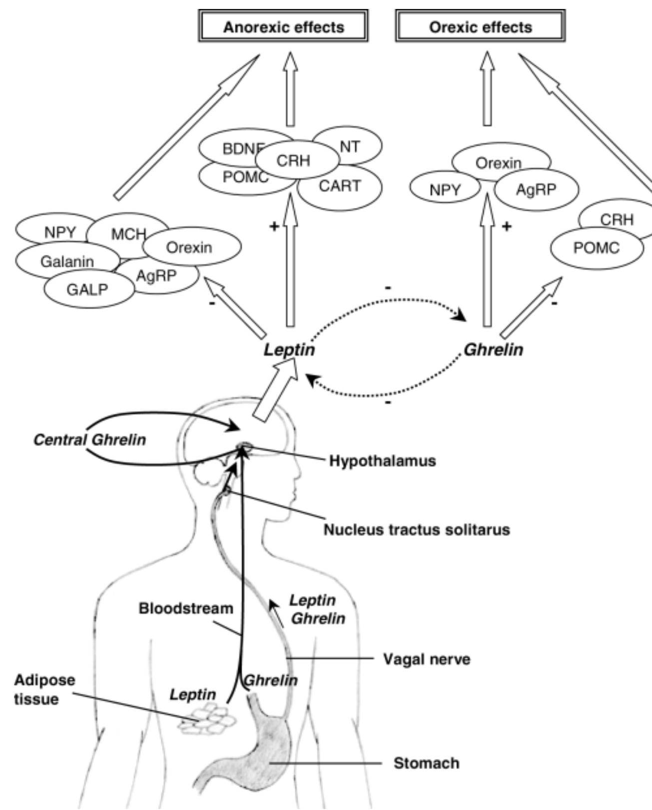

Figure 7. Pathways by which leptin and ghrelin may have effect on energy balance in humans

This schematic drawing shows the pathways by which leptin and ghrelin may reach the hypothalamus in order to have an effect on food intake and body weight. Leptin is secreted by adipose tissue and ghrelin is secreted by the stomach. Both hormones may enter the brain through the bloodstream (long arrow with straight line). In addition, ghrelin and gastric leptin may reach the hypothalamus through the vagal nerve and nucleus of the solitary tract (short arrows with straight line). In addition, central ghrelin may affect the energy center in the hypothalamus (curved arrow). Leptin and ghrelin both stimulate (+) and suppress (–) hypothalamic neurons containing various neuropeptides, resulting in anorexigenic or orexigenic effects on energy balance (open arrows). Studies on the effect of leptin on circulating ghrelin levels produced conflicting results. Ghrelin`s potential influence on circulating leptin levels has not yet been studied (curved arrows with dashed line). (AgRP) agouti-related protein, (BDNF) brain-derived neurotrophic factor, (CART) cocaine- and amphetamine-regulated transcript, (CRH) corticotropin-

releasing hormone, (GALP) galanin-like peptide, (MCH) melanin-concentrating hormone, (NPY) neuropeptide Y, (NT) neurotensin, (POMC) pro-opiomelanocortin (Klok et al., 2007).

Integrative physiology of eating behavior

Maintaining physiological parameters (e.g. blood glucose levels) is one of the body's major challenges, and is called homeostasis. Indeed, in humans and many species, nutrient intake is not continuous over time, while organs have continuous energy requirements. To overcome this discrepancy, there exist many mechanisms for regulating blood glucose levels in order to store excess glucose or generate glucose from reserves, in the form of glycogen and triglycerides. Changes in reserves, mainly in the form of lipids, and glucose levels, will thus modulate hunger signals in order to prevent a deficiency or excess of nutrients.

Based on these hunger signals, four phases of feeding behavior can be established: the appetite phase where hunger signals are high and induce eating; the food intake phase where hunger signals decrease; the aversive phase where hunger signals are at a minimum; and the stimulus-free phase (Craig, 1918). We will particularly highlight the physiological mechanisms of the appetite phase and the food intake phase leading to aversion, at which point we will focus on the factors that can induce an alteration of these mechanisms of normal hunger regulation.

Appetite phase

In animals, the appetite phase is marked by a feeding behavior which is itself caused by an increase in central and peripheral appetite signals. The appetite phase is the result of a gradual increase in peripheral orexigenic signals, such as ghrelin, and a decrease in anorexigenic signals, such as leptin and insulin (Cummings et al., 2001). The increase in ghrelin levels is induced by low glucose

and decreases in the amount of nutrients in the stomach, both resulting from the fasting period (Toshinai et al., 2001). Conversely, since glucose levels are at a basal level or slightly lower than normal at this time, insulin secretion is reduced to its lowest levels as well. These low blood glucose and insulin levels are thought to cause a decrease in plasma leptin levels during fasting (Boden et al., 1996).

The three hormones mentioned above, ghrelin, leptin, and insulin, will bind to receptors in the central nervous system. Ghrelin specifically activates orexigenic neurons and inhibits anorexigenic Arc neurons. In addition, a competitive interaction between orexigenic and anorexigenic signals has been found in Arc neurons (Nakazato et al., 2001), in which ghrelin suppresses the anorexigenic effect of leptin via activation of NPY and AgRP neurons, leading to inhibition of POMC neurons (Klok et al., 2007). Finally, this activation of NPY neurons of the Arc may induce an inhibition of the CRF secreting neurons in the hypothalamus (Cowley et al., 1999), thus enhancing the anorexigenic effect of the CRF. In summary, in the appetite phase, the activation of the hypothalamus by the signals of the periphery induces a behavior that leads to the food intake phase.

Food intake phase

The food intake phase is marked by the ingestion of nutrients and, consequently, the gradual increase in satiety signals at the peripheral and central levels. It has been found that these signals could be represented as a cascade of satiety involving sensory, cognitive, post-ingestion, and post-absorption stimuli (Blundell and Halford, 1994).

Perception of food by sight, smell, or taste results in a first peak of insulin that is thought to be dependent on the VN (Sjöström et al., 1980, Just et al., 2008). The arrival of nutrients in the stomach will induce the secretion of anorexigenic factors such as CCK. The VN, stimulated by gastric distension, stimulates the NTS. VN stimulation is potentiated by CCK, whose action can itself be potentiated by leptin and induce greater activation of NTS (Barrachina et al., 1997). The VN is also stimulated by increased plasma glucagon levels in the hepatic portal vein

(Geary and Smith, 1983). This anorexigenic response prevents meal overeating and is called short-term regulation (Fig. 8) (Havel, 2001).

The gradual absorption of glucose stimulates insulin secretion. Insulin binds to its receptors in the liver and muscles to store glucose as glycogen. It also couples with receptors in the liver and fat tissue to store glucose as lipids, resulting in a decrease in blood glucose and a return to normal blood glucose levels. In addition, GLP-1 enhances the effects of insulin and stimulates its secretion, and it is believed to be involved in the stimulation of the VN to induce satiety (Näslund et al., 1999). Ghrelin secretion decreases with gastric distension and nutrient absorption (Tschöp et al., 2001). Its role in satiety mechanisms is thought to be reflected in the removal of inhibition of the action of other anorexigenic factors such as leptin and insulin (Nakazato et al., 2001).

As in the appetite phase, various peripheral signals will act directly on the brain. In the Arc, leptin and insulin stimulate POMC neurons and inhibit NPY neurons. In addition, leptin can stimulate CRF neurons, which contribute to its anorexigenic effect (Uehara et al., 1998).

PYY 3-36 has a similar role to GLP-1, as increasing levels of PYY 3-36 at the peripheral and central level also decrease food intake (Batterham et al., 2002). However, this effect remains controversial (Tschöp et al., 2004). Finally, the increase in glucose inhibits NPY neurons and stimulates POMC neurons in the Arc (Parton et al., 2007), thus inducing an energy-releasing effect marked by glucose storage and thermogenesis.

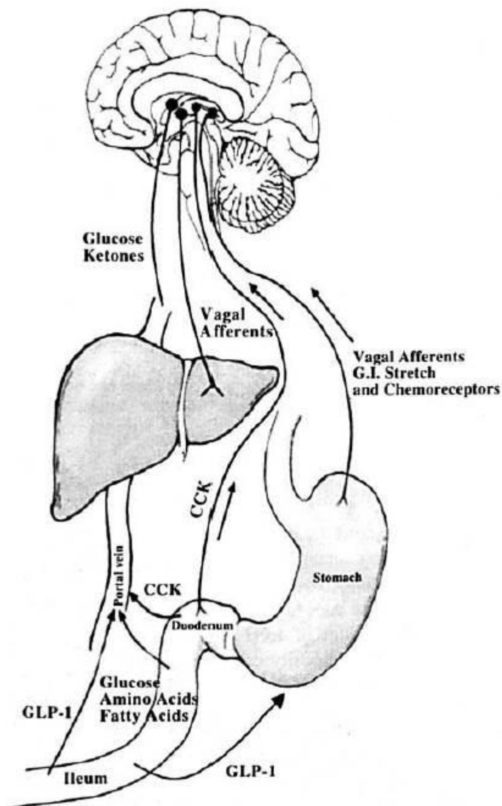

Figure 8. Short-term signals regulating food intake

Signals from the GI tract and the liver are involved in short-term regulation of feeding. Afferent signals travel in vagal nerve fibers from stretch receptors, and chemoreceptors activated by the presence of nutrients in the stomach and proximal small intestine are involved in inducing meal termination. Nutrients arriving via the portal vein may also trigger vagal afferent signals from the liver. Glucose can modulate food intake by acting on glucose-responsive neurons in the CNS. Ketones appear to decrease appetite. In response to nutrient stimulation, the proximal intestine releases cholecystokinin (CCK), which reaches the liver via the portal vein and the CNS via the systemic circulation; CCK may act on CCK-A receptors at both sites to inhibit food intake.

Adapted from (Havel, 2001)

Contrary to the short-term signals, long-term alterations in energy intake will also be sustained. Body weight and body fat in particular are largely regulated via leptin, positively correlating with plasma leptin levels. However, an increase in body fat reduces the anorexigenic effect of leptin. Plasma insulin levels are also positively correlated with body fat, similarly contributing to regulation of long-term food intake and body weight (Baskin et al., 1999). Finally, ghrelin is an orexigenic factor involved in the central regulation of long-term food intake. Indeed, its circulating levels vary according to energy stocks and changes in body weight (Cummings, 2006).

Food restriction condition

Food restriction is a condition in which access to food is limited and, as previously mentioned, is a positive criteria for anorexia nervosa (American Psychiatric Association, 2013). The two main ways of inducing it are by limiting the time of access to food, which corresponds to food-deprivation, or by limiting the amount of food available. In most cases, the daily limitation of the amount of food leads to a decrease in body weight, or lessens the body weight gain in a growing individual (Rothwell and Stock, 1982). This effect is not always observed with time-limited access to food because the individual temporarily deprived of food may be able to ingest a cumulative quantity of food comparable to the unrestricted subject once he or she has free access to food again (Levitsky et al., 1976).

In addition to the effect on body weight, limiting access to food over time is a stronger homeostatic challenge. Indeed, in the case of a dietary restriction that is sufficiently limited in time, the individual quickly moves from a state of fasting to a state where the body's ability to ingest food is pushed to its maximum. Fasting stimulates the production of hyperglycemic factors, such as glucagon, which in the long term promote a decrease in muscle mass (Daniel et al., 1977), while re-feeding is marked by a sharp increase in glucose and insulin levels (Strubbe et al., 1988). Thus, limiting the time of food consumption can affect body weight and promote an imbalance in peripheral factors.

Energy metabolism is also affected in both types of restrictions. Although time-limited access to food leads to an increase in energy metabolism, food deprivation and chronic dietary restriction lead to a decrease in energy metabolism and thermogenesis (Cumming and Morrison, 1960, Rothwell and Stock, 1982, Hayashi and Nagasaka, 1983). These physiological effects could be explained by the alteration of the expression of certain neuropeptides in the hypothalamus. Indeed, expression of the anabolic or energy storage-inducing neuropeptide NPY is increased while expression of catabolic or energy release-inducing neuropeptides, such as POMC and CRF, is decreased (Brady et al., 1990). In addition, food deprivation is reported to be accompanied by a decrease in the excitatory efferences of VMH to the POMC neurons of the Arc, thus decreasing the catabolic effect of these neurons (Scott et al., 2005). As in the appetite phase, the plasma leptin levels are particularly low during fasting (Cecil et al., 2008) and are show to have less stimulation of Arc POMC neurons (Mizuno et al., 1998).

Finally, dietary restriction may induce an increase in plasma corticosterone levels (Woodward et al., 1991) and could be described as a physical stressor (Holly et al., 2016), leading us to focus on the effect of stress and its hormones on food intake.

Hedonic feeding

Under certain conditions, homeostatic control of food intake may be exceeded by non-homeostatic signals related to hedonism whose purpose, conscious or unconscious, is the search for a reward that typically generates pleasure (Berthoud, 2004). Hedonism is traditionally defined as the association of three behavioral phenomena (Berridge, 2004). The first phase, essential for the subsequent recognition of the food and the activation of other neuronal circuits involved in hedonism, is the learning phase. This phase mainly involves the central nucleus of the amygdala. The motivational aspect of hedonism, or "wanting," includes the development of the strategy to achieve the hedonic value associated with the stimulus. Even if this approach seems largely voluntary and therefore

conscious, there is also an unconscious element, called "incentive salience." This aspect of hedonism involves dopaminergic connections between the VTA and the NAc. The third aspect of hedonism is "liking." It illustrates the conscious or unconscious pleasure associated with a stimulus. It involves GABA neurons and the opioid system of the NAc. The regulation of food intake by this system can be summarized by Figure 9. In addition to being important actors in homeostatic control of food intake, insulin and leptin inhibit food intake by modulating dopaminergic and opioidergic pathways of the reward system (Figlewicz and Benoit, 2009).

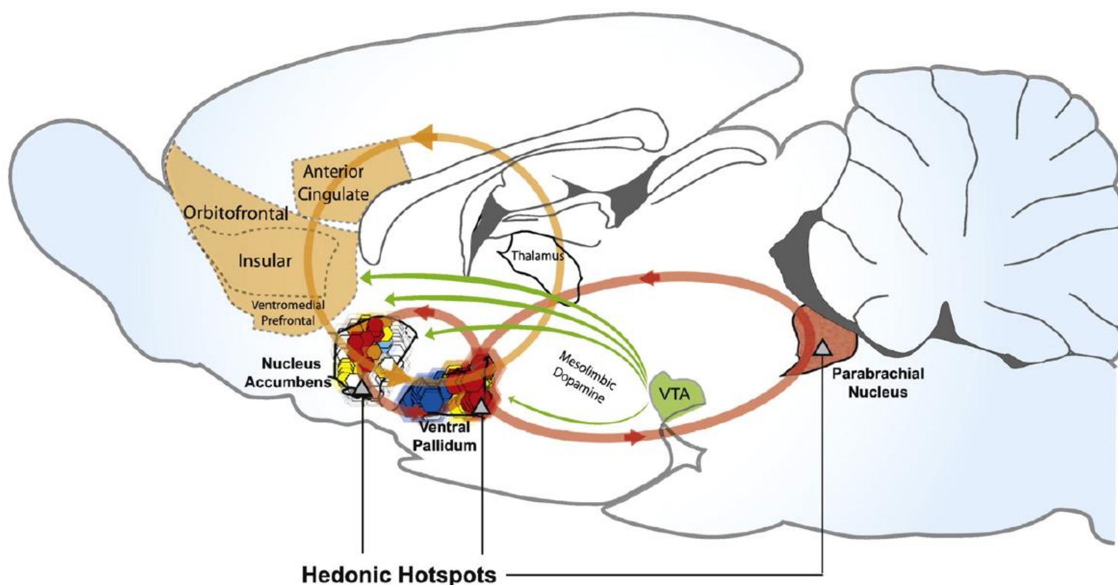

Figure 9. Hedonic hotspots and circuits

Hedonic hotspots are shown in the nucleus accumbens, ventral pallidum, and brainstem parabrachial nucleus, where opioid or other signals cause amplification of core “liking” reactions to sweetness.

Adapted from (Berridge et al., 2010)

Palatability and food dependency

In addition to the effect of sex hormones on stress and food intake, the palatability of food may also play a role in these two responses of the body. Indeed, it has been shown that the palatability of food increases food intake in rats (Corbit and Stellar, 1964). Furthermore, the consumption of palatable foods decreases activation of the stress axis in stressed rats and CRF expression in unstressed rats (Pecoraro et al., 2004).

The increase in food intake due to palatable foods stems from an increase in hunger signals and a decrease in satiety signals (Erlanson-Albertsson, 2005). Consumption of palatable foods is also reinforced by the activation of reward systems in the brain, leading to true food dependence. Indeed, active brain regions in cases of food dependence are similar to those in drug dependence (Pelchat et al., 2004, Wang et al., 2004). BED is a typical example of food dependence that is characterized by a pressing need to consume large amounts of food and a withdrawal period when this need is not met (Avena, 2010).

The development of binge-like eating in animal models is mainly induced by manipulating some of the environmental factors that cause the eating disorders described above, particularly diet and stress (Hardaway et al., 2015), in such a way that allows measuring of their impact on food intake (Patterson and Abizaid, 2013).

Diet can be modulated by the availability of food, but also by its quality (e.g. palatability and nutritional value). Male rats with intermittent access to sucrose followed by a 12-hour fast for about a month develop binge-like eating towards sucrose (Avena, 2010). Similarly, male rats with free access to regular chow combined with limited high-fat and high-sugar diet develop binge-like eating towards this palatable food (Berner et al., 2008).

Stress and/or dietary restriction appear to be important factors in the development of binge-like eating in female rats. Indeed, female rats that have experienced dietary restriction develop binge-like eating following certain types of

acute stress episodes and in the presence of palatable food, high in fat and sugar (Hagan et al., 2002). Additionally, it has been shown that stress episodes without dietary restriction were sufficient to induce binge-like eating in female rats (Calvez et al., 2016b, Micioni Di Bonaventura et al., 2014).

Relaxin-3/RXFP3 system

In addition to all components mentioned above that influence the regulation of food intake, relaxin-3, a new neuropeptide, was discovered in 2001 and its role in regulating food intake and stress has been highlighted in recent years. However, thus far there are relatively few studies dealing with this molecule, the main characteristics of which will be detailed in this section.

Relaxin-3 family and superfamily

The neuropeptide relaxin-3 (RLN3, aka insulin-7 or INSL7) is a 6kDa peptide which belongs to the relaxin peptide family along with other six members: relaxin-1, relaxin-2 and insulin-like peptides-3, -4, -5 and -6 (Bathgate et al., 2002, Burazin et al., 2002). Unlike other relaxin- family peptides that display considerable species heterogeneity, RLN3 sequences are well-conserved across species including fish, frogs, rodents, macaques and humans, suggesting it plays an important biological role (Wilkinson et al., 2005). In addition, RLN3 is the ancestral member of the insulin superfamily, composed of insulin, insulin-like growth factors I / II and, relaxin peptides (Fig. 10) (Bathgate et al., 2002, Burazin et al., 2002).

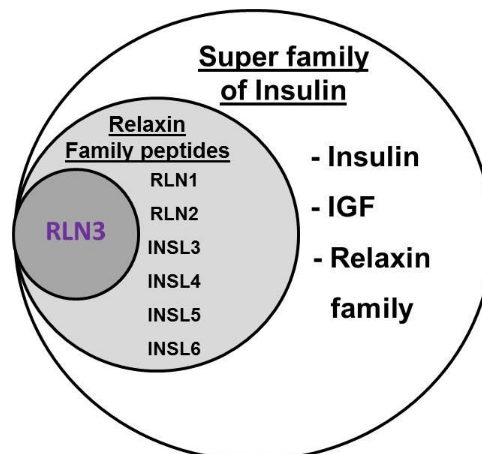

Figure 10. The relaxin-3 (RLN3) peptide (purple) belongs to the relaxin family peptides which is part of the superfamily of insulin

Relaxin-1, RLN1; relaxin-2, RLN2; insulin-like peptides-3, INSL3; insulin-like peptides-4, INSL4; insulin-like peptides-5, (INSL5); and insulin-like peptides-6, INSL6.

All mature peptides of the insulin superfamily contain two amino acid chains (A and B) containing three disulfide bonds on six conserved cysteine residues and are synthesized from preprohormones (Shabanpoor et al., 2009) (Fig. 11), a feature shared by relaxin-3 that is evidence of the shared origin discussed above.

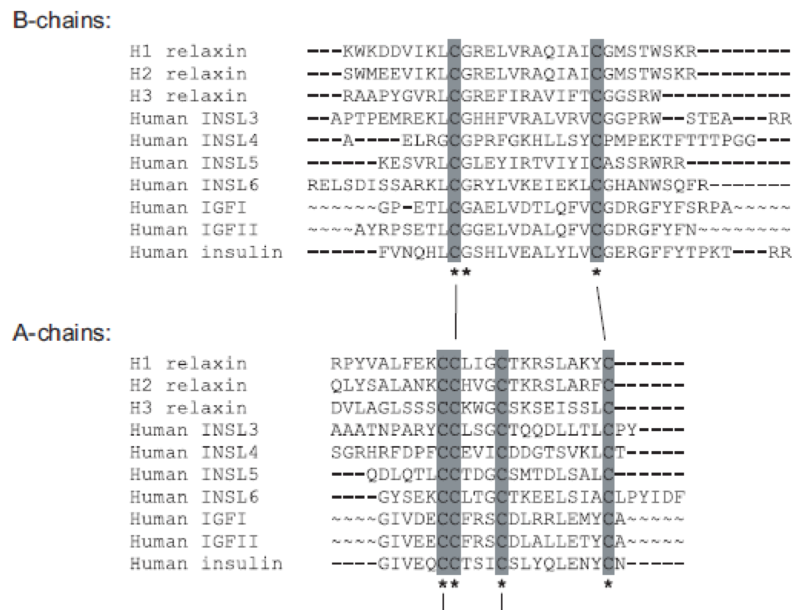

Figure 11. Relationship between relaxin family peptides, insulin, and insulin-like peptides

Relaxin and insulin family peptide sequences are from the UniProt/SwissProt database: human relaxin-1 (P04808), human relaxin-2 (P04090), human relaxin-3 (Q8WXF3), human INSL3 (P51460), human INSL4 (Q14641), human INSL5 (Q9Y5Q6), human INSL6 (Q9Y581), human IGFI (P01343), human IGFII (P01344), and human insulin (P01308). Conserved cysteine residues are black with gray background. Solid black lines indicate disulfide bonds. Identical amino acid residues are indicated by *. The insulin-like growth factors are not cleaved

from the pro-hormone state, thus indicating that the sequence of these peptides is continuous.

Adapted from (Bathgate et al., 2013)

Expression of relaxin-3 in the brain

Relaxin-3 is mainly produced in the brain, in the nucleus incertus (NI) or the "uncertain nucleus" at the pons of the brainstem. In an adult rat, the nucleus extends for ~0.7mm from just caudal to Bregma −9.12mm to Bregma −9.84mm (Fig. 12A) (Bathgate et al., 2002, Ma et al., 2007)**.** The NI presents medium size neurons and is histologically divided in two parts: *pars compacta (NIc)* and *pars dissipata (NId)* (Goto et al., 2001, Ma et al., 2007) (Fig. 12B). It is estimated that ~2000 cells in the NI express RLN3. In addition, several neuropeptides have been described in the NI: neuromedin B (NMB), cholecystokinin (CCK), etc., but RLN3 is the one most highly restricted to the area (Tanaka et al., 2005).

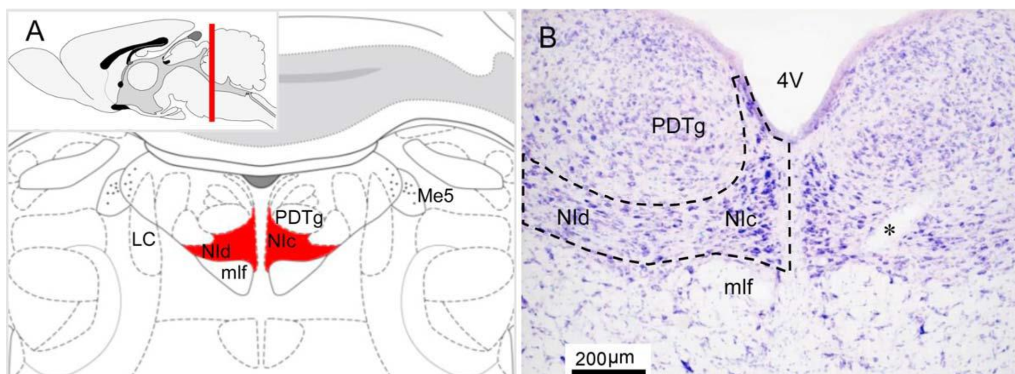

Figure 12. Anatomical location of the nucleus incertus in the rat brain

The nucleus incertus is located in the 'prepontine region of the hindbrain' (inset) in the ventral region of the periventricular gray, medial to the locus coeruleus (LC) and caudal to the dorsal raphe nucleus. (B) Nissl stained section through the mid-rostrocaudal extent of the nucleus incertus, located adjacent to the posterior dorsal tegmental nucleus (PDTg) and dorsal to the medial longitudinal fasciculus (mlf). Approximate borders of the pars compacta (NIc) and the pars dissipata (NId) are

outlined. Abbreviations: Me5, trigeminal nerve nucleus, 4V, fourth ventricle. *Blood vessel or tear in the section.

Adapted from (Ryan et al., 2011)

Relaxin-3 is expressed by neurons in the brain stem but nerve fibres containing relaxin-3 largely innervate the prosencephalon including the limbic system and hypothalamus (Burazin et al., 2002). Neuroanatomic studies in rats and mice revealed that relaxin-3 was strongly expressed in NI neurons, while smaller populations were also present in the pontine raphe (PnR), periaqueductal gray (PAG) and an area dorsal to the substantia nigra (Bathgate et al., 2002, Burazin et al., 2002, Smith et al., 2010).

Figure 13. Localization of relaxin-RXFP1 and relaxin-3-RXFP3 sites in rat brain

The figure shows a sagittal section of rat brain with indications of the areas containing cells staining positive for relaxin mRNA or immunoreactivity or relaxin-3 mRNA or immunoreactivity. The regions containing RXFP1 binding sites determined using radiolabeled relaxin (blue) or RXFP3 binding sites obtained using labeled 125I-R3/I5 (yellow) are outlined together with sites containing both receptors (green). (Acb) nucleus accumbens, (Ahi) amygdalohippocampal area,

(Amb) nucleus ambiguous, (Amy) amygdala, (AON) anterior olfactory nucleus, (AP) anterior pituitary, (APT) anterior pretectal nucleus, (Arc) arcuate nucleus of hypothalamus, (ARP) area postrema, (BST) bed nucleus of stria terminalis, (C) cerebellum, (CC) corpus callosum, (CM) central medial thalamic nucleus, (CPut) caudate putamen, (Cx) cerebral cortex, (DBB) diagonal band of Broca, (DG) dentate gyrus, (DMH) dorsomedial nucleus of hypothalamus, (DR) dorsal raphe nucleus, (DTg) dorsal tegmental nucleus, (DTT) dorsal taenia tecta, (GP) globus pallidus, (Hi) hippocampus, (Hpt) hypothalamus, (IC) inferior colliculus, (IGL) intergeniculate leaflet, (IL) intermediate lobe of pituitary, (IO) inferior olive, (IPN) interpeduncular nucleus, (LH) lateral hypothalamus, (LS) lateral septum, (ME) median eminence, (MPA) medial preoptic area, (ND) nucleus of Darkschewitsch, (NI) nucleus incertus, (NL) neural lobe of pituitary, (NST) nucleus of solitary tract, (OB) olfactory bulb, (Orb) orbital cortex, (PAG) periaqueductal gray, (PBN) parabrachial nucleus, (PF) posterior hypothalamus, (Pir) piriform cortex, (PnR) pontine raphe, (PP) peripeduncular nucleus, (PVA) paraventricular thalamic area, (PVN) paraventricular nucleus of hypothalamus, (Re) reuniens thalamic nucleus, (S) septum, (SC) super colliculus, (SFO) subfornical organ, (Shy) septohypothalamic nucleus, (SN) substantia nigra, (SON) supraoptic nucleus, (Sp5) spinal trigeminal tract, (Sub) subiculum, (SuM) supramammillary nucleus, (Th) thalamus, (VMH) ventromedial nucleus of hypothalamus, (VP) ventral pallidum, (VR) ventral raphe nuclei, (VTT) ventral taenia tecta. Piriform cortex; septohypothalamic nucleus; arcuate nucleus; horizontal/vert diagonal band of Broca; median preoptic region; 3rd ventricle lining (not included in schematic);
Adapted from (Bathgate et al., 2013)

The majority of neurons expressing relaxin-3 produces the inhibitory neurotransmitter GABA and co-express the type 1 CRF receptor. CRF icv injection induces expression of the c-fos neuronal activation marker in relaxin-3 neurons of the NI (Tanaka et al., 2005). The identification of densely aggregated peptide

vesicles in neuronal soma and in synaptic terminations of relaxin-3 neurons strongly suggests that relaxin-3 plays a neurotransmitter role (Tanaka et al., 2005).

Relaxin-3 neurons project largely to the hypothalamus, septum, cortex and limbic regions of the brain (Ma et al., 2007). Immunohistochemistry, *in situ* hybridization and autoradiography have shown that the distribution of RXFP3 mRNA largely covers relaxin-3 binding sites and the distribution of relaxin-3 positive axonal terminations (Sutton et al., 2004, Ma et al., 2007).The density of relaxin-3 fibers, as well as RXFP3 mRNA and binding sites, are elevated in the brainstem and hypothalamic regions such as NTS, NI, PVN, SO, periventricular hypothalamus, LHA, septum, hippocampus, central and medial tonsil and PVT (Smith et al., 2011). This wide distribution of the relaxine-3/RXFP3 system suggests multiple functional implications for relaxine-3. Thus, studies have shown that the relaxin-3 pathway is involved in stress and anxiety (Watanabe et al., 2011b, Ryan et al., 2013a), food intake and metabolism (Hida et al., 2006, McGowan et al., 2006), motivation, reward and wakefulness (Smith et al., 2012, Ryan et al., 2013b, Smith et al., 2014).

Relaxin-family peptide receptors

The peptides of the relaxin family bind to a G-protein coupled receptor (GPCR). So far, four relaxin receptors, relaxin family receptors (RXFP) type 1-4, have been identified (Bathgate et al., 2006). RXFP3 and RXFP4 bind strongly with relaxin-3 and INSL5 (Liu and Lovenberg, 2008, Bathgate et al., 2013a). The RXFP1 and RXFP2 receptors have a broad extracellular domain rich in leucine and are the specific receptors for relaxin (relaxin-2 in primates and relaxin-1 in other mammals) and INSL3 (Bathgate et al., 2013a).

Relaxin-family peptide 3 (RXFP3) is the cognate receptor for relaxin-3. RXFP3 is an inhibitory G-protein coupled receptor, Gα (i/o) and its signaling leads to inhibition of neurons through inhibition of adenylyl cyclase (AC) and cAMP accumulation (Fig.12)**.** In vitro studies showed that RLN3 also couples with low affinity to the relaxin RXFP1 and to the Insulin like peptide 5 (INSL5) receptors,

RXFP4. RXFP4 is a non-functional pseudogene in rats (Chen et al., 2005) that limits RLN3 brain action to RXFP1 and RXFP3. Although RXFP3 and RXFP1 are GPCRs, they present different responses once activated. RXFP1 is excitatory (Gαs) and it promotes cAMP increase and depolarization of subsequent neurons. The relaxin-3 neurons projections in the brain show an overlapping expression of RXFP1 and RXFP3 in several nuclei (Fig. 14), however, it has been shown that these two receptors are distributed differently in the cells and do not co-localize, leading to different action/activation timings (Ma et al., 2006).

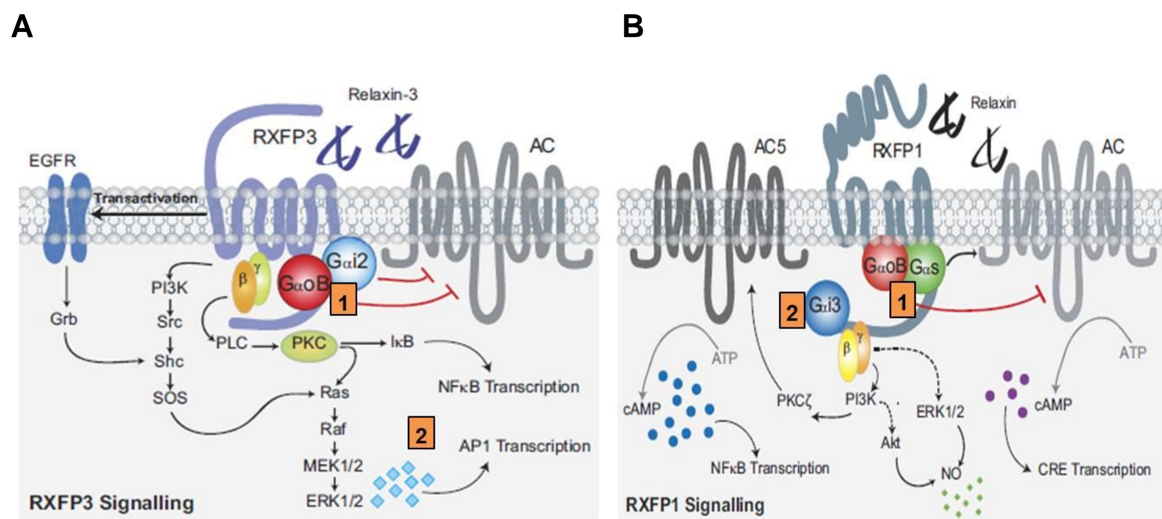

Figure 14. RXFP3 and RXFP1 signaling

(A) **Number 1 indicates:** Recruitment of $G\alpha_{i2}$ and $G\alpha_{OB}$ and inhibition of adenylyl cyclase (AC) and. **Number 2 indicates:** Activation of mitogen-activated protein kinases (MAPK) signaling and nuclear factor - kappa B (NFκB) transcription.

(B) **Number 1 indicates 1.** Recruitment of $G\alpha_{s}$ to activate AC and $G\alpha_{OB}$ to modulate this effect and, **Number 2 indicates:** RXFP1 also couples to $G\alpha_{i3}$ and subunits from this interaction to activate Phosphoinositide 3-kinases (PI3K) to cause translocation of protein kinase C (PKC), which in turn activates AC5 cAMP.

Adapted from (Bathgate et al., 2013a)

Role of relaxin-3/RXFP3 system in stress-induced hyperphagia

The role of this orexigenic neuropeptide has been integrated with other neural systems involved in food intake, such as regions specifically involved in appetite regulation, the stress axis and regions involved in motivation. However, as demonstrated in the introduction, food intake results from the integration of the expression of a myriad of molecules on which RLN3 could have a stimulating or inhibitory effect. For example, the first-order neurons of the Arc and many other hypothalamic regions have not been highlighted in the regulation of food intake in our animal models. There are two reasons for this.

First, the specific effect of RLN3 on food intake may not be mediated by these regions. Indeed, while the injection of RLN3 in certain hypothalamic regions such as the Arc induces an orexigenic response, the immunohistochemistry of the Fos protein has not revealed neuronal activation of these regions (McGowan et al., 2007). In addition, the expression of neuropeptides secreted by first-order neurons in food intake, such as POMC, NPY and AgRP, has not been affected by the chronic expression of an RXFP3 agonist in PVN (Ganella et al., 2013a). These results suggest that RLN3 would act independently of these neural networks.

Secondly, the RLN3/RXFP3 system does not appear to have as major of a role in regulating food intake as the system involving NPY and POMC neurons of the Arc. It has been proposed that its role would instead result in a fine modulation of food intake regulation, in parallel with existing systems (Ganella et al., 2012). Indeed, the orexigenic effect of RLN3 is weaker than that of other orexigenic peptides such as NPY (Morley et al., 1987) and the invalidation of the relaxin-3 gene in most mouse lines does not induce an effect on food intake and body weight gain (Smith et al., 2009, Watanabe et al., 2011b). However, although several systems governing food intake appear to operate in parallel, the fact remains that the brain integrates all this information to generate an appropriate behavioral response. Neurons expressing RLN3 in the NI project towards areas involved in the non-homeostatic (e.g. BNST) and homeostatic feeding (e.g. LHA),

suggesting its role on food intake by finely modulating the activity of each of these regions (Kumar et al., 2017).

Behavioral studies have demonstrated the importance of the role of RLN3 in regulating food intake (McGowan et al., 2005, Ganella et al., 2012). The icv administration of RLN3 increases food consumption for one hour after its administration at the beginning of light or dark phases (McGowan et al., 2005). A similar increase in food intake has been demonstrated after the icv injection of specific RXFP3 agonists (e.g. R3/I5, RXFP3 analogue 2) (Kuei et al., 2007). Blocking the orexigenic effect of RLN3 through RXFP3-specific antagonists [e.g. R3(B_23-27)R/I5 or analog RXFP3 analogue 3] has shown that the increase in food intake induced by RLN3 is mediated by RXFP3 (Kuei et al., 2007, Haugaard-Kedstrom et al., 2011, Shabanpoor et al., 2012). The neuronal population involved in the orexigenic effects of RLN3 would include some hypothalamic regions, as the administration of RLN3 in PVN, Arc, SO, and MPOA strongly stimulates food intake (McGowan et al., 2006, McGowan et al., 2007). However, unlike the effects observed in rats, icv or intra-PVN administration of RXFP3 agonists does not increase food intake in mice that are satiated or only moderately deprived of food, while administration of RXFP3 antagonists decreases food intake (Smith et al., 2014). These inters species variations suggest a possible difference in the baseline level of RLN3 or RXFP3 expression in the regions involved in regulating food intake in the brains of mice and rats.

In addition to hyperphagia, chronic and subchronic icv or intra-PVN injection of human RLN3 or a specific RXFP3 agonist induces an increase in lipid accumulation and body weight gain (Hida et al., 2006, McGowan et al., 2006, Sutton et al., 2009). Bilateral intra-PVN injection of recombinant adeno-associated virus (rAAV), expressing the R3/I5 agonist specific for RXFP3, increases daily food intake and body weight in adult rats (Ganella et al., 2013a). The suppression of RLN3 induced by the infusion of specific rAAV does not alter food intake or body weight of chow-fed male rats (Callander et al., 2012). However, the effect of

silencing RLN3 positive neurons in the NI on body weight and food intake of female rats is still not known.

RLN3 is also involved in the response to stress. It has been shown that RLN3 is strongly stimulated by stress (Tanaka et al., 2005, Watanabe et al., 2011a) and stimulates the HPA axis by increasing the expression of CRF in the PVN and corticosterone concentration in plasma in rats (Lenglos et al., 2014, McGowan et al., 2014). Indeed, the expression of RLN3 can be disrupted by homeostatic challenges, such as stressful conditions. Forced swimming stress increases the expression of the marker of c-fos neuronal activation (Tanaka et al., 2005) and relaxin-3 (Banerjee et al., 2010) in NI neurons (Fig. 15). This effect would be mediated by type 1 CRF receptors because the blocking of these receptors prevents the stimulation of stress-induced RLN3 expression (Banerjee et al., 2010).

Figure 15. The nucleus incertus responds to stress and contributes to stress responses

Stressors (yellow box) directly or indirectly (activating the PVN and increasing CRF released) activate the NI, as indicated by c-Fos induction or enhanced expression of relaxin-3 mRNA (blue box). Micro-infusion of CRF or electrical stimulation of NI suppressed firing of mPFC neurons and LTP in the HP-mPFC pathway. Stimulation of NI is also known to increase theta activity in the dorsal hippocampus (green box). Behavioral or pharmacological manipulations such as food anticipation, exposure to spontaneous alternation tasks (SAT), and pharmacological treatments (FG-7142, PCPA, and antipsychotics) have been shown to induce c-Fos in the NI (purple box). (dH) dorsal hippocampus, (vH) ventral hippocampus, (PCPA) para-chlorophenylalanine.
Adapted from (Kumar et al., 2017)

Pharmacological effects of the central administration of RXFP3 receptor ligands and inhibition of the NI

Preclinical studies have explored relaxin-3 signaling and the function of the NI. The following figure (Fig. 16) is a summary of the effects of lesions or manipulations (electrical or pharmacological) of the NI, effects of central microinfusions of ligands (RLN3, its agonist, or its antagonist) on physiology and behavior, and the impact of RLN3 gene deletion or knock-down.

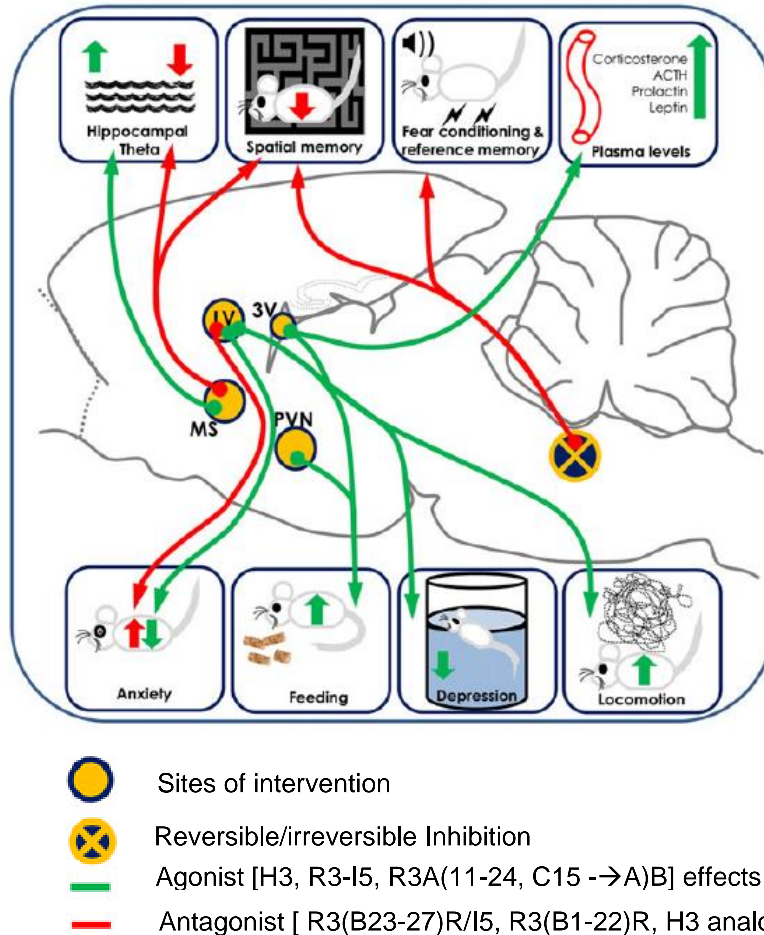

Figure 16. Pharmacological effects of RXFP3 receptor ligands and inhibition of the NI. Agonist effects are shown in green and antagonist effects are shown in red. Infusion of relaxin-3 or RXFP3 receptor agonists in the lateral ventricle (LV) causes increased feeding and locomotion and decreased anxiety and depressive-like behavior. Infusion of relaxin-3 into hypothalamic centers, especially the PVN, increased feeding behavior. Infusion of relaxin-3 into the third ventricle (3V) increased both feeding behavior and plasma levels of corticosterone, ACTH, prolactin and leptin. Infusion of RXFP3 receptor antagonist in the medial septum (MS) impaired spatial memory and decreased hippocampal theta activity, whereas infusion of agonist in the MS increased hippocampal theta activity. Finally, reversible inhibition (by lidocaine) of the NI causes impairment in spatial and reference memory and irreversible inhibition (by CRF-saporin or electrolytic

lesions) of theNI causes derangements in fear conditioning. *Adapted from* (Kumar et al., 2017)

Sex-specific effects of RLN3/RXFP3

In rats, the RLN3/RXFP3 system is regulated differently depending on sex. Acute and chronic icv injections of RLN3 have stronger orexigenic effects in females than in males (Lenglos et al., 2014, Calvez et al., 2016a). The chronic RLN3 treatment blunted the HPG axis in females only, as well as led to a greater increase in body weight gain and in adipose tissue accumulation (Calvez et al., 2016a). At the neuronal level, females showed an increase in the expression of CRF mRNA in BNST. This nucleus contains orexin-positive neurons projecting to the LHA. In males, the same treatment induces an increase in CRF mRNA in the PVNp, promoting an anorexigenic effect. In addition, corticosterone levels were not altered by RLN3 in females, while an increase was found in males (Lenglos et al., 2014).

On a non-pharmacological level, only female rats subjected to chronic stress and dietary restriction experience hyperphagia and increased relaxin-3 expression. Female subjects also displayed an imbalance of the HPA axis and an increase in mRNA expression of RLN3 in the NI (Lenglos et al., 2013), suggesting a likely role for this peptide in stress-induced hyperphagia in female rats.

Objectives and hypothesis

Study 1: Effects of silencing relaxin-3 production in *nucleus incertus* neurons on food intake, body weight, anxiety-like behaviour and limbic brain activity in female rats

Objectives

To examine the effects of miRNA-induced depletion (knock-down) of RLN3 mRNA/(peptide) production in neurons of the brainstem nucleus incertus (NI) in female rats on a range of physiological, behavioral, and neurochemical indices, including food intake, body weight, anxiety, plasma corticosterone levels, mRNA levels of key neuropeptides in the paraventricular nucleus of hypothalamus (PVN), and limbic neural activity patterns (reflected by *c-fos* mRNA)

Hypothesis

The depletion of RLN3-positive neurons of the NI will affect body weight and food intake through the prevention of inhibition of anorexigenic neuropeptides such as ocytocin and vasopressine.

Study 2: Differential effects of relaxin-3 and a selective relaxin-3 receptor agonist on food and water intake and hypothalamic neuronal activity in rats

Objectives

To examine the involvement of RXFP3 and RXFP1 receptors in food and water intake, hypothalamic-pituitary-adrenal (HPA) and gonadal (HPG) axes, and neural activity patterns in the hypothalamus (reflected by *c-fos* mRNA)

Hypothesis

The effect of RLN3 on food intake and the HPA and HPG axes will be mediated by its cognate receptor, RXFP3. RXFP1 will mediate water intake.

Chapter 1. Effects of silencing relaxin-3 production in *nucleus incertus* neurons on food intake, body weight, anxiety-like behaviour and limbic brain activity in female rats*

Camila de Ávila[1-3], Sandrine Chometton[1], Sherie Ma[2]§, Lola Torz Pedersen[3], Elena Timofeeva[1]**†, Carlo Cifani[1,4]** and Andrew L. Gundlach[2]**

1 *CRIUCPQ, Faculty of Medicine, Department of Psychiatry and Neuroscience, Université Laval, Québec (Québec), Canada*

2 *The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria, Australia*

3 *NNF CBMR, Nutrient and Metabolite Sensing, Faculty of Health and Medical Science, University of Copenhagen, Copenhagen, Denmark*

4 *Pharmacology Unit, School of Pharmacy, University of Camerino, Camerino, Sicily, Italy*

* Dedicated to Elena Timofeeva, a fine scientist, colleague, supervisor and mentor

** ET, CC and ALG jointly supervised this research

† Deceased 28/09/2017

§ Current Address: Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia

Corresponding authors

Camila de Ávila (email: camila.avila@criucpq.ulaval.ca)

Andrew L. Gundlach (email: andrew.gundlach@florey.edu.au)

1.1. Résumé

Les troubles alimentaires sont souvent déclenchés par le stress et sont plus fréquents chez les femmes que chez les hommes. Les premiers signes apparaissent souvent au début de l'adolescence, mais le fondement biologique de ce dimorphisme sexuel reste inconnu. L'injection intra-cérébrale du peptide endogène de la relaxine-3 (RLN3) ou de ses analogues chimériques/tronqués a différents effets sur la prise alimentaire et l'activité de l'axe AHP chez les rats adultes mâles et femelles, mais le rôle précis de la signalisation endogène de la RLN3 dans le contrôle métabolique et neuroendocrinien est mal défini.

Par conséquent, nous avons examiné les effets de l'appauvrissement (knock-down) de la production d'ARNm/(peptide) RLN3 dans les neurones du noyau incertus (NI) du tronc cérébral, chez les rats femelles, sur une gamme de testes physiologiques, comportementaux et neurochimiques, dont la prise alimentaire, le poids corporel, l'anxiété, la corticostérone plasmatique, les taux d'ARNm des principaux neuropeptides dans le noyau paraventriculaire de l'hypothalamus (PVN) et les modèles d'activité neurale limbique (reflétés par les ARNm c-fos). Le knock-down des neurones RLN3 dans le NI des rats femelles (n = 8) a entraîné une diminution légère mais soutenue (~2 %) du poids corporel, un déséquilibre dans l'apport alimentaire et une augmentation des comportements anxieux dans le «large open field», mais pas dans le «elevated plus-maze ou dans le «light/dark box». De plus, le knock-down de la RLN3 du NI a perturbé la régulation de la corticostérone, a augmenté l'oxytocine et l'argininine vasopressine, mais pas l'ARNm du facteur de libération de la corticotropine dans le PVN, et a diminué les niveaux basaux d'ARNm c-fos dans le PVN parvocellulaire et magnocellulaire, le noyau du lit de la strie terminalis et l'hypothalamus latéral, régions du cerveau qui sont impliquées dans le stress et dans la prise alimentaire. Ces résultats soutiennent le rôle des neurones RLN3 du NI dans le réglage précis des réponses au stress et des réponses neuroendocriniennes ainsi que dans la régulation de la prise alimentaire chez les rats femelles.

1.2. Abstract

Eating disorders are frequently triggered by stress and are more prevalent in women than men. First signs often appear during early adolescence, but the biological basis for the sex-specific differences is unknown. Central administration of native relaxin-3 (RLN3) peptide or chimeric/truncated analogues produces differential effects on food intake and HPA axis activity in adult male and female rats, but the precise role of endogenous RLN3 signaling in metabolic and neuroendocrine control is unclear.

Therefore, we examined the effects of microRNA-induced depletion (knock-down) of RLN3 mRNA/ (peptide) production in neurons of the brainstem nucleus incertus (NI) in female rats on a range of physiological, behavioural and neurochemical indices, including food intake, body weight, anxiety, plasma corticosterone, mRNA levels of key neuropeptides in the paraventricular nucleus of hypothalamus (PVN), and limbic neural activity patterns (reflected by c-*fos* mRNA). Validated depletion of RLN3 in NI neurons of female rats (n = 8) produced a small, sustained (~2%) decrease in body weight, an imbalance in food intake, and an increase in anxiety-like behaviour in the large open field, but not in the elevated plus-maze or light/dark box. Furthermore, NI RLN3 depletion disrupted corticosterone regulation, increased oxytocin and arginine vasopressin, but not corticotropin-releasing factor, mRNA, in PVN, and decreased basal levels of c-*fos* mRNA in parvocellular and magnocellular PVN, bed nucleus of stria terminalis and the lateral hypothalamic area, brain regions involved in stress and feeding. These findings support a role for NI RLN3 neurons in fine-tuning stress and neuroendocrine responses and food intake regulation in female rats.

1.3. Highlights

- Relaxin-3/RXFP3 signalling is implicated in stress, feeding and metabolism
- *Nucleus incertus* (NI) neurons that broadly innervate limbic forebrain express relaxin-3
- Relaxin-3 silencing in NI of female rats disrupted food intake and plasma corticosterone levels
- Relaxin-3 silencing in NI produced a sustained reduction in body weight gain
- Anxiety-like behaviour was unaltered in paradigms with escape areas
- Oxytocin/vasopressin mRNA in PVN was increased in the hypothalamus

1.4. Introduction

The lifetime prevalence of eating disorders is three times higher in women than men (Hoek and Van Hoeken, 2003, Hudson et al., 2007, Preti et al., 2009), and the first signs appear during early adolescence, but the biological basis for the sex-specific differences involved in this problematic is unknown. However, it is likely that a disruption of neural circuits within the brain are associated with this condition, and stress, which has multiple effects on brain function, is a key factor in triggering eating disorders such as anorexia, bulimia and hyperphagia (Lo et al., 2007).

Stress produces activation of the 'HPA axis' composed of the paraventricular nucleus of the hypothalamus (PVN), and the pituitary and adrenal glands. Corticotropin-releasing factor (CRF) is the key hypothalamic regulator of the HPA axis. Following stress, CRF released from PVN neuronal axons/terminals located in the median eminence activates corticotropic cells of the anterior pituitary gland and stimulates the release of adrenocorticotrophin (ACTH) into the circulation. Following release, ACTH stimulates the release of glucocorticoids from the adrenal cortex, cortisol in humans and corticosterone in rodents (Herman and Cullinan, 1997). The anorexigenic effects of stress include a reduction of physiological activities not related to an immediate stress 'fight or flight' reaction, such as food intake or digestion, and mobilization of all the resources needed to respond appropriately to the 'threatening' situation. CRF is a putative anorexigenic peptide involved in the inhibition of food intake induced by stress (Krahn et al., 1986, Smagin et al., 1999).

Conversely, glucocorticoids stimulate food intake to help to restore energy resources depleted during the stress response (Dallman et al., 2004). The balance of anorexigenic and then orexigenic regulation of food intake in response to acute stress is an important mechanism for survival. Nevertheless, chronic stress or a highly traumatic incident can lead to an imbalance in HPA axis activity that can markedly disrupt feeding behaviour (Timofeeva and Calvez, 2014), and indeed,

cause an eating disorder (Lo et al., 2007, Timofeeva and Calvez, 2014). In particular, stress or negative emotions were documented as the main causes of food compulsive episodes (American Psychiatric Association, 2013, Black and Grant, 2014, Timofeeva and Calvez, 2014) and women are more likely than men to eat due to stress (Laitinen et al., 2002, Zellner et al., 2006).

In our laboratory, we discovered that chronic stress of rats with access to palatable foods, led to increased food intake in female rats with no difference in male rats (Lenglos et al., 2013). Female subjects also displayed an imbalance of the HPA axis, and an increase in the brainstem *nucleus incertus* (NI) of the expression of relaxin-3 (RLN3) (Lenglos et al., 2013), a neuropeptide that is regulated by stress and CRF (Banerjee et al., 2010, Ma et al., 2013) and that activates feeding following central injection (McGowan et al., 2005) suggesting a likely role for this peptide in stress-induced hyperphagia in female rats.

RLN3 belongs to the insulin superfamily, which also includes relaxin-1, relaxin-2 and insulin-like peptides-3, -4, -5 and -6 (Bathgate et al., 2002, Bathgate et al., 2012, Kumar et al., 2017). The RLN3 gene is well-conserved across several species, including fish, frogs, rodents, macaque and humans, suggesting it plays an important biological role (Wilkinson et al., 2005). As mentioned, the neuropeptide is strongly expressed in NI neurons (Burazin et al., 2002, Smith et al., 2010, Ganella et al., 2013b) and NI RLN3 neurons project to many brain areas including the PVN, the lateral hypothalamic area or lateral hypothalamus (LHA) and the bed nucleus of the stria terminalis (BNST) (Sutton et al., 2004, Ma et al., 2007). The cognate G-protein-coupled receptor for RLN3 is the relaxin-family peptide 3 receptor (RXFP3) (Liu et al., 2003, Bathgate et al., 2013), but RLN3 also binds to the relaxin receptor, RXFP1, with lower affinity (Gundlach et al., 2009). RXFP1 and RXFP3 mRNA are expressed in multiple brain regions of rat brain, with a high density of each in the PVN and the supraoptic nucleus (SON), while some regions such as the LHA contain only RXFP3 mRNA (Sutton et al., 2004, Ma et al., 2006, Ma et al., 2007, Ganella et al., 2013).

The hypothalamus and BNST are key components of circuits involved in regulation of food intake, motivation, stress responses and arousal, and RLN3 is implicated in these physiological functions, (Smith et al., 2014, Ma et al., 2018). Indeed, injection of RLN3 into the lateral cerebral ventricle or directly into the PVN stimulates chow consumption in satiated rats, with a stronger effect in female than in male rats (McGowan et al., 2005, Kuei et al., 2007, Lenglos et al., 2014, Calvez et al., 2015, De Ávila et al., 2017). RLN3 has also been demonstrated to influence food anticipation behaviour and consumption of highly palatable food.

Thus, RLN3 expression in the NI was positively correlated with the intake of rewarding substances such as sucrose or alcohol (Ryan et al., 2014), and central injection of RXFP3 antagonists inhibits alcohol intake in rats (Ryan et al., 2013a) and the consumption of palatable food in mice (Smith et al., 2014). RLN3 expression is also strongly stimulated by stress in male rats (Tanaka et al., 2005, Banerjee et al., 2010, Watanabe et al., 2011a) and icv injection of the neuropeptide stimulated the HPA axis by increasing the expression of CRF in the PVN and the concentration of corticosterone in the plasma in male rats (McGowan et al., 2014, De Ávila et al., 2017). In female rats, icv injection of RLN3 increased CRF expression in the BNST and orexin expression in the LHA (Calvez et al., 2015), although the direct or indirect nature of these effects and the relative involvement of RXFP3 and/or RXFP1 is not known.

In summary, eating disorders are frequently triggered by stress and are more prevalent in women than men, while RLN3 has differential effects on food intake and HPA axis activity in male and female rats. Therefore, in studies to better understand the relationship between RLN3, feeding behaviour and stress, we investigated the effect of depletion of RLN3 mRNA and peptide from neurons in the NI of female rats on food intake and body weight, on anxiety-like behaviour, and on plasma levels of corticosterone. In addition, the brain areas involved in the regulation of food intake and the HPA axis were analysed using *in situ* hybridization to detect mRNA levels.

1.5. Materials and methods

1.5.1. Animals

Experiments described were conducted with the approval of The Florey Institute of Neuroscience and Mental Health Animal Ethics Committee and according to ethical guidelines issued by the National Health and Medical Research Council of Australia. All efforts were made to minimize the number of rats used. Female Sprague-Dawley rats (n=21) were obtained from the Animal Resources Centre (Canning Vale, WA, Australia). They were group-housed prior to surgery in a temperature and humidity controlled room (21°C ± 2°C) under a 12 h light/dark cycle initiated at 0700h with *ad libitum* access to standard pelleted food and water.

1.5.2. Surgical procedures

Rats (225-250 g) were anesthetized by inhalation of 4% isoflurane delivered in air (1 litre/min) and the head was positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) using ear and incisor bars. Nose position was 16 degrees down. Anesthesia was maintained with 1-2% isoflurane delivered at 200 ml/min in air using a rat inhalation mask (Kopf Instruments). Rats received bilateral injections of 0.2 μl of either the knock-down virus (rAAV1/2 EmGFP miR499, 1×10^{12} viral particles/ml, n = 12) or control virus (rAAV1/2 EmGFP miRC, 3.8×10^{12} viral particles/ml, n = 9) into the NI (AP: -2.5 mm from lambda, ML: ± 0.1 mm, DV: -6.4 mm, according to a stereotaxic rat brain atlas (Paxinos and Watson, 2006). Injections were made at a rate of 0.02 μl/12 s over 120 s via a 30-G needle connected to a 10 μl Hamilton Syringe mounted on an injection pump (Harvard Apparatus, Holliston, MA, USA). The dose and time course are based on previous evidence (Callander et al., 2012) and viral particles were kindly provided by Prof Ross Bathgate (Florey Institute of Neuroscience and Mental Health, Parkville, Australia). Following the injection, the needle was left in place for 10 min, retracted 1 mm and kept in place for a further 1 min, before complete withdrawal, to maximize viral diffusion within the NI. The incision was sutured and each rat

received an injection of meloxicam (3 mg/kg, s.c.), 0, 24 and 48 h post-surgery. Rats were allowed to recover for 7 days.

1.5.3. Validation of the knock-down virus specificity for RLN3 neurons

Two female Sprague-Dawley rats from the rAAV1/2 EmGFP miR499 treated group were used to confirm the site of injection and the specificity of the RLN3 knock-down. After 7 days recover, these rats were sacrificed and their brains were collected. Coronal sections (40 μm) through the rostrocaudal brain axis were cut on a cryostat at -18°C (Cryocut 1800, Leica Microsystems, Heerbrugg, Switzerland) and collected in phosphate-buffered saline (PBS), pH 7.4. An immunohistochemical (IHC) analysis of RLN3 was conducted to confirm the specificity of the viral transfection. Free-floating sections were incubated in blocking buffer (0.05 M Tris-buffered saline containing 0.3% (v/v) Triton X-100 (TBX), 10% (v/v) normal horse serum (NHS) and 2% bovine serum albumin (BSA)) for 1 h with shaking at RT. Sections were then incubated in monoclonal RLN3 antiserum (Tanaka et al., 2005, Ma et al., 2007a) diluted 1:5 in 0.1 M PBS, pH 7.3, 2% NHS, overnight at room temperature. Sections were then washed 3 × 10 min in 0.1 M PBS, pH 7.3. For immunofluorescence, sections were incubated in the secondary antibody, donkey anti-mouse conjugated to AlexaFluor-488 (1:500) + PBS for 1 h, followed by 3 × 5 min washes in PBS. Sections were mounted onto glass microscope slides and coverslipped using Fluoromount-G (Southern Biotech, Birmingham, AL, USA).

1.5.4. Body weight and food intake measurements

Body weight (g) of rats in the knock-down (n = 10) and control (n = 9) groups was measured daily during the light phase, between 0900-1100 h. Three weeks after surgery, food consumption was measured every 24 h in the home cage from week 4 to 6. Pre-weighed chow in powder form (Teklad Global 18% Protein Rodent Diet, Envigo++++, Specialty Feeds, Western Australia, Australia) was provided daily following regular body weight measurements. Any food remaining after 24 h (including any uneaten food in the cage) was removed and weighed.

1.5.5. Behavioural tests

Behavioural tests were conducted in week 7 during the light phase, with a 24 h interval between each test and in the following order: Light/Dark box (L/D), Elevated Plus Maze (EPM) and Large Open Field (LOF). Rats were habituated to the experimental behavioural room 1-3 h prior to the L/D and EPM tests, but due to the high light level within the entire room for the LOF test, rats were not habituated prior to the test.

Light/dark box (L/D)

The L/D box test aims to detect anxiety behaviour in rodents reflected by avoidance of bright areas and a decrease in exploration (Bourin and Hascoët, 2003). A 41 cm × 41 cm × 41 cm clear-walled locomotor cell (Tru-Scan Photobeam Arena, E63-20; Coulbourn Instruments, Holliston, MA, USA) was divided in two compartments; one defined as 'dark', the other as 'light', created by placing an opaque box (20.5 cm × 41 cm × 41 cm), impermeable to visible light, but not to locomotor cell photobeams in one side of the cell (Light/Dark Box, E63-26, Coulbourn Instruments). The 'light side' was lit by an array of diodes (500 lux in the centre, 450 lux in the corners), creating an aversive arena/stimulus. A small hole (765 mm) was present in the bottom centre of the dark box to allow rats free passage between the light and dark sides. Each rat was placed in the light side and monitored for 10 min. The floor and walls of the arena were cleaned with 70% ethanol and water between each rat. Parameters measured included time spent in and distance travelled in the light and dark compartments.

Elevated plus maze (EPM)

The EPM test aims to detect anxiety behaviour in rodents reflected by avoidance of open area exploration (Rodgers and Dalvi, 1997). The EPM consisted of four arms (44 cm long × 12 cm wide) projecting from a central square (12 cm × 12 cm), with a height of 72 cm from the ground. Two opposing arms have high 10 cm walls and are designated 'closed' arms. A small 0.6 cm ledge was present on either side of the 'open' arms to prevent rats falling off as they turn around. The

apparatus was placed in the middle of the experimental room under low lux (~50 lux closed arms, ~70 lux open arms). Rats were placed in the central square, facing an open arm, and were allowed to explore the apparatus for 10 min, while being recorded by EthoVision® software (EthoVision®, Version 3.0.15, Noldus Information Technology, Wageningen, The Netherlands). All four limbs had to be with an arm to be recorded as an entry. The apparatus was cleaned and dried with 70% ethanol, followed by water, between rats. Parameters measured included time spent in and distance travelled in the open and closed arms.

Large open field (LOF)

The LOF test aims to detect anxiety behaviour in rodents reflected by avoidance of bright areas and decreased exploration and locomotion. The apparatus consisted of a 1.2 m diameter circular arena on the floor of an experimental room, surrounded by a 61 cm high aluminium wall. The arena was divided into three equal sized zones (centre circle, and middle and outer bands). The arena was lit by a flood light mounted on the ceiling above the centre of the arena, and provided light of 3000 lux in the centre, and 2000 lux at the periphery.

Rats were tracked using EthoVision®, Version 3.0.15 (Noldus Information Technology), which provided measures of time spent in the centre, middle and outer zones, in addition to locomotor activity. Rats were placed in the centre of the arena at the start of the trial, and recorded for 5 min. In between tests, the floor and walls of the arena were cleaned with 70% ethanol, and the flood light was turned off to avoid overheating of the apparatus.

1.5.6. Saphenous vein blood samples

Blood samples were collected after each test, via the lateral saphenous vein. The back of the hind leg was shaved with an electric trimmer three days before sampling, to minimize stress on the behavioural test day. Hair-removal cream was applied around the saphenous vein. After each test, rats were gently restrained by the experimenter, the leg was immobilized and slight pressure was gently applied above the knee joint. The vein was punctured using a 20G needle

and an adequate volume of blood was collected with a capillary tube. The puncture site was compressed to stop the bleeding and capillary tubes were stored on ice before centrifugation. After blood centrifugation plasma was collected and stored at -80°C.

1.5.7. Blood and brain preparation

At the end of the experiments (week 8), during the early light phase, rats were deeply anesthetized in a separate room with sodium pentobarbital (0.5 ml of 200 mg/ml Lethabarb, Virbac Pty Ltd, Milperra, NSW, Australia), intra-cardiac blood was collected, and rats were perfused intra-cardially with 100 ml of saline followed by 200 ml of a 4% formaldehyde solution. Brains were removed and post-fixed in 4% formaldehyde for one week. Brains were then transferred to a solution containing 4% formaldehyde and 20% sucrose, prior to sectioning using a sliding microtome (Histoslide 2000, Heidelberg, Germany). Coronal sections (30 μm) were collected and stored at -20°C in a cold sterile cryoprotection solution containing 50 mM sodium phosphate buffer, 30% ethylene glycol and 20% glycerol. Coronal sections were used for determining mRNA levels of c-*fos*, RLN3, CRF, OT and AVP.

1.5.8. Determination of plasma corticosterone concentrations

Blood from saphenous or intra-cardiac samples was centrifuged (3000 g for 15 min at 4°C) and plasma was stored at -80°C until the determination of corticosterone levels in duplicate, using a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI, USA; sensitivity, 5.0 pg/ml).

1.5.9. *In situ* hybridization (ISH)

Levels of RLN3 mRNA in the NI; *c-fos* mRNA in the paraventricular hypothalamic nucleus (PVN), the bed nucleus of the stria terminalis (BNST) and the lateral hypothalamus (LHA); and oxytocin (OT), vasopressin (AVP) and CRF mRNA in the PVN, were measured using *in situ* hybridization, as described (Martin and Timofeeva, 2010, Mitra et al., 2011). In short, sections (30 μm) from brains prepared as described (section 2.7) were mounted on poly-L-lysine-coated slides

and fixed for 20 min in fresh 4% formaldehyde, digested for 30 min at 37°C with proteinase K (10 µg/ml in 100 mM Tris-HCl containing 50 mM EDTA, pH 8.0), acetylated with acetic anhydride (0.25% in 0.1 M trietholamine, pH 8.0) and dehydrated through graded concentrations of ethanol. Sections were incubated overnight at 60°C with antisense [^{35}S]-labelled cRNA probes for relaxin-3 mRNA , for *c-fos* mRNA [generated from a 2116-bp fragment of rat c-*fos* cDNA; GenBank accession number X06769.1; Dr I Verma, Salk Institute, La Jolla, CA, USA], for OT mRNA [generated from a 177-bp fragment of rat OT cDNA; Gen-Bank X12792; courtesy of Dr D Richard, Université Laval, Quebec, Canada], for AVP mRNA [generated from a 230-bp fragment of rat AVP cDNA; GenBank NM016992.1; courtesy of Dr D Richter, Clinical Neurobiological University, Hamburg, Germany), or for CRF mRNA [generated from a 1063-bp fragment of rat CRF cDNA; GenBank accession number NM_031019; Dr K Mayo, Northwestern University, Evanston, IL, USA). Slides were rinsed with sodium chloride-sodium citrate solution, digested with RNase-A (20 µg/ml), washed in descending concentrations of sodium chloride-sodium citrate solutions and dehydrated in an ethanol gradient. Finally, slides were defatted in toluene, dipped in NTB2 nuclear emulsion (Eastman Kodak, Rochester, NY, USA) and exposed for 3 days (OT and AVP), 1 week (RLN3 and CRF) or 11 days (*c-fos*), before being developed. Tissues were counterstained with thionine, dehydrated through graded ethanols, cleared in toluene and coverslipped with mounting medium.

1.5.10. Image analyses

Viral transduction of neurons was assessed on an Olympus IX fluorescence microscope (Olympus, Mt Waverley, VIC, Australia). Slides subjected to ISH were examined with dark-field microscopy using an Olympus BX61 microscope (Olympus Canada, Richmond Hill, ON, Canada). Images were acquired with a DVC-2000C digital camera (DVC Company Inc., Austin, TX, USA) and analyzed with Stereo Investigator software version 9.13 (MBF Bioscience, Williston, VT, USA). The system was calibrated for each set of analyses to prevent saturation of the integrated signal. Mean optical density (OD) was obtained from measurements

of pixels of positive hybridization signal on 2-4 sections through the *pars compacta* and *pars dissipata* of the NI (NIc and NId), respectively; from 9.6 mm to 9.8 mm caudal to bregma), two or three consecutive brain sections (four to six values per rat) of the parvocellular and magnocellular PVN (PVNp and PVNm, respectively; from 1.72 mm to 1.80 mm caudal to bregma), six consecutive brain sections (twelve values per rat) of the lateral and perifornical LHA (from 2.9 mm to 3.4 mm caudal to bregma) and 3-4 consecutive brain sections (six to eight values per rat) of the ventral and dorsal parts of the BNST (BNSTv and BNSTd, respectively; from 0.3 mm rostral to bregma to 0.3 mm caudal to bregma). Background optical density readings taken from the areas immediately surrounding the region analyzed were subtracted.

Data in the Results are expressed in OD, except for *c-fos* expression, where the OD values were somewhat lower. In this case, the individual score of each rat was normalized to the mean value of the control-injected group (i.e., mean value of a rat/mean value of the control group) to obtain the relative mRNA expression level.

1.5.11. Statistical analysis

Results are presented as mean ± SEM. An unpaired Student's t-test was used to assess effects of the treatment on central (immediate-early gene and neuropeptide mRNA levels) and peripheral (corticosterone) indices, as well as in behavioural tests. Two-way ANOVA was used to detect any main and interactive effects of the treatment (knock-down *vs* control) and time (weeks) on body weight and food intake. *Post-hoc* comparisons between the groups were performed using the Bonferroni test, when the main or interactive ANOVA effects were significant. Statistical analyses were performed using PRISM statistical software, V6.04 (GraphPad Software Inc., La Jolla, CA, USA). Results were considered significant with * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

1.6. Results

The current study investigated the effects of the miRNA-induced depletion (knock-down) of RLN3 mRNA/peptide production in neurons of the brainstem NI in female rats on a range of physiological, behavioural and neurochemical indices, including food intake, body weight, anxiety-like behaviour, corticosterone levels, limbic neural activity patterns (reflected by *c-fos* mRNA), and levels of hypothalamic CRF, OT and AVP mRNA in the PVN. In brief, lower levels of *c-fos* mRNA were observed in the PVN, BNST and LHA of knock-down than control rats. Conversely, levels of OT and AVP mRNA were increased in the hypothalamus of NI RLN3 knock-down rats, with no differences in CRF mRNA in the two groups.

Anxiety-like behaviour was not altered in RLN3 knock-out rats in the L/D box and EPM paradigms, whereas knock-out rats displayed a decrease in time spent and distance travelled within the centre area of the LOF. Corticosterone levels were similar in knock-down and control rats immediately after the different behavioural tasks, but were lower in the RLN3 knock-down group when normalized to basal levels. Further details of these findings are provided below.

1.6.1. Confirmation of the specific knock-down of RLN3 in NI neurons

In studies designed to determine whether rAAV1/2 EmGFP miR499 could successfully transduce RLN3-containing neurons in the NI, as described (Callander et al., 2012) an initial assessment was conducted using two female rats, prior to any subsequent functional analysis. One week after stereotaxic injection of rAAV1/2 EmGFP miR499, expression of RLN3 (Fig. 1.1A) and EmGFP (Fig. 1.1A′) was observed in the NI. The co-localization of RLN3-LI and EmGFP (Fig. 1.1A″) demonstrated that the rAAV1/2 vector and the protocol used was capable of effectively transducing RLN3-expressing neurons in the NIc *and* NId.

At the end of the behavioural experiments, brains from control rats (n = 9) and knock-down rats (n = 10) were processed to detect RLN3 mRNA levels in the NI. The injection sites in two ‘knock-down’ rats were *outside* the NI, so these rats were not included in the data analysis. RLN3 mRNA levels were significantly suppressed

in knock-down rats (n = 8) compared to control rats (n = 9; Fig. 1.1B), with significant differences in NIc ($t_{(5)} = 4.711$, $p = 0.0053$) and NId ($t_{(5)} = 4.709$, $p = 0.0053$) of rostral NI, as well as in medial NIc ($t_{(8)} = 7.221$, $p < 0.0001$) and NId ($t_{(8)} = 9.924$, $p < 0.0001$) and caudal NIc ($t_{(9)} = 7.773$, $p < 0.0001$) and NId ($t_{(8)} = 6.737$, $p = 0.0001$).

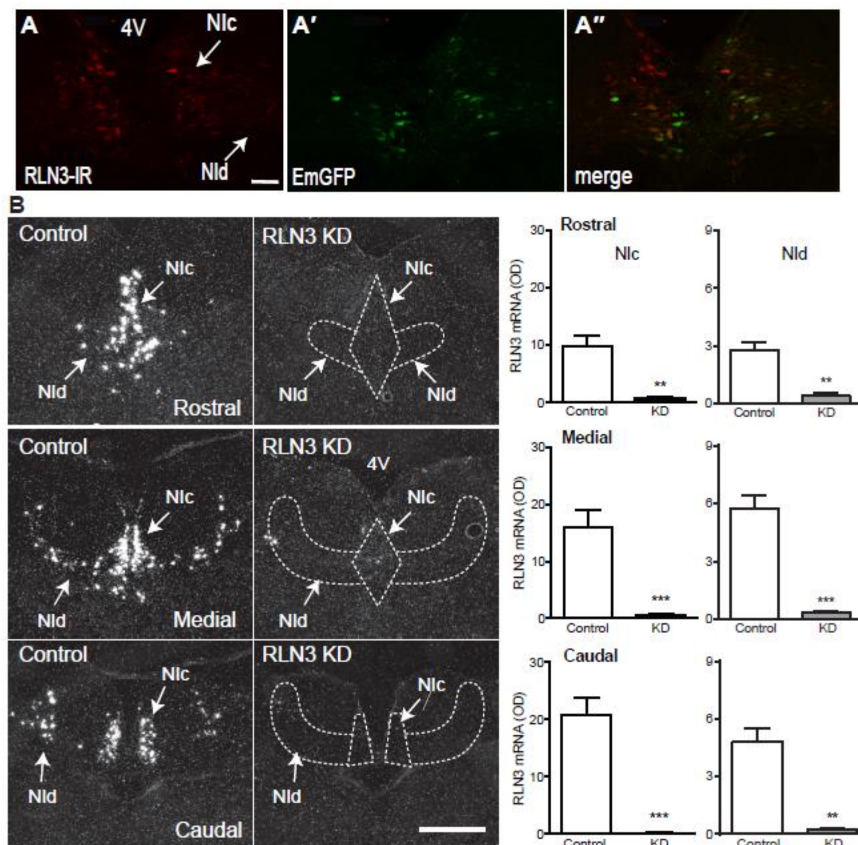

Figure 1.1. Validation of the selectivity and effectiveness of the viral RLN3 knock-down protocol

(A) One week after injection of rAAV1/2 499 EmGFP into the *nucleus incertus* (NI) of female rats (n = 2), brains were processed for detection of EmGFP fluorescence and RLN3 immunoreactivity (IR) within the NI. (A′) The viral injection site and effective viral expression was localized within the NI by the presence of EmGFP expression (green). (A″) EmGFP fluorescence (green) was co-localized with RLN3-IR (red), demonstrating that rAAV1/2 499 EmGFP transduced the target RLN3-containing neurons in both the NI *pars compacta* (NIc) and *pars dissipata* (NId). (B) At the completion of the behavioural experiments, brains from the two groups of

rats were processed for the detection of RLN3 mRNA using radioactive *in situ* hybridization (Martin and Timofeeva, 2010, Mitra et al., 2011). Levels of RLN3 mRNA were significantly decreased in *pars compacta* and *pars dissipata* of the rostral, medial and caudal NI of the knock-down (KD, n = 8) rats, relative to control (n = 9) rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Dark-field photomicrographs of coronal brain sections (left) illustrate the positive hybridization signal for RLN3 mRNA in the rostral, medial and caudal NIc and NId of control and KD rats. Scale bar A, 50 μm; B, 500 μm. 4V, fourth ventricle.

1.6.2 Effect of knock-down of RLN3 in NI neurons on body weight and food intake

At the start of the experiment (one day before surgery), the mean body weights of the eventual control and knock-down groups were 239.8 ± 4.6 g and 240.6 ± 3.7 g, respectively. Following surgery and recovery, rats in both groups increased their body weight over the following weeks, but RLN3 knock-down rats weighed significantly less than control rats (Fig. 1.2A). Two-way ANOVA revealed a significant difference in body weight over time ($F_{(7,24)} = 108.1$; $p < 0.0001$), treatment ($F_{(7,24)} = 280.2$; $p<0.0001$) and interaction ($F_{(7,24)} = 9.867$; $p < 0.0001$). Bonferroni's multiple comparison tests revealed a modest, but sustained ~2.2% lower mean body weight of RLN3 knock-down rats at week 2 ($p = 0.0014$) which persisted until week 7 ($p < 0.0001$; Fig. 1.2A). As it was assumed that for a substantial period after the surgery, the viral treatment would not have a major impact on RLN3 expression, food intake was measured *only* from week 4 to 6.

During week 7, rats were subjected to behavioural tests and food intake was not monitored due to a possible (likely) disturbance of intake associated with the testing. Two-way ANOVA revealed a significant decrease in food intake (g/100 body weight) in the knock-down group compared to the control group during week 4 (interaction $p = 0.04$, $F_{(2,16)} = 3.961$, Bonferroni's multiple comparison, $p = 0.0219$), with no difference in food intake during week 5 and 6 (**Fig. 1.2B**). Notably,

as a further ‘control’, the two rats that had misplaced injection sites displayed a similar body weight and food intake profile to the control group rats.

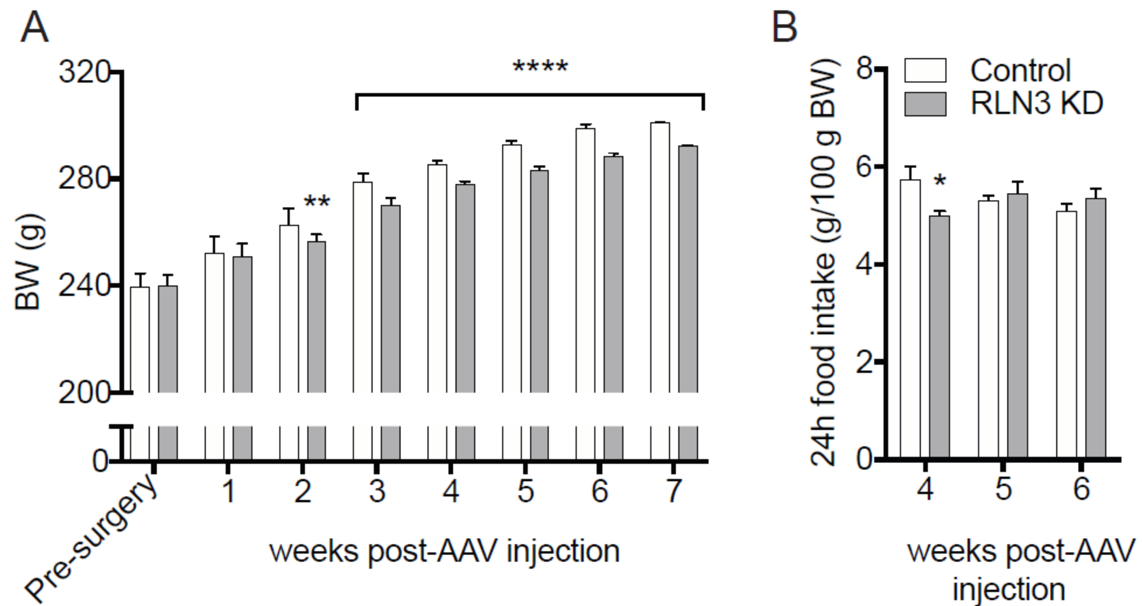

Figure 1.2. Viral depletion of RLN3 from NI neurons produced a decrease in body weight and an imbalance in food intake in female rats

(A) Body weight (BW, g) was consistently lower in knock-down (KD, n = 8) compared to control (n = 9) rats from two weeks after the surgery until the end of the experiment in week 8 post-treatment. No difference in mean BW was observed between groups before the surgery or during week 1, ** $p < 0.01$, **** $p < 0.0001$. (B) An imbalance in chow intake relative to BW was observed between KD (n = 8) and control (n = 9) rats at week 4 (* $p < 0.05$), followed by a normalization of relative food intake thereafter.

1.6.3. Effect of knock-down of RLN3 in NI neurons on anxiety-like behaviour

Large open field (LOF)

The time spent (s) and distance travelled (m) in the outer, middle and centre zones of the LOF were analyzed (Fig. 1.3), which revealed that RLN3 knock-down rats spent more time (Fig. 1.3A) and travelled further (Fig. 1.3B) in the outer zone than the control rats ($t_{(15)} = 2.759$, $p = 0.0146$ and $t_{(15)} = 2.563$, $p = 0.0216$, n = 8-9 rats per group), but similar time and distance in the middle zone ($t_{(15)} = 0.1665$, $p = 0.8700$ for time and $t_{(15)} = 0.2181$, $p = 0.8303$ for distance, n = 8-9 rats per group; Fig. 1.3C and D). Conversely, the time and distance travelled in the center zone was lower in knock-down than control rats ($t_{(15)} =3.478$, $p < 0.05$ and $t_{(15)} =2.488$, $p = 0.251$ respectively, n = 8-9 rats per group; (Fig. 1.3E and F). These data are consistent with an increase in anxiety-like behaviour in RLN3 knock-down compared to control rats.

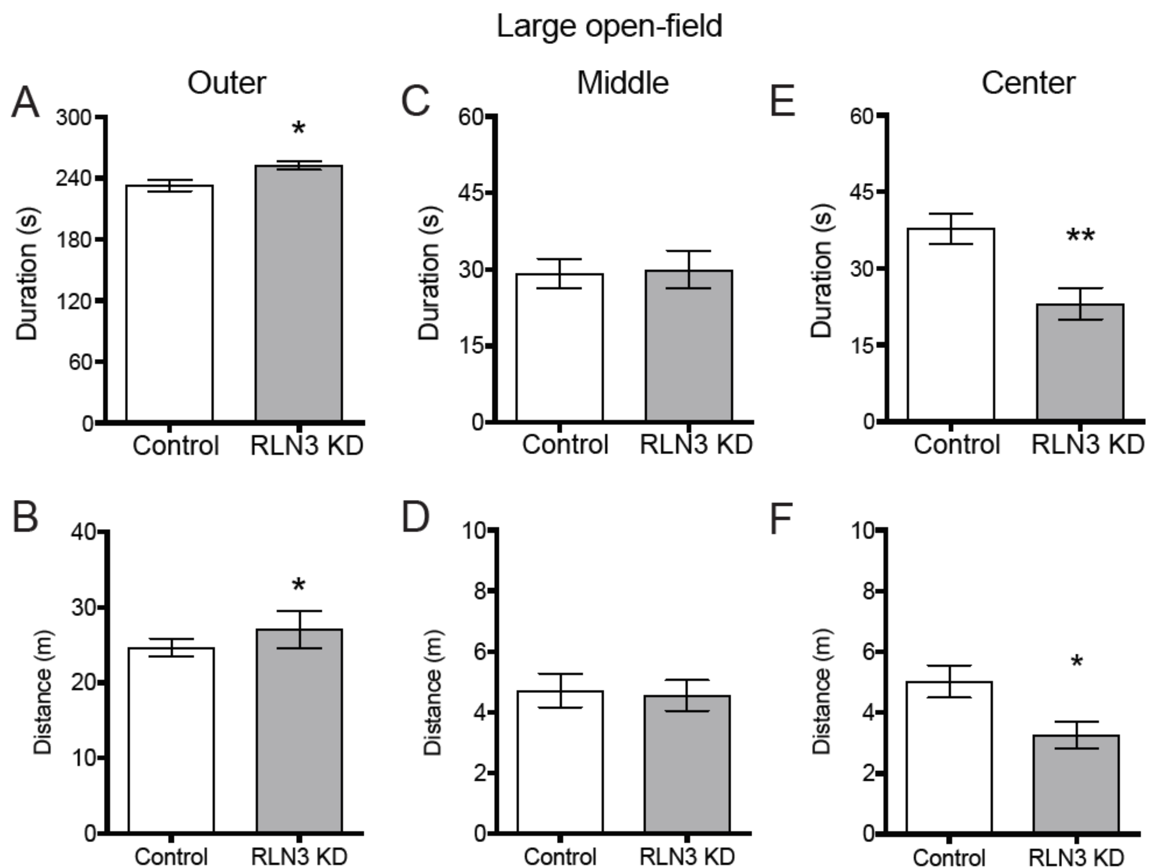

Figure 1.3. Effect of knock-down of RLN3 mRNA/(peptide) in NI neurons of female rats on anxiety-like behaviour in the LOF test

(A, B) An increase in duration (A, s) and distance travelled (B, m) by knock-down (KD) compared to control rats in the outer zone of the LOF (* $p < 0.05$). (C, D) No difference in duration (C, s) or distance travelled (D, m) by KD and control rats in the middle zone of the LOF. (E, F) A decrease in duration (E, s) and distance travelled (F, m) by KD compared to control rats in the centre zone of the LOF (* $p < 0.05$, ** $p < 0.01$, KD, $n = 8$ and control, $n = 9$).

Light/dark box (L/D)

Analysis of the time spent (s) and distance travelled (m) in the light and dark compartments of the L/D box, by the RLN3 knock-down and control rats (**Fig. 4**), revealed no differences between the groups in time (**Fig. 4A and E**, $t_{(13)} = 0.4610$, $p = 0.6524$ and $t_{(14)} = 0.3668$, $p = 0.6588$ respectively, n = 8-9 rats per group) or distance travelled (**Fig. 4B and F**, $t_{(13)} = 0.3280$, $p = 0.7481$ and $t_{(13)} = 0.2672$, $p = 0.7939$ respectively, n = 8-9 rats per group) in both the light and dark compartments. Also, the number of entries and vertical counts in the light zone did not differ between groups (**Fig. 4C and D**, $t_{(15)} = 1.694$, $p = 0.1109$ and $t_{(14)} = 0.2658$, $p = 0.7943$ respectively, n = 8-9 rats per group). Therefore, in contrast to the LOF test, these data indicate a similar level of anxiety-like behaviour in RLN3 knock-down and control rats.

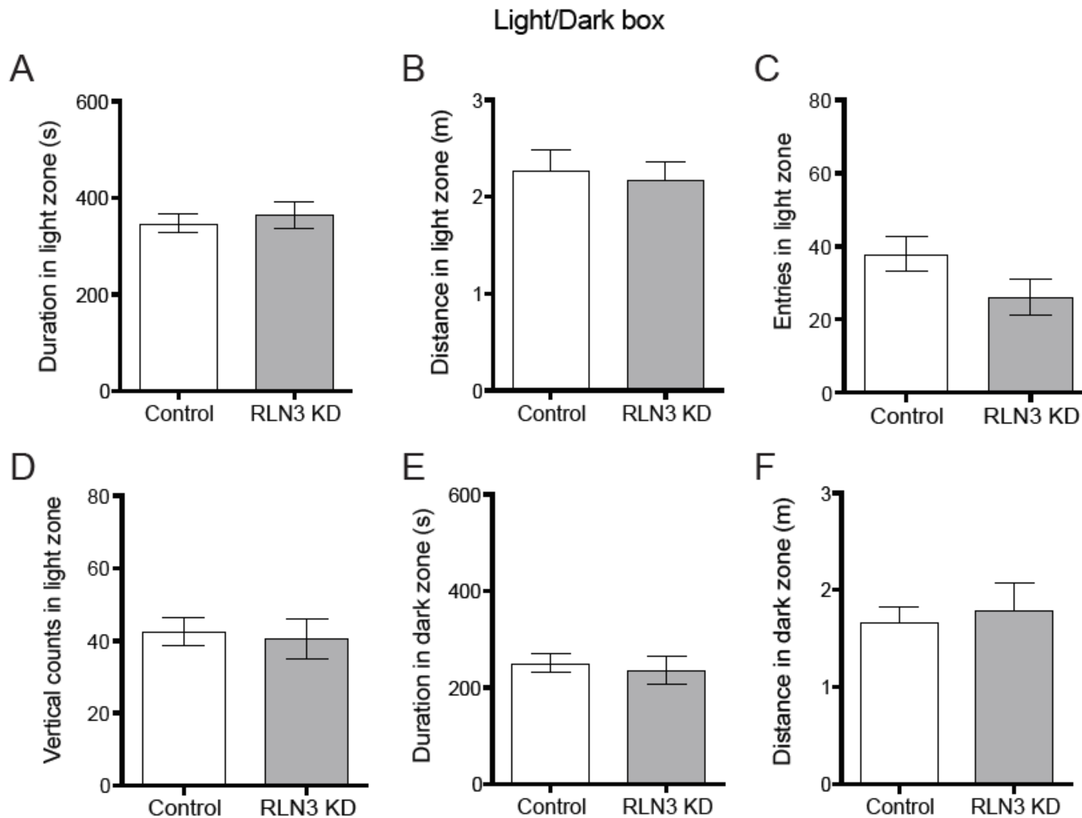

Figure 1.4. Effect of knock-down of RLN3 in NI neurons of female rats on anxiety-like behaviour in the L/D test

(A-E) No difference in duration (s) of knock-down (KD) group compared to control was observed in the light (A) and dark (E) compartments. (B-F) No difference in distance travelled (m) of KD compared to control group was observed in the light (B) and dark (F) compartments. (C-D) No difference in the number of entries (C) and in the vertical counts in the light zone (D). KD (n = 8) and control (n = 9).

Elevated plus maze (EPM)

Analysis of the time spent (s) and the distance travelled (m) in the open and closed arms and entries in the open arms of the EPM by the RLN3 knock-down and control rats revealed no difference between groups in time ($t_{(15)} = 0.6274$, $p = 0.5398$ for closed arms and $t_{(15)} = 0.1049$, $p = 0.9179$ for open arms, n = 8-9 rats per group), distance travelled ($t_{(15)} = 0.8671$, $p = 0.3996$ for closed arms and $t_{(15)} =$

0.05566, $p = 0.9536$ for open arms; n = 8-9 rats per group) or entries ($t_{(15)} = 0.1763$, $p = 0.8624$ for open arms; data not shown, n = 8-9 rats per group). These data further suggest a lack of markedly increased anxiety in the RLN3 knock-down rats.

1.6.4. Effect of knock-down of RLN3 in NI neurons on plasma corticosterone levels

This study was designed to assess whether RLN3 silencing in the NI altered the activity of the HPA axis at the level of the adrenal glands. Corticosterone levels in plasma samples collected from knock-down and control group rats at the completion of the behavioural tests were analyzed (**Fig. 1.5**). No difference in corticosterone levels was observed between groups following the LOF test ($t_{(10)} = 0.3741$, $p = 0.7162$, n = 5-7 rats per group), L/D test ($t_{(7)} = 0.4521$, $p = 0.6648$, n = 4-5 rats per group) or EPM (data not shown, $t_{(7)} = 0.3895$, $p = 0.7085$, n = 4-5 rats per group). However, plasma corticosterone levels were significantly decreased in the knock-down compared to the control group, when measured at the time the rats were killed with no prior behavioural stimulus (referred as basal state/levels) ($t_{(11)} = 3.871$, $p = 0.0026$, n = 6-7 rats per group).

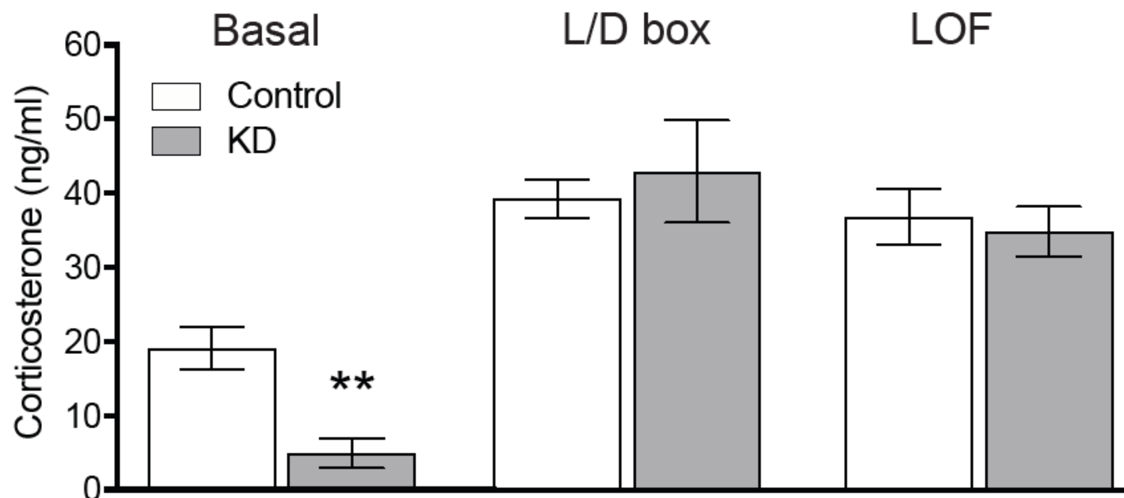

Figure 1.5. Effect of knock-down of RLN3 in NI neurons of female rats on plasma corticosterone levels under basal conditions and after anxiety-like behaviour tests
A difference in basal corticosterone levels (ng per ml) was observed between knock-down (KD, $n = 6$) and control rats ($n = 7$, ** $p < 0.01$), whereas no difference in corticosterone levels (ng per ml) was observed between knock-down and control rats after the L/D test ($n = 5$ and $n = 4$, respectively) and the LOF test.

1.6.5. Effect of knock-down of RLN3 in NI neurons on forebrain *c-fos* mRNA levels

In studies to assess the possible effect of depletion of RLN3 in NI neurons on forebrain activity, basal levels of *c-fos* mRNA were analyzed in knock-down and control rats in forebrain regions that receive projections from RLN3 neurons and are involved in stress and/or food intake regulation (Fig. 1.6A). Basal *c-fos* mRNA expression was decreased in the parvocellular (p) and magnocellular (m) PVN ($t_{(15)} = 2.873$, $p = 0.0116$, and $t_{(15)} = 2.722$, $p = 0.0157$, respectively, n = 8-9 rats per group), in the dorsal (d) and ventral (v) parts of the bed nucleus of the stria terminalis (BNST) ($t_{(15)} = 3.972$, $p = 0.0012$ and $t_{(15)} = 5.413$, $p < 0.0001$, respectively, n = 8-9 rats per group), and in the perifornical (pf) and lateral (l) zones of the lateral hypothalamus area (LHA) ($t_{(14)} = 2.699$, $p = 0.0173$, $n = 8$ rats per group and $t_{(15)} = 2.946$, $p = 0.01$, respectively, n = 8-9 rats per group) in knock-down compared to control rats (Fig. 1.6B).

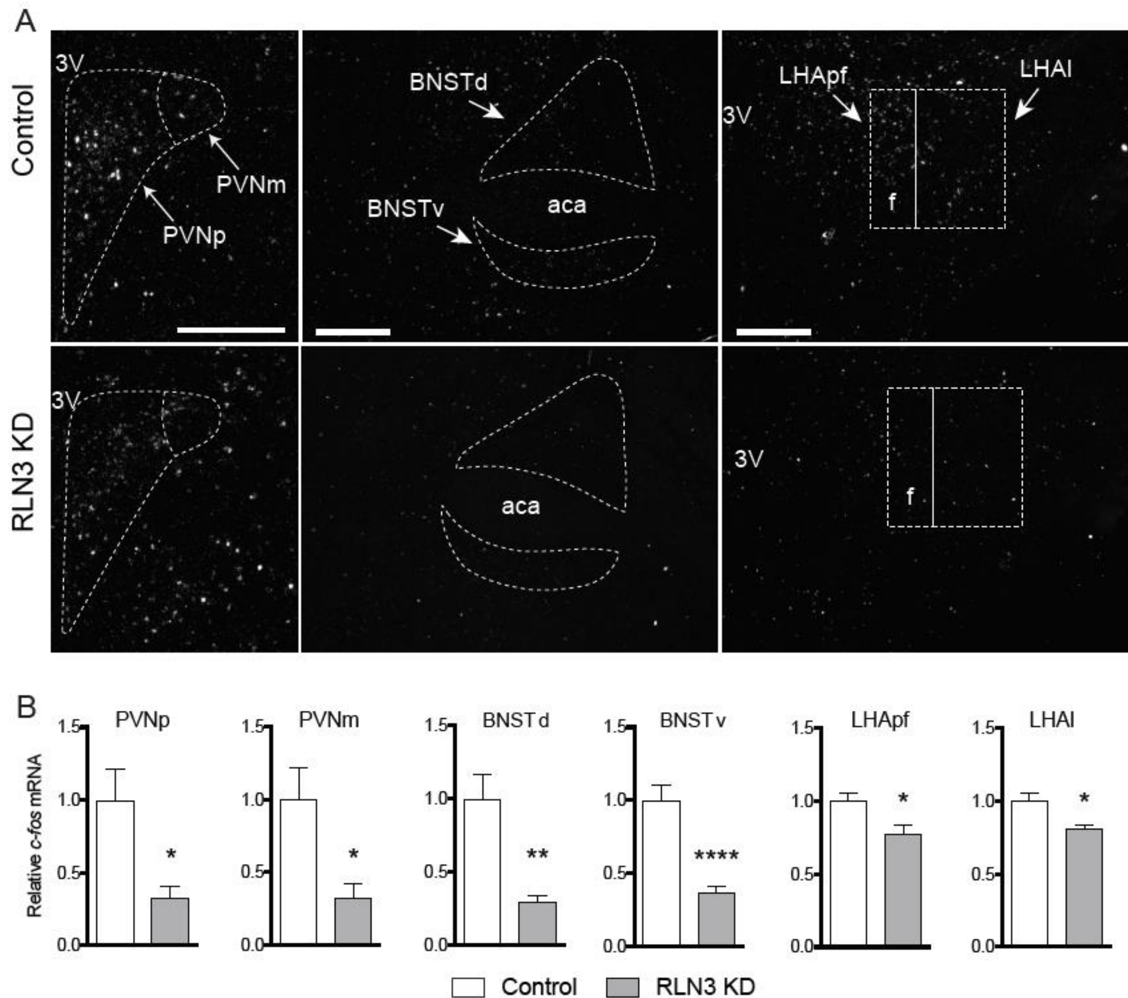

Figure 1.6. Knock-down of RLN3 in NI neurons in female rats produced a decrease in relative levels of *c-fos* mRNA expression in limbic brain regions

(A) Dark-field photomicrographs of representative coronal brain sections from control and RLN3 knock-down (KD) rats illustrate *c-fos* mRNA hybridization in the magnocellular (m) and parvocellular (p) paraventricular nucleus of the hypothalamus (PVN), in the dorsal (d) and ventral (v) bed nucleus of the stria terminalis (BNST) and in the perifornical (pf) and lateral (l) lateral hypothalamus (LHA). 3V, third ventricle; aca, anterior part of anterior commissure; f, fornix. Scale bars, 500 µm. (B) Relative *c-fos* mRNA expression was significantly decreased in the PVNp and PVNm, BNSTd and BNSTv, and LHApf and LHAl in knock-down (KD, n = 8) rats compared to control (n = 9) rats. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

1.6.6. Effect of knock-down of RLN3 in NI neurons on CRF, OT and AVP mRNA levels in the PVN

In view of previous studies that revealed a marked decrease in OT and AVP mRNA levels in hypothalamus following the local, chronic activation of RXFP3 (Ganella et al., 2013a), and to further explore the possible (direct/indirect) impact of RLN3/RXFP3 signalling on CRF neurons (Sutton et al., 2009, Tanaka, 2010), we assessed the effect of depletion of RLN3 in NI neurons on OT, AVP and CRF mRNA levels in the PVN of knock-down and control rats, using radioactive *in situ* hybridization detection of these transcripts as described (Martin and Timofeeva, 2010, Mitra et al., 2011). These experiments revealed that CRF mRNA levels in the PVNp* of knock-down and control rats were not different (Fig. 1.7A; $t_{(15)} = 0,6607$, $p = 0.5188$, n = 8-9 rats per group). However, knock-down rats displayed a significant ~16% and ~28% increase in levels of OT mRNA in the PVNp and PVNm, compared to control rats (Fig. 1.7B; $t_{(15)} = 2.200$, $p = 0.0439$ and $t_{(15)} = 2.169$, $p = 0.0465$, respectively, n = 8-9 rats per group). AVP mRNA levels were also significantly increased in the PVNp of knock-down rats relative to control, and there was a trend towards an increase in the PVNm (Fig. 1.7C; $t_{(13)} = 2.370$, $p = 0.0339$ and $t_{(13)} = 2.019$, $p = 0.0646$ respectively, n = 7-8 rats per group). *[There was only a small number of CRF mRNA positive neurons detected within the PVNm, and these were not assessed (Simmons and Swanson, 2009; Jiang et al. 2018)].

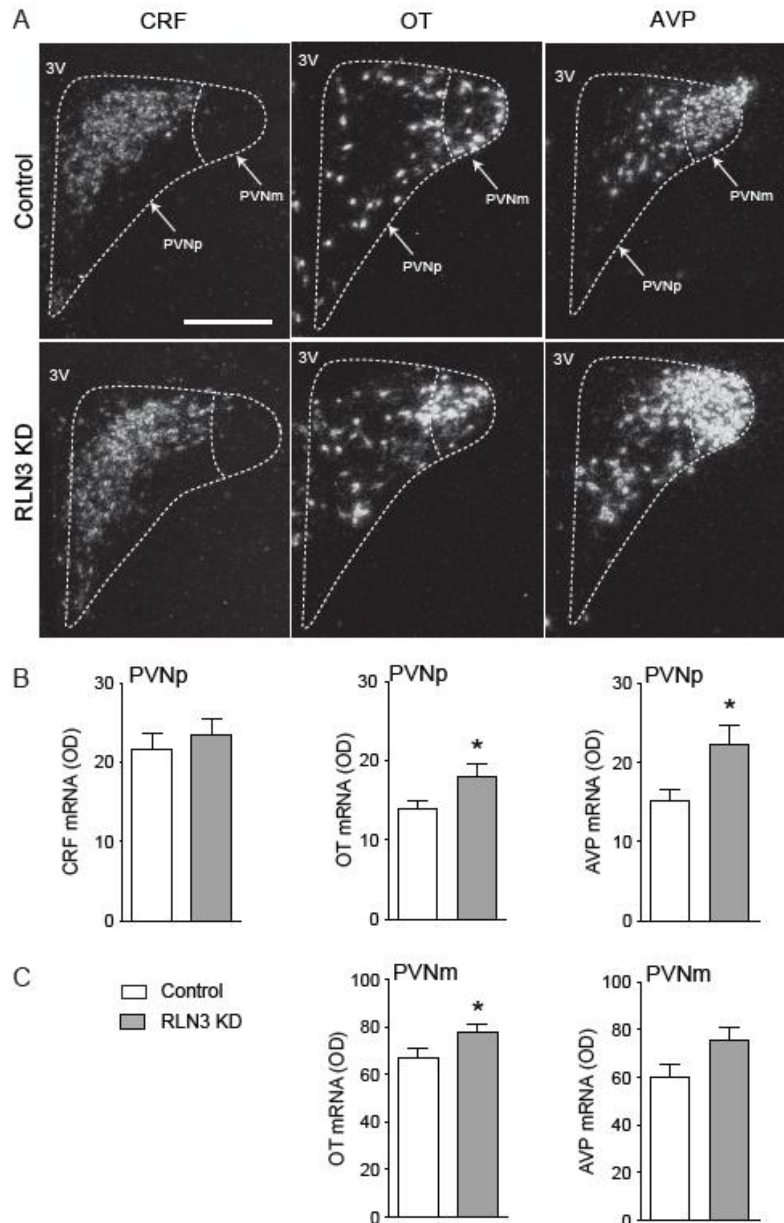

Figure 1.7. Knock-down of RLN3 in NI neurons in female rats increased oxytocin (OT) and arginine-vasopressin (AVP), but not corticotropin-releasing factor (CRF) mRNA in the paraventricular nucleus of the hypothalamus (PVN)

(A) Dark-field photomicrographs of representative coronal brain sections from control and RLN3 knock-down (KD) rats illustrate CRF mRNA hybridization in the PVN parvocellular (p) and OT and AVP mRNA hybridization in the magnocellular (m) and PVNp. 3V, third ventricle. (B) CRF mRNA levels were not different in the PVNp of KD and control rats. OT mRNA levels were significantly increased in the PVNm and PVNp of KD compared to control rats. AVP mRNA levels were

significantly increased in the PVNp of KD compared to control rats, and in the material examined, there was a trend towards an increase in the PVNm (p = 0.0646).

1.7. Discussion

The central RLN3/RXFP3 signalling is involved in the modulation of motivational and affective states, and in homeostasis of metabolism (Ganella et al., 2013, Ma et al., 2018). Important species and sex differences in RLN3/RXFP3 biology have been identified and further studies are required to better elucidate the physiological role, cellular and molecular targets, and translational potential of this highly-conserved, peptide/receptor system (Kumar et al., 2017, Ma et al., 2017). In this regard, the current study investigated the effects of miRNA-induced depletion (or knock-down) of RLN3 mRNA/peptide production in neurons of the brainstem NI in female rats on a range of physiological, behavioural and neurochemical indices, including food intake, body weight, anxiety-like behaviour, corticosterone levels, limbic neural activity patterns (reflected by *c-fos* mRNA), and on levels of hypothalamic peptide (CRF, OT and AVP) mRNA expression in the PVN. Further details of our findings and their implications are provided below.

Earlier studies have demonstrated that RLN3/RXFP3 signalling can alter food intake and body weight in male *and* female rodents. Several independent studies demonstrated an orexigenic effect of acute and chronic central administration of the native relaxin-3 peptide or an RXFP3-selective agonist peptide, in satiated rats (McGowan et al., 2005, Hida et al., 2006, McGowan et al., 2006, Kuei et al., 2007, Shabanpoor et al., 2012, Ganella et al., 2013, Lenglos et al., 2014, Calvez et al., 2015, De Ávila et al., 2017). Furthermore, the increase in food intake was associated with an increase in body weight and white fat mass that was sex-specific, with female rats more sensitive to the orexigenic effect of RLN3 than males (Calvez et al. 2016). The effects of RXFP3 activation on feeding can be inhibited by prior administration of RXFP3 antagonist peptides, although acute

RXFP3 blockade alone did not significantly alter food intake in male rats (Haugaard-Kedstrom et al., 2011, Shabanpoor et al., 2012, Calvez et al., 2016c).

Our current results indicate that depletion of RLN3 from NI neurons in female rats produced a moderate but sustained reduction in body weight gain and induced a temporary imbalance in food intake. Indeed, a significant decrease in food intake was observed during week 4 post-treatment, after which levels of food consumption were similar to the control group during week 5 and 6. A possible explanation for this profile is that during the first weeks post-treatment, the depletion of RLN3 in NI neurons induces a decrease in food intake and body weight gain in knock-down female rats compared to controls, but as food consumption and homeostatic body weight maintenance is important for survival, neural circuits downstream of relaxin-3/RXFP3 signalling might begin to compensate for the changes induced by RLN3 knock-down. Furthermore, in a previous study we observed that RLN3 knock-down in NI neurons of *male* rats did not alter either food intake or body weight (Callander et al., 2012). Together these data further support the idea that RLN3 acts differently in male and female rats.

Previous research also implicates RLN3/RXFP3 signalling in the modulation of elevated anxiety-like behaviour and stress-responses in rats and mice (Watanabe et al., 2011b, Ryan et al., 2013a, Zhang et al., 2015, Lawther et al., 2018). RLN3 neurons are activated by stressors via the corticotropin-releasing hormone receptor-1 (CRF_1) (Tanaka et al., 2005, Ma et al., 2013). Several studies have described an anxiolytic effect of icv injection of RLN3 or a selective RXFP3 agonist in the EPM, in male rats and mice (Ryan et al., 2013a, Zhang et al., 2015). A similar effect was also observed in the L/D, but notably, not in the LOF. Furthermore, central administration of an RXFP3 antagonist in adult male mice increased an index of innate anxiety-like behaviour in the EPM test, however no difference was found in L/D box and LOF. (Zhang et al., 2015). Distinct implications of the endogenous RLN3/RXFP3 system in anxiety-like behaviour have been reported in different species; with a more subtle role under basal conditions in mice than with elevated (drug-induced) anxiety (Zhang et al., 2015), and a clear role in

the response to high levels of stress in rats (Banerjee et al., 2010a, Ma et al., 2013, Ryan et al., 2013a, Ryan et al., 2013b).

In the present study, female control rats and rats with RLN3 depleted from NI neurons displayed similar behaviour in the L/D box and the EPM tests, whereas in the LOF the assessed parameters: duration and distance travelled in the center area, indicated a slight increase in anxiety levels in the RLN3-knock-down rats compared to controls. It is important to emphasise that even though these paradigms are routinely used to assess anxiety-like behaviour and stress responses, there are relevant differences between the tests that should be considered. In the EPM and L/D, rodents have access to sheltered or dark compartments respectively, for coping or escaping from the stressful context, instead of exploring the open and lit exposed environment. In the LOF, rats are placed in a circular apparatus in which the light is 6 times and 4 times stronger in the center and outer parts respectively, than the light level in the L/D. Therefore, rats exposed to this paradigm may be coping differently than in the other two tests, due to a lack of escape areas, which makes the LOF a non-equivalent, stressful, anxiogenic challenge compared to the EPM and L/D.

Thus, our data suggest that in female rats, depletion of NI RLN3 may slightly increase anxiety-like behaviour in a stressful environment with no escape (LOF), but does not modify anxiety levels in an environment with the possibility to escape (EPM and L/D). These findings might further reflect the potential differences in exogenous vs endogenous RLN3/RXFP3 signalling in anxiety responses in male and female rats (Ryan et al., 2013a).

Interestingly, plasma corticosterone levels assessed after each of these behavioural tests were similar between groups, and a lack of difference in corticosterone levels after the LOF is inconsistent with the possible increase in anxiety levels in RLN3 knock-down rats; although anxiety-like behaviour has been reported to be independent of the HPA axis (Pich et al., 1993). The similarity between plasma corticosterone in knock-down and control rats after behavioural tests raises a question regarding the change in the basal corticosterone levels

observed in the two groups of rats. Indeed, basal corticosterone levels in RLN3 knock-down rats were 4-fold lower than those in control rats, and when corticosterone levels measured after the behavioural tests were normalized relative to the basal state, a significant increase was observed in all tests. Notably, the basal levels detected were similar to previous reports (Lenglos et al., 2014).

In an effort to understand the effects of NI RLN3 depletion on basal corticosterone levels and food consumption, *c-fos* mRNA levels was analyzed in some major brain regions involved in stress and food intake that receive putative RLN3-containing projections. The PVNp is a key nucleus in the HPA axis that regulates stress responses and corticosterone production, as neurons in this area produce CRF (Herman and Cullinan 1997). A recent study observed that acute icv human RLN3 administration increased *c-fos* mRNA levels in the PVNp of male, but not female, rats (Lenglos et al., 2014). In contrast, in the current study, we observed a decrease in basal *c-fos* mRNA levels in the PVNp in female knock-down rats. This differential response could be the consequence of experimental conditions, and/or the ability of RLN3 delivered as a pharmacological agent to activate RXFP1 in the PVN (Ma et al., 2006a). Interestingly, CRF mRNA expression was increased in the PVNp of males, but not female, rats after icv RLN3 injection (Lenglos et al., 2014), and this is in accordance with our current data, whereby RLN3 knock-down in NI neurons in female rats did not affect basal CRF mRNA expression in the PVNp. However, the basal plasma levels of corticosterone were significantly decreased in knock-down rats and increased two-fold in control and eight-fold in RLN3 knock-down rats to reach a similar level, after the anxiety tests. In this regard, it has been shown that ACTH and corticosterone release can be observed in rodents without CRF stimulation (Muglia et al., 2001). For example, in female *and* male rats, the intracerebral injection of an OT antagonist induced an increase in basal secretion of ACTH and corticosterone into the bloodstream (Neumann et al., 2000).

Similarly, in the current study, we observed that OT and AVP mRNA levels were significantly increased in the PVNp of RLN3 knock-down rats relative to

control. In a previous study, mRNA encoding RXFP3, the cognate $G_{\alpha i/o}$-protein-coupled receptor for RLN3, was co-localized with OT and AVP immunoreactivity in neurons of the PVN, and RXFP3 activation directly inhibited both types of neurons *in vitro* (Kania et al., 2017). Therefore, a lack of inhibition of these neurons by the NI RLN3/RXFP3 system following the depletion of RLN3 may explain the elevated OT and AVP mRNA levels in the PVN, which in turn may contribute to the inhibition of corticosterone secretion and the reduced plasma levels. While not addressed here, future analysis of CRF, AVP and OT expression in the PVNp after a stressor in female rats with RLN3 depletion should be performed to better understand the corticosterone response and the involvement of RLN3 in its regulation.

The BNST receives projections from RLN3 neurons (Ma et al., 2007a) and is involved in anxiety (Walker et al., 2003, Lee et al., 2008) and reward processes (Ryan et al., 2013a, Avery et al., 2016). Our results indicate that depletion of RLN3 is associated with a decrease in *c-fos* mRNA in the BNST, suggesting that under basal conditions and in the absence of RLN3, the BNST is less activated than in rats with an intact RLN3-positive innervation. This region contains GABAergic neurons (Gafford et al., 2012), and CRF neurons (Moga et al., 1989). In accordance with our data, an earlier study revealed that icv RLN3 administration increased CRF expression in the BNST of female rats (Lenglos et al., 2014).

In light of the complex responses observed for the two experimental groups in the EPM, L/D and LOF, further analysis of CRF expression patterns in the BNST of female rats with RLN3 depletion should be performed after different stressful stimuli to better understand the involvement of RLN3/RXFP3 signalling in BNST in the regulation of anxiety.

The LHA and the magnocellular PVN are two nuclei involved in food intake regulation and homeostasis (Schwartz et al., 2000, Morton et al., 2006). Previous studies have revealed that icv injection of RLN3 in female rats induces an increase in *c-fos* mRNA in these structures (Lenglos et al., 2014, Calvez et al., 2015b), while our studies reveal that depletion of NI RLN3 induced a decrease in basal levels of *c-fos* mRNA expression in the LHA and the PVNm. The LHA contains orexigenic

orexin and melanin-concentrating hormone (MCH) neuron populations, and GABAergic neurons (Saper et al., 2002, Stuber and Wise, 2016). The increase in *c-fos* expression observed after icv RLN3 administration did not involve MCH neurons, but orexin mRNA expression was increased (Calvez et al., 2015). Furthermore, in the PVNm, AVP neurons were not involved in the response, but OT mRNA levels were increased after RLN3 administration (Calvez et al. 2015).

Our results revealed that depletion of RLN3 induced an increase in OT mRNA in PVNm and a tread for increased AVP mRNA levels. These differences may also be due to the acute vs chronic nature of the different studies, but OT neurons in the PVNm are anorexigenic (Schwartz et al., 2000), so an increase in OT expression in RLN3 knock-down rats might explain the decrease in food intake observed during the first weeks of our experiments. After the fourth week, other signalling systems may have rebalanced the food intake decrease. Notably, the brains analyzed for gene expression patterns were collected during the early light (inactive) phase, when rats were satiated, and therefore it would be of interest in future to analyze neuropeptide expression in hungry female rats with NI RLN3 depletion.

1.8. Conclusions

In summary, the current study has revealed that depletion of RLN3 mRNA/peptide expression in NI neurons of female rats produced a decrease in body weight gain, an imbalance in food intake, and an increase in anxiety levels in a behavioural paradigm with no escape. Furthermore, the depletion of NI RLN3 disrupted corticosterone regulation and levels of *c-fos*, OT and AVP mRNA in brain regions involved in stress and food intake regulation, consistent with an important role for RLN3-positive neurons in the NI in fine-tuning stress responses and food intake regulation.

1.9. Acknowledgements

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1.10. Conflict of Interest

The authors report no conflict of interest.

1.11. References

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Chapitre 2. Differential effects of relaxin-3 and a selective relaxin-3 receptor agonist on food and water intake and hypothalamic neuronal activity in rats

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Camila de Ávila*[a], Sandrine Chometton*[a], Christophe Lenglos[a], Juliane Calvez[a], Andrew L. Gundlach[b], Elena Timofeeva[a]

*These authors contributed equally to this research.

[a] Faculté de Médecine, Département de Psychiatrie et de Neurosciences, Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec, Université Laval, Québec (QC), G1V 0A6, Canada.

[b] The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria 3052, Australia; and Florey Department of Neuroscience and Mental Health, The University of Melbourne, Melbourne, Victoria 3010, Australia.

2.1. Résumé

Le neuropeptide relaxine-3 (RLN3) se lie avec une forte affinité à son récepteur spécifique, le récepteur peptidique de la famille de la relaxine 3 (RXFP3), et avec une affinité plus faible à RXFP1, le récepteur spécifique pour relaxine. L'injection intracérébroventriculaire (icv) de la RLN3 chez le rat augmente fortement la prise alimentaire et la prise d'eau, et modifie l'activité des axes hypothalamo-hypophyso-surrénalien (HPA) et gonadique (HPG), mais l'implication de RXFP3 et de RXFP1 dans ces effets est inconnue. Par conséquent, les effets d'injections de quantités équimolaires (1,1 nmol) de la RLN3 et de l'agoniste sélectif de RXFP3, RXFP3-A2, sur la prise alimentaire et d'eau, les concentrations plasmatiques de corticostérone, testostérone et l'expression des ARNm de oxytocine et de c-*fos* dans les régions hypothalamiques clés chez les rats mâles ont été comparés.

La prise alimentaire a été augmenté à la fois par la RLN3 et le RXFP3-A2, mais les effets orexigèniques de RXFP3-A2 ont été significativement plus forts que ceux du RLN3, 30 et 60 minutes après injection. La prise d'eau et les taux plasmatiques de corticostérone et de testostérone ont augmenté de façon significative avec la RLN3, mais pas avec RXFP3-A2. Inversement, RXFP3-A2 a diminué les taux plasmatiques d'ocytocine, mais pas RLN3. RLN3 a augmenté l'expression d'ARNm c-*fos* dans les noyaux paraventriculaires parvocellulaire (PVNp) et magnocellulaire (PVNm), et supraoptique (SON) de l'hypothalamus, dans l'aire préoptique médiane ventrale (MPAv) et dans l'organum vasculosum de la lamina terminalis (OVLT), mais pas RXFP3-A2. Une augmentation significative de l'expression de l'ARNm c-*fos* a été induite dans l'aire périfornicale de l'hypothalamus latéral (LHApf) par la RLN3 et par RXFP3-A2. Ces résultats suggèrent que la stimulation de RXFP1 par la RLN3 est impliqué dans la prise d'eau et l'activation des axes HPA et HPG. La diminution de la stimulation de la prise alimentaire par la RLN3 par rapport à RXFP3-A2 peut être liée à l'activation des circuits orexigène et anorexigène par RLN3.

2.2. Abstract

The neuropeptide relaxin-3 (RLN3) binds with high affinity to its cognate receptor, relaxin-family peptide receptor 3 (RXFP3), and with lower affinity to RXFP1, the cognate receptor for relaxin. Intracerebroventricular (icv) administration of RLN3 in rats strongly increases food and water intake and alters the activity of the hypothalamic-pituitary-adrenal (HPA) and gonadal (HPG) axes, but the relative involvement of RXFP3 and RXFP1 in these effects is not known. Therefore, the effects of icv administration of equimolar (1.1 nmol) amounts of RLN3 and the RXFP3-selective agonist RXFP3-A2 on food and water intake, plasma levels of corticosterone, testosterone, and oxytocin and c-fos mRNA expression in key hypothalamic regions in male rats were compared.

Food intake was increased by both RLN3 and RXFP3-A2, but the orexigenic effects of RXFP3-A2 were significantly stronger than RLN3, 30 and 60 min after injection. Water intake and plasma corticosterone and testosterone levels were significantly increased by RLN3, but not by RXFP3-A2. Conversely, RXFP3-A2 but not RLN3 decreased oxytocin plasma levels. RLN3, but not RXFP3-A2, increased c-fos mRNA levels in the parvocellular (PVNp) and magnocellular (PVNm) paraventricular and supraoptic (SON) hypothalamic nuclei, in the ventral medial preoptic area (MPAv), and in the organum vasculosum of the lamina terminalis (OVLT). A significant increase in c-fos mRNA expression was induced in the perifornical lateral hypothalamic area (LHApf) by RLN3 and RXFP3-A2. These results suggest that RXFP1 is involved in the RLN3 stimulation of water intake and activation of the HPA and HPG axes. The reduced food intake stimulation by RLN3 compared to RXFP3-A2 may relate to activation of both orexigenic and anorexigenic circuits by RLN3.

2.3. Introduction

Relaxin-3 (RLN3) is the ancestral member of the insulin-relaxin peptide superfamily (Bathgate et al., 2002, Burazin et al., 2002), which is comprised of seven peptides known initially for their major roles in reproduction, growth and development (Bathgate et al., 2013, Halls et al., 2015, Patil et al., 2017).

The peptides of this superfamily contain two amino acid chains (A and B) connected by three disulfide bonds (Shabanpoor et al., 2009). In humans there are three relaxin genes: *RLN1*, encoding human relaxin-1 (H1), *RLN2* encoding human relaxin-2 (H2), and *RLN3* encoding human relaxin-3 (Smith et al., 2011, Bathgate et al., 2013). Humans and higher primates have all three relaxin genes, whereas in non-primates, only two relaxin genes are present: *RLN1* and *RLN3*. The product of the *RLN1* gene in non-primates, relaxin, is equivalent to the *RLN2* gene product in humans, H2. In mammals, relaxin is produced in several tissues as a paracrine and autocrine factor, and it circulates in the blood during pregnancy (Bathgate et al., 2013). Unlike other relaxin-family peptides that display considerable species heterogeneity, RLN3 sequences are well-conserved across species (Wilkinson et al., 2005, Yegorov et al., 2009). Also, unlike other relaxin-family peptides that are strongly expressed in the peripheral organs, RLN3 is primarily a neuropeptide produced almost exclusively in the brain (Bathgate et al., 2002), with a particularly strong expression in the pontine nucleus incertus (NI) (Ma et al., 2007).

NI neuron projections were detected throughout the brain, including areas of the brainstem, hypothalamus, septum, hippocampus, and cerebral cortex (Ma et al., 2007). RLN3 binds with high affinity to its cognate receptor, known as relaxin-family peptide receptor 3 (RXFP3), and with a lower affinity to RXFP1, the cognate receptor for relaxin (Liu et al., 2003, Halls et al., 2015). RLN3 also binds to RXFP4, the cognate receptor for insulin-like peptide 5 (INSL5), but this receptor is a non-functional pseudogene in rats, which therefore limits the brain actions of RLN3 to RXFP3 and RXFP1 in this species (Sutton et al., 2005).

RXFP3 and RXFP1 are G-protein-coupled receptors. The activation of RXFP3 by RLN3 induces the inhibition of cAMP accumulation *via* $G_{i/o}$-protein coupling, while the activation of RXFP1 stimulates adenylate cyclase and increases intracellular cAMP (Liu et al., 2003, Halls et al., 2007). An increase in cAMP in response to RXFP1 activation stimulates the cAMP-response element that triggers the expression of the immediate early gene c-*fos*, a molecular marker of neuronal activation (Fukuchi et al., 2015, Ma et al., 2017). The activation of RXFP3 by RLN3 may stimulate the protein kinase C-dependent (PKC) pathway that drives the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 (van der Westhuizen et al., 2007) and subsequently activates c-*fos* gene expression (Gutierrez-Mecinas et al., 2011). RXFP1 and RXFP3 mRNAs are expressed in several different brain areas, with an overlapping expression in the paraventricular (PVN) and the supraoptic (SON) hypothalamic nuclei; however, other brain regions, such as the lateral hypothalamic area (LHA), only express RXFP3 (Osheroff and Phillips, 1991, Sutton et al., 2004, Ma et al., 2007, Ganella et al., 2013).

RLN3 plays a role in several physiological functions. The icv administration of this neuropeptide increases food intake (McGowan et al., 2007c, Calvez et al., 2015a, Lenglos et al., 2014) and water intake (McGowan et al., 2005b, Bathgate et al., 2006, Otsubo et al., 2010) in rats. Central RLN3 administration also induces the expression of c-Fos protein in the corticotropin-releasing factor (CRF) neurons of the PVN and increases plasma corticosterone levels (Watanabe et al., 2011a, Lenglos et al., 2014). RLN3 treatment of hypothalamic explants from male rats stimulates the release of gonadotropin-releasing hormone (GnRH) (McGowan et al., 2008). In addition, chronic icv administration of RLN3 increases plasma levels of luteinizing hormone (LH) and follicular-stimulating hormone (FSH) in male rats (Calvez et al., 2016a); however, the precise involvement of RXFP3 and/or RXFP1 in these physiological functions is not clearly understood. A specific antagonist of RXFP1 would be of interest, however this molecule it is not produced. Recently, a high-affinity RXFP3 agonist [R3A(11–24,C15→A)B], referred to as RXFP3-selective analogue 2 (RXFP3-A2), was produced (Shabanpoor et al., 2012).

Similar to RLN3, the icv administration of RXFP3-A2 increased food intake in satiated rats (Shabanpoor et al., 2012). The application of RLN3 and RXFP3-A2 in patch clamp studies produced inhibitory effects on oxytocin and vasopressin PVN neurons *in vitro* (Kania et al., 2017). However, the application of RLN3 in the presence of RXFP3 blockade caused an increase in action potential firing, which might represent an excitatory action of RLN3 on these neurons that is mediated by RXFP1 (Kania et al., 2017).

So far, there is no clear understanding of the differential effects of the specific activation of RXFP3 by its selective agonist *versus* the simultaneous activation of RXFP3 and RXFP1 by RLN3 on food and water intake, plasma hormones, or on the induction of c-*fos* mRNA expression. Given the possible involvement of RLN3 signalling in binge eating, memory, and stress (Banerjee et al., 2010, Watanabe et al., 2011a, McGowan et al., 2014, Calvez et al., 2016a, Haidar et al., 2017, Kumar et al., 2017), further elucidation of the relative contribution of RXFP1 and RXFP3 to the pharmacological effects of RLN3 is required.

Therefore, this study was designed to compare the effects of RLN3, a non-selective agonist at RXFP3 and RXFP1, and RXFP3-A2, a truncated RLN3 analogue with a high selectivity for RXFP3 over RXFP1 and strong agonist activity (Shabanpoor et al., 2012), on food and water intake and on plasma levels of corticosterone, testosterone, oxytocin, vasopressin, epinephrine, and norepinephrine in male rats. In addition, the activation by RLN3 and RXFP3-A2 of the key hypothalamic regions involved in the regulation of food intake and the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes activity were investigated by assessing the levels of *c-fos* mRNA expression.

2.4. Materials and methods

2.4.1. Animals

Male Sprague-Dawley rats (postnatal days 57-59, 250-275 g, n = 32) were purchased from the Canadian Breeding Laboratories (St-Constant, QC, Canada). The rats were housed in individual plastic cages lined with wood shavings and maintained on a 12:12 h dark-light cycle (lights on between 6:00 and 18:00 h), with an ambient temperature of 23 ± 1°C. Rats were left undisturbed for at least 1 week to adapt to the new environment. Rats had free access to standard laboratory rat chow (2018 Teklab Global 18% Protein Rodent Diet; 3.1 kcal/g, Harlan Teklab, Montreal, QC, Canada) and tap water. All rats were cared for and handled according to the *Canadian Guide for The Care and Use of Laboratory Animals*, and the present protocol was approved by our institutional animal care committee.

2.4.2. Peptides

Rats were infused icv with artificial cerebrospinal fluid (aCSF) or 1.1 nmol of human RLN3 (Pharmaceuticals, Belmont, CA, USA) or 1.1 nmol of RXFP3-A2 (Dr MA Hossain, The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria, Australia). RLN3 and RXFP3-A2 were dissolved in aCSF. The dose used was chosen based on an earlier report on the icv administration of RLN3 and RXFP3-A2 in rats (Shabanpoor et al., 2012).

2.4.3. Icv injections

Each rat was deeply anesthetized using 4% isoflurane and implanted with a 26-gauge stainless steel guide cannula (Plastics One, Roanoke, VA, USA) aimed at the lateral ventricle using the coordinates: 0.8 mm caudal to the bregma, 1.5 mm lateral to the midline, and 3.5 mm ventral from the skull surface. After 7 days of postoperative care, all rats were habituated to regular handling over 5 – 7 days. Rats were randomly divided into three groups and all peptides were administered using a between-subject design. Icv injections of 5 µl of RLN3 (n = 10), RXFP3-A2

(n = 12) or aCSF (n = 10) were administered over 1 min via stainless steel injectors (27-gauge) that projected 1 mm below the tip of the guide cannula. After injection, the injector was left in place for 1 min, and then the rats were returned to their home cages. All injections were performed with *ad libitum*-fed rats in the early light phase (8:00 – 11:00). Body weight (BW) was measured immediately before the icv injections to assess the food and water intake in milligram per gram BW. Each rat received three injections separated by one week of the same assigned peptide to measure chow intake, water intake, and c-*fos* mRNA expression. One week before the injections, the cannula position was verified by a positive dipsogenic response to human angiotensin II injection (30 ng per rat, Sigma-Aldrich, St. Louis, MO). A dipsogenic response was validated if the rat started to drink water without delay and drank continuously for at least 2 min after injection; with a volume of water consumed during the 30 min post-injection >5 ml. Cannula placements were ultimately confirmed histologically at the end of the study. Only rats with correct cannula placement were included in the data analysis.

2.4.4. Water and chow intake

Rats were injected icv with RLN3 (n = 10), RXFP3-A2 (n = 12) or aCSF (n = 10) and water intake was measured at 0, 30, 60 and 120 min post-injection in home cages without access to chow. Water intake was measured by calculating the difference in the weight of the cage water container before and after the test. One week later, the rats were icv injected with RLN3 (n = 10), RXFP3-A2 (n = 12) and aCSF (n = 10) and chow intake was measured at 0, 30, 60 and 120 min post-injection in home cages without access to water. Food intake was measured by calculating the difference in the weight of the chow remaining and that provided immediately before the test.

2.4.5. Blood sample and brain preparation

At the end of experiment, rats were sacrificed 30 min after the final icv injection of RLN3, RXFP3-A2 or aCSF during the early light phase. Immediately after injection, the rats were transferred to their home cages, where they had no

access to chow and water in the 30 min before euthanasia. Rats were deeply anesthetized (60 mg/kg ketamine plus 7.5 mg/kg xylazine), intracardial blood was collected, and they were perfused intracardially with 100 ml of saline followed by 200 ml of a 4% paraformaldehyde solution. Brains were removed and post-fixed in 4% paraformaldehyde for one week. Brains were then transferred to a solution containing 4% paraformaldehyde and 20% sucrose, before being sectioned 12 h later using a sliding microtome (Histoslide 2000, Heidelberg, Germany). Thirty-micron-thick coronal sections were collected and stored at –20°C in a cold sterile cryoprotection solution containing 50 mM sodium phosphate buffer, 30% ethylene glycol, and 20% glycerol.

2.4.6. Determination of plasma concentrations of testosterone, corticosterone, oxytocin, vasopressin, epinephrine, and norepinephrine

Blood samples were centrifuged (3,000 g for 15 min at 4°C) and plasma was stored at -80°C until the determination of corticosterone, testosterone, oxytocin, vasopressin, epinephrine, and norepinephrine plasma levels in duplicate using commercial ELISA kits of corticosterone (Cayman Chemical, Ann Arbor, MI, USA; sensitivity, 5.0 pg/ml), testosterone (Enzo Life Science, Farmingdale, NY, USA; sensitivity, 5. 7 pg/ml), oxytocin (Phoenix Pharmaceuticals, Burlingame, CA, USA; sensitivity: 0.13 ng/ml), vasopressin (Enzo Life Science, Farmingdale, NY, USA; sensitivity: 2.84 pg/ml), epinephrine and norepinephrine (Abnova, Taipei City, Taiwan; sensitivity: 10 pg/ml and 36 pg/ml, respectively).

2.4.7. *In situ* hybridization for c-*fos* mRNA

Levels of c-*fos* mRNA expression in the hypothalamus were detected using *in situ* hybridization, as described (Mitra et al., 2015, Calvez et al., 2016d). Briefly, the sections were mounted on poly-L-lysine coated slides and fixed for 20 min in 4% paraformaldehyde, digested for 30 min at 37°C with proteinase K (10 µg/ml in 100 mM Tris-HCl containing 50 mM EDTA, pH 8.0), acetylated with acetic anhydride (0.25% in 0.1 M trietholamine, pH 8.0), and dehydrated through graded

concentrations of ethanol. Sections were incubated overnight with antisense [^{35}S]-labeled cRNA probes (10^7 cpm/ml) for c-*fos* (generated from the 2116-bp fragment of rat c-*fos* cDNA; GenBank accession number X06769.1; Dr I Verma, Salk Institute, La Jolla, CA) at 60°C. Slides were rinsed with sodium chloride-sodium citrate solution, digested with ribonuclease-A (20 μg/ml), washed in descending concentrations of sodium chloride-sodium citrate solutions, and dehydrated through an ethanol gradient. Thereafter, slides were defatted in toluene, dipped in nuclear track beta-2 nuclear emulsion (Eastman Kodak, Rochester, NY, USA), and exposed for 2 weeks before developing. Tissues were counterstained with thionine, dehydrated through graded concentrations of ethanol, cleared in toluene and coverslipped with mounting medium.

2.4.8. Image analyses

Slides were examined with dark-field microscopy using an Olympus BX61 microscope (Olympus Canada, Richmond Hill, ON, Canada). Images were acquired with a DVC-2000C digital camera (DVC Company Inc., Austin, TX, USA) and analyzed with Stereo Investigator software (MBF Bioscience, Williston, VT, USA). The system was calibrated for each set of analyses to prevent saturation of the integrated signal; and the optical density (OD) reading was normalized to a scale where minimum (0) and maximum (1) values represent pure dark (opaque) and white pixels, respectively. Mean OD was obtained by measuring the OD of pixels of the positive hybridization signal in sections of the parvocellular PVN (PVNp) and the magnocellular PVN (PVNm) (from 1.72 mm to 1.80 mm caudal to bregma), the SON (from 1.32 to 1.56 caudal to bregma) and the OVLT (from 0.15 mm to 0.00 mm rostral to bregma) (Paxinos and Watson, 2007), and subtracting the background readings taken from the areas immediately surrounding the analyzed region. Because of the scattered hybridization signals of c-*fos* mRNA in the MPA (from 0.3 rostral to bregma to 0.4 mm caudal to bregma) and the LHA (from 2.9 to 3.4 mm caudal to bregma) (Paxinos and Watson, 2007), c-*fos* mRNA expression was assessed using the cumulative area of the positive hybridization

signal. A threshold depicting positive hybridization signal was set for pixels with OD >5 times the background for all micrographs captured with the same luminosity.

The OD measurements were performed in the regions of interest outlined bilaterally on two or three consecutive brain sections (four to six values per rat). The individual score of each rat was normalized to the mean value of the aCSF-injected group (mean value of a rat/mean value of aCSF group) to obtain the relative expression of c-*fos* mRNA.

2.4.9. Statistical analyses

Results are presented as mean ± SEM. For cumulative food intake and water intake analysis, two-way repeated-measures ANOVA was used to detect the main and interactive effects of treatment (RLN3, RXFP3-A2 and aCSF) and time (0, 30, 60 or 120 min). For plasma hormones and for c-*fos* mRNA analyses, one-way ANOVA was used to detect the effect of treatment. *Post-hoc* comparisons between the groups were performed using Fisher's protected least significant difference test when the main and interactive ANOVA effects were significant. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using PRISM statistical software, V6.04 (GraphPad Software Inc., La Jolla, CA, USA).

2.5. Results

2.5.1. Food and water intake

The cumulative food intake was significantly increased by the icv administration of RLN3 (5.7 ± 1.3 mg/g BW, 8.9 ± 1.2 mg/g BW, and 12.6 ± 2.6 mg/g BW after 30, 60, and 120 min, respectively) and RXFP3-A2 (11.3 ± 1.6 mg/g BW, 12.6 ± 1.4 mg/g BW, and 13.3 ± 1.4 mg/g BW after 30, 60, and 120 min, respectively) compared to the control injections of aCSF (0.5 ± 0.2 mg/g BW, 0.9 ± 0.5 mg/g BW, and 3.2 ± 1.2 mg/g BW after 30, 60, and 120 min, respectively) (Fig. 2.1A). The two-way repeated-measures ANOVA revealed significant effects of time

($F_{3, 116} = 31.63$, $p < 0.0001$) and treatment ($F_{2, 116} = 43.16$, $p < 0.0001$) and a significant interaction between time and treatment ($F_{6, 116} = 5.555$, $p < 0.0001$) on food intake. *Post-hoc* analyses revealed that compared to the aCSF control group, the injection of RLN3 significantly increased the cumulated food intake after 30 min ($p < 0.0054$), 60 min ($p < 0.0001$), and 120 min ($p < 0.0001$). Similarly, the administration of RXFP3-A2 significantly increased the cumulative food intake after 30, 60, and 120 min ($p < 0.0001$ at each time-point) compared to the aCSF control. Compared to RLN3, the administration of RXFP3-A2 produced significantly stronger orexigenic effects after 30 min ($p < 0.0023$) and 60 min ($p < 0.0384$), but not after 120 min ($p > 0.05$) following icv injections.

The cumulative water intake was significantly increased by RLN3 (12.2 ± 2.7 mg/g BW, 17.4 ± 2.8 mg/g BW, and 27.9 ± 4.9 mg/g BW after 30, 60, and 120 min, respectively) but not by RXFP3-A2 (1.1 ± 0.5 mg/g BW, 5.6 ± 1.4 mg/g BW, and 10.6 ± 2.3 mg/g BW after 30, 60, and 120 min, respectively) compared to the aCSF-injected group (2.8 ± 0.8 mg/g BW, 5.6 ± 1.2 mg/g BW, and 9.5 ± 1.5 mg/g BW after 30, 60, and 120 min, respectively) (Fig. 2.1B). The two-way repeated-measures ANOVA revealed significant effects of time ($F_{3, 108} = 32.22$, $p < 0.0001$) and treatment ($F_{2, 108} = 30.88$, $p < 0.0001$) and a significant interaction between time and treatment ($F_{6, 108} = 4.342$, $p < 0.0006$) on water intake. *Post-hoc* analyses revealed that compared to the aCSF control group, the injection of RLN3 significantly increased water intake after 30 min ($p < 0.0018$), 60 min ($p < 0.0001$), and 120 min ($p < 0.0001$). Conversely, no significant difference ($p > 0.05$) was detected between RXFP3-A2 and aCSF-injected groups at any time point. The cumulative water intake was significantly higher in RLN3-injected rats after 30 min ($p < 0.0003$), 60 min ($p < 0.0001$), and 120 min ($p < 0.0001$) compared to RXFP3-A2 administration.

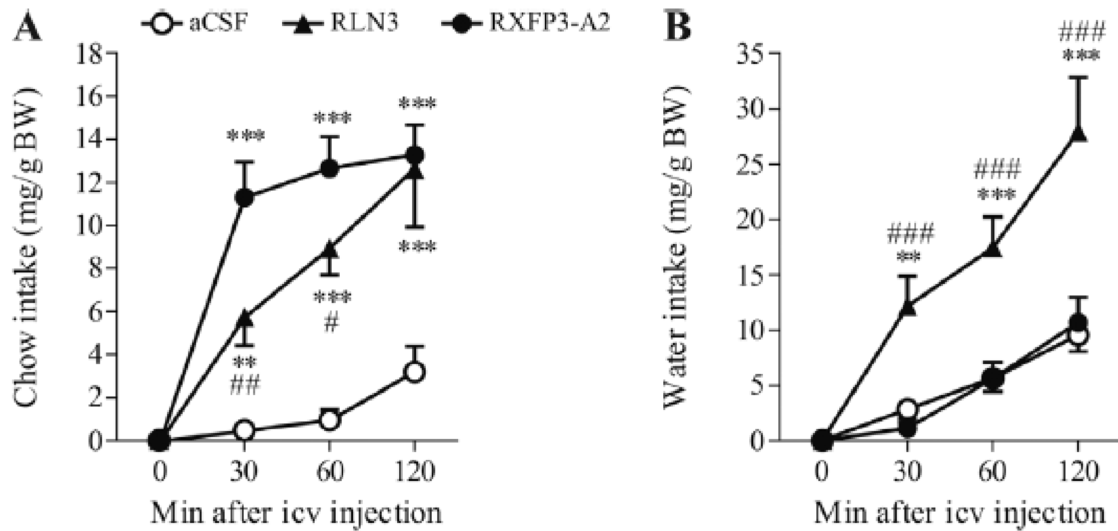

Figure 2.1. Chow (*A*) and water (*B*) intake relative to body weight (BW) at 30, 60, and 120 min following icv administration of aCSF (n = 10), RLN3 (n = 10), or RXFP3-A2 (n = 12)

**, $p < 0.01$; ***, $p < 0.001$: significantly different from the aCSF-injected rats. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$: significantly different from the RXFP3-A2-injected rats.

2.5.2. Plasma concentrations of testosterone, corticosterone, oxytocin, vasopressin, epinephrine and norepinephrine

The levels of plasma hormones were measured at 30 min after the icv administration of RLN3 or RXFP3-A2. Testosterone was increased by RLN3 (11.9 ± 1.3 ng/ml) but not by RXFP3-A2 (5.3 ± 0.5 ng/ml) compared to the aCSF-injected group (6.1 ± 0.7 ng/ml) (Fig. 2.2A). The one-way ANOVA revealed a significant effect of the treatment on plasma testosterone ($F_{2, 29} = 17.32$, $p < 0.001$). *Post-hoc* analyses detected significant differences between the RLN3 and aCSF groups ($p < 0.0001$) and between the RLN3 and RXFP3-A2 groups ($p < 0.0001$), but not between the aCSF and RXFP3-A2 groups ($p > 0.05$).

The plasma corticosterone levels were increased by RLN3 (51.9 ± 6.2 ng/ml) but not by RXFP3-A2 (17.1 ± 3.4 ng/ml) compared to the aCSF-injected

group (18.4 ± 5.6 ng/ml; Fig. 2.2B). The one-way ANOVA revealed significant effects of treatment on plasma corticosterone ($F_{2,\ 29}$ = 14.83, $p < 0.0001$). According to the *post-hoc* analyses, the plasma corticosterone levels were significantly higher after RLN3 administration compared to RXFP3-A2 ($p < 0.0001$) and aCSF ($p < 0.0001$), but they were not different between the RXFP3-A2- and aCSF-injected groups ($p > 0.05$).

Oxytocin plasma levels were significantly affected by treatments ($F_{2,\ 29}$ = 3.89, p = 0.0326, one-way ANOVA; Fig. 2.2C). *Post-hoc* analyses revealed that oxytocin levels were significantly ($p < 0.01$) decreased by RXFP3-A2 (8.4 ± 0.4 ng/ml) but not by RLN3 ($p > 0.05$; 9.3 ± 0.4 ng/ml) relative to levels in aCSF-injected group (10.8 ± 0.9 ng/ml). However, there was no significant difference ($p > 0.05$) between oxytocin levels in the RLN3- and RXFP3-A2-injected groups.

Although there was a tendency for a decrease in plasma vasopressin after injection of RLN3 (16.4 ± 14.6 pg/ml) or RXFR3-A2 (49.0 ± 18.7 pg/ml) relative to aCSF control injection (103.3 ± 49.3 pg/ml), these changes did not reach significance (Fig. 2.2D). One-way ANOVA ($F_{2,\ 29}$ = 1.95, p = 0.1609) and *post-hoc* analyses did not reveal significant effects of treatments on plasma vasopressin. The wide variation if vasopressin data is a likely explanation for the lack of difference between groups. Similarly, plasma epinephrine ($F_{2,\ 29}$ = 0.35, p = 0.7096; Fig. 2.2E) and norepinephrine ($F_{2,\ 29}$ = 1.832, p = 0.1817; Fig. 2.2F) levels were not affected by icv injections of RLN3 or RXFP3-A2.

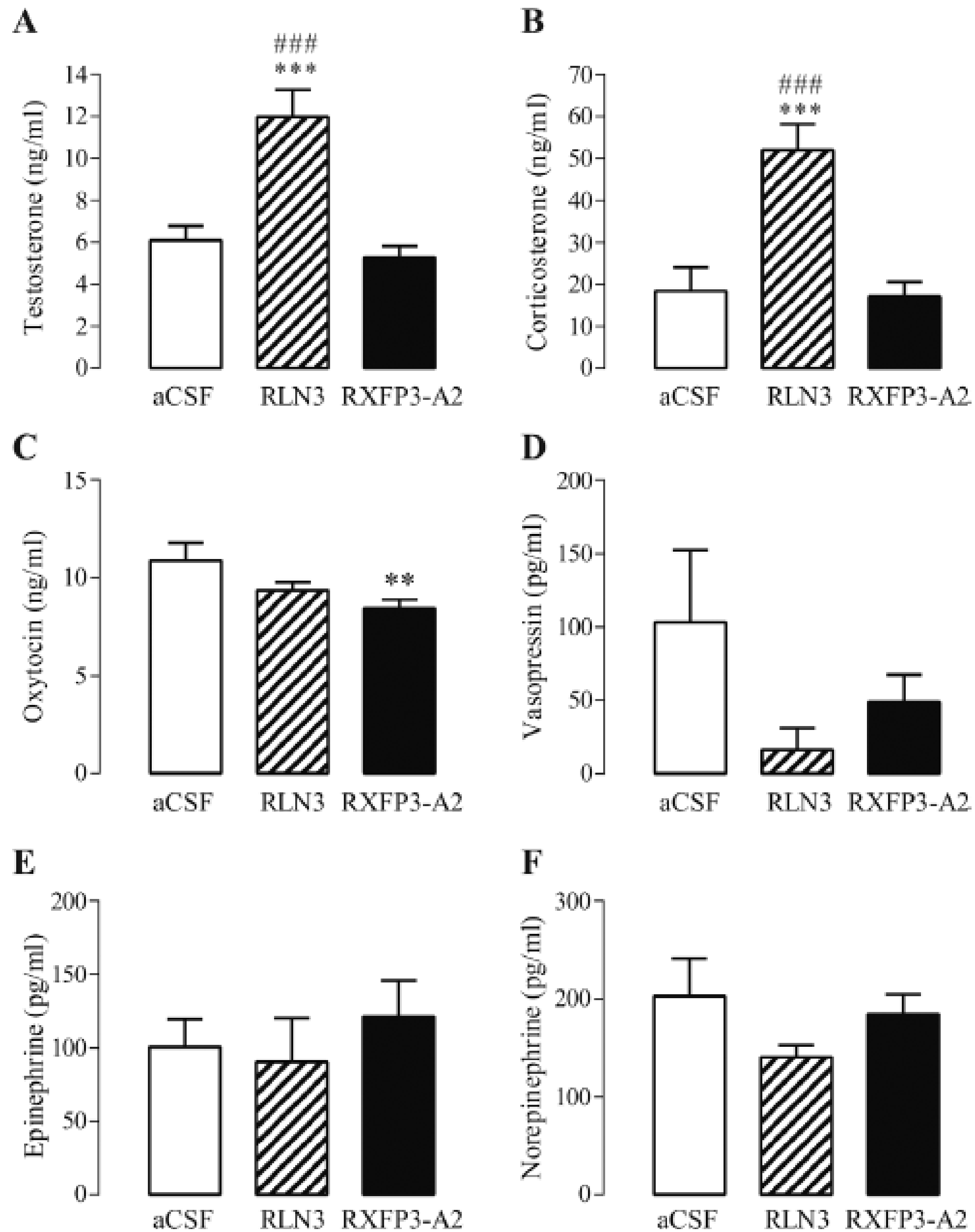

Figure 2.2. Plasma levels of testosterone (A), corticosterone (B), oxytocin (C), vasopressin (D), epinephrine (E), and norepinephrine (F) measured 30 min after icv administration of aCSF, RLN3 or RXFP3-A2

**, $p < 0.01$; ***, $p < 0.001$: significantly different from the aCSF-injected rats. ###, $p < 0.001$: significantly different from the RXFP3-A2-injected rats.

2.5.3. c-*fos* mRNA expression in the PVN and SON

At 30 min after the icv administration of RLN3, the expression of c-*fos* mRNA was increased in the magnocellular (Fig. 2.3 A, D) and parvocellular (Fig. 2.3 B, D) parts of the PVN and in the SON (Fig. 3C, E) compared to aCSF-injected rats. However, no difference in the levels of c-*fos* mRNA was observed in these regions between rats that received icv injections of RXFP3-A2 and aCSF. One-way ANOVA revealed a significant effect of the treatment on c-*fos* mRNA for the magnocellular ($F_{2,\ 29}$ = 26.93, $p < 0.0001$) and parvocellular ($F_{2,\ 29}$ = 3.867, $p = 0.0324$) parts of PVN and for the SON ($F_{2,\ 29}$ = 14.32, $p < 0.0001$). The *post-hoc* analyses revealed a significant increase in c-*fos* mRNA expression in the RLN3 group compared to the aCSF group ($p < 0.0001$ for the PVNm, $p < 0.05$ for the PVNp, and $p < 0.001$ for the SON) and compared to the RXFP3-A2 group ($p < 0.0001$ for the PVNm and the SON; $p < 0.05$ for the PVNp) for these three regions. Conversely, no significant variation was observed in the c-*fos* mRNA expression in the RXFP3-A2 group compared to the aCSF-injected rats ($p > 0.05$ for the PVNm, PVNp, and SON).

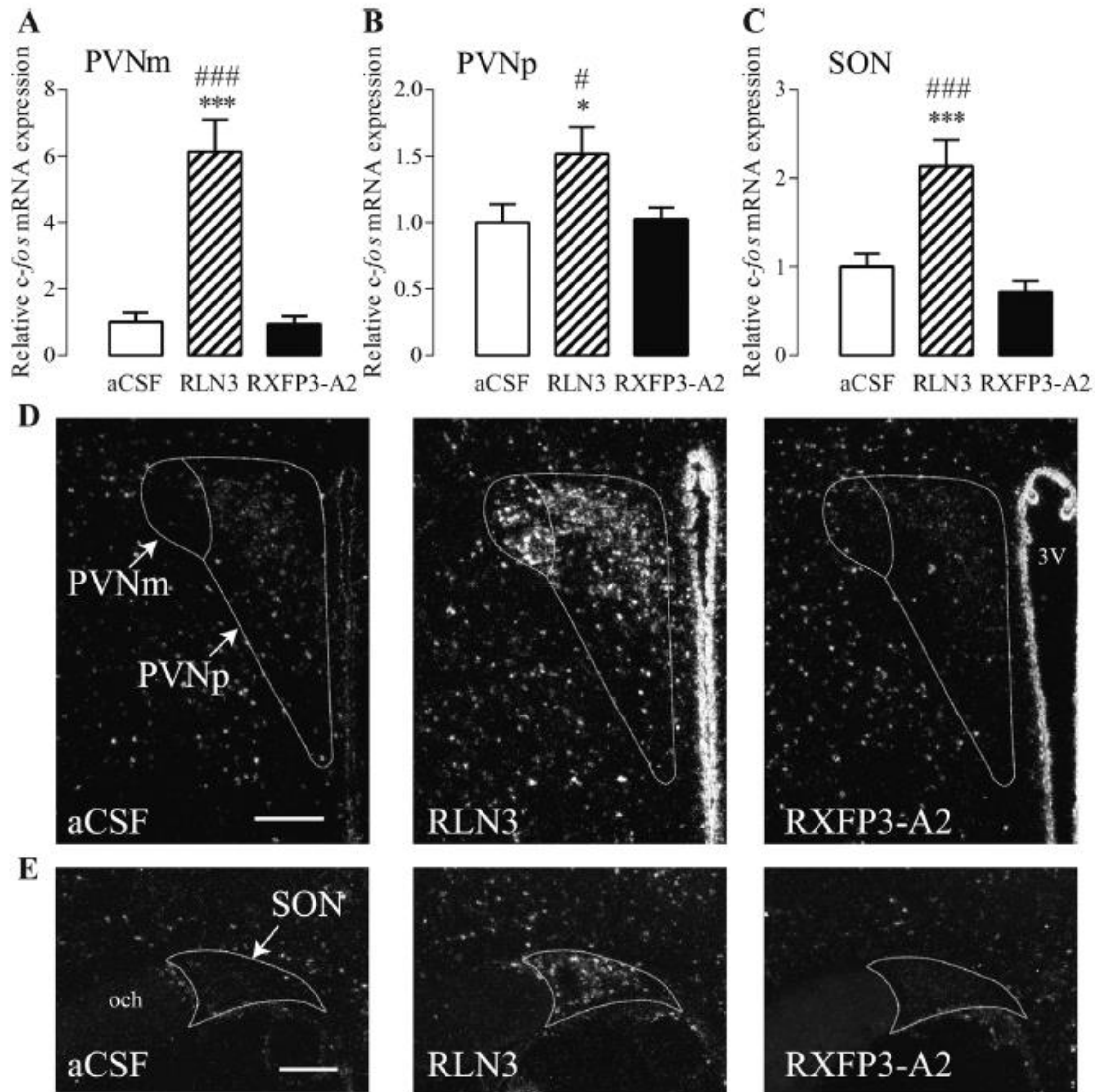

Figure 2.3. Relative levels of c-*fos* mRNA expression in the magnocellular (PVNm, *A*) and parvocellular (PVNp, *B*) hypothalamic paraventricular nucleus and the supraoptic (SON, *C*) hypothalamic nucleus 30 min after icv administration of aCSF (n = 10), RLN3 (n = 10), or RXFP3-A2 (n = 12)

Dark-field micrographs illustrate the positive hybridization signal of c-*fos* mRNA in the PVNm and PVNp (*D*) and SON (*E*). *, $p < 0.05$; ***, $p < 0.001$: significantly different from the aCSF-injected rats. #, $p < 0.05$; ###, $p < 0.001$: significantly different from the RXFP3-A2-injected rats. 3V, third ventricle; och, optic chiasm. Scale bars, 250 µm.

2.5.4. c-*fos* mRNA expression in the LHA

Icv injections of RLN3 and RXFP3-A2 induced c-*fos* mRNA expression in the perifornical LHA (Fig. 2.4 B, C), but not in the lateral LHA (Fig. 2.4 A, C). One-way ANOVA revealed a significant effect of the treatment on the c-*fos* levels in the perifornical part ($F_{2, 23} = 7.547$, $p = 0.003$), but not the lateral part ($F_{2, 26} = 1.123$, $p = 0.3406$) of the LHA. The *post-hoc* analyses revealed a significantly larger increase in c-*fos* mRNA expression in the RLN3 ($p < 0.001$) and the RXFP3-A2 ($p < 0.05$) groups compared to aCSF-injected rats in the perifornical LHA but not lateral LHA ($p > 0.05$). No significant differences in c-*fos* mRNA were observed between the RLN3 and RXFP3-A2 groups in the perifornical ($p > 0.05$) or lateral ($p > 0.05$) LHA.

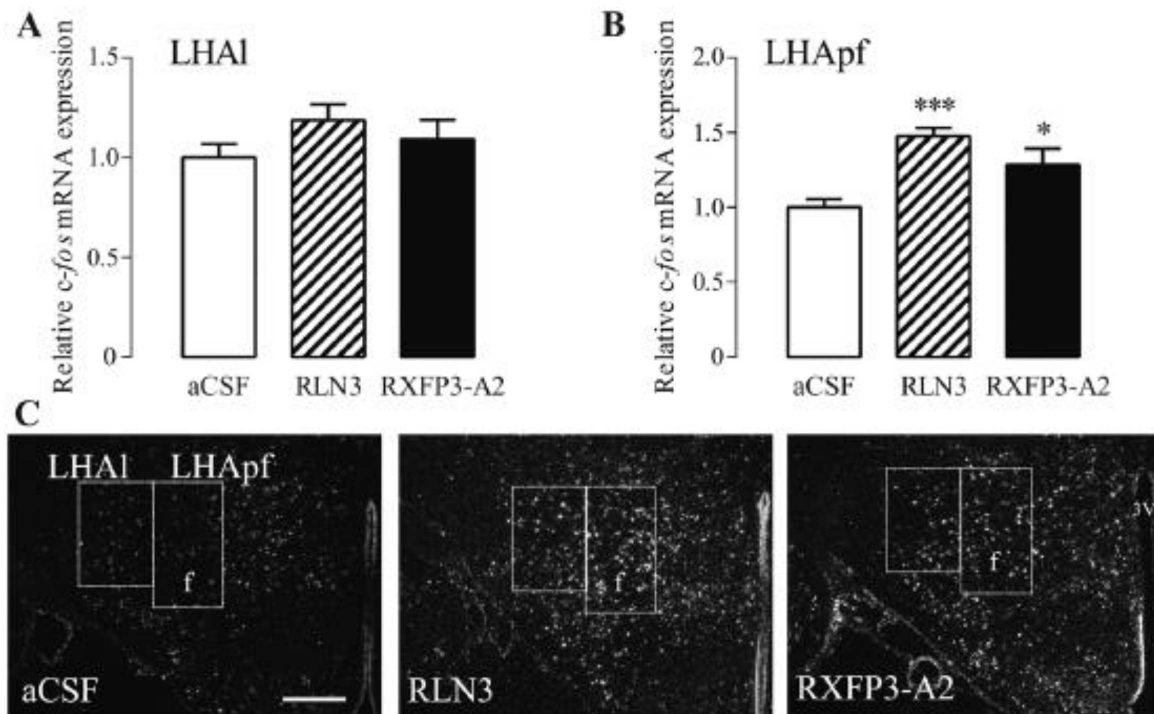

Figure 2.4. Relative levels of c-*fos* mRNA expression in the lateral (LHAl, *A*) and perifornical (LHApf, *B*) regions of the lateral hypothalamic area 30 min after icv administration of aCSF (n = 10), RLN3 (n = 10), or RXFP3-A2 (n = 12)

Dark-field micrographs illustrate the positive hybridization signal of c-*fos* mRNA in the LHAl and LHApf (*C*). *, $p < 0.05$; ***, $p < 0.001$: significantly different from the aCSF-injected rats. 3V, third ventricle; f, fornix. Scale bar, 500 µm.

2.5.5. c-*fos* mRNA expression in the ventral MPA and OVLT

The expression of c-*fos* mRNA in the ventral MPA (Fig. 2.5 A, C) and the OVLT (Fig. 2.5 B, C) was altered by the icv administration of RLN3. One-way ANOVA revealed a significant effect of the treatment on the c-*fos* expression for the ventral MPA ($F_{2, 28} = 5.155$, $p = 0.0124$) and the OVLT ($F_{2, 28} = 35.95$, $p < 0.0001$). The *post-hoc* analyses detected a significant increase in c-*fos* mRNA expression in these regions in the RLN3-injected group compared to the aCSF group ($p < 0.01$ for the ventral MPA and $p < 0.001$ for the OVLT) and RXFP3-A2 group ($p < 0.05$ for the ventral MPA and $p < 0.001$ for the OVLT). No significant differences were observed between the RXFP3-A2 and aCSF-control groups in the ventral MPA or OVLT ($p > 0.05$).

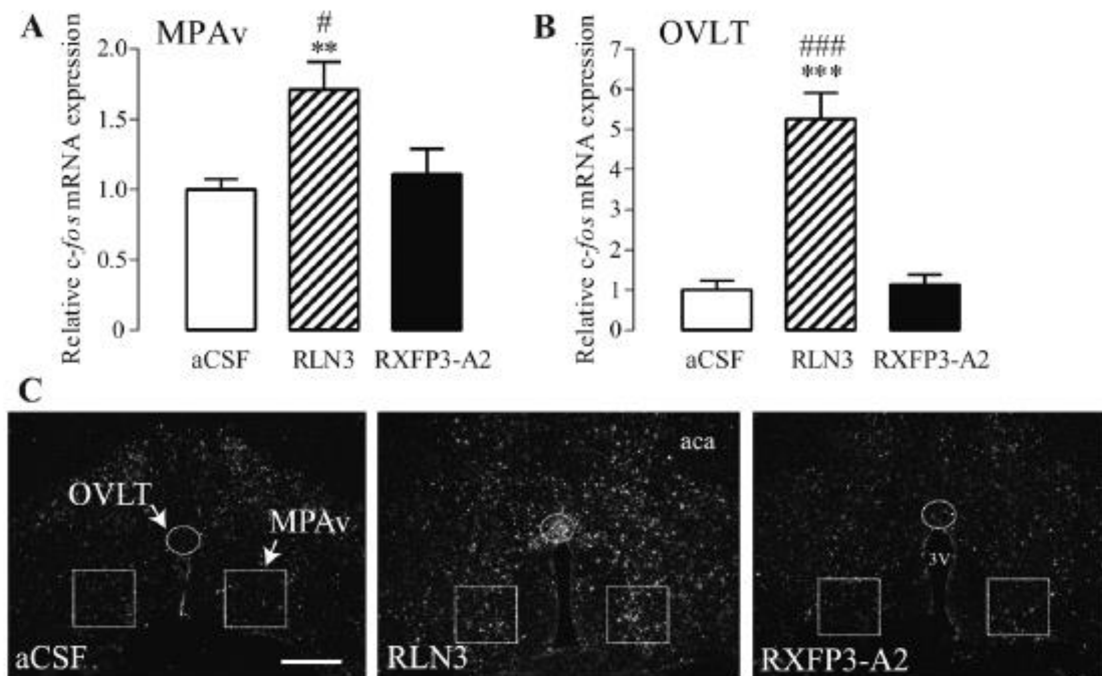

Figure 2.5. Relative levels c-*fos* mRNA expression in the ventral region of the medial preoptic area (MPAv, *A*) and the organum vasculosum of the lamina terminalis

(OVLT, *B*) 30 min after icv administration of aCSF (n = 10), RLN3 (n = 10), or RXFP3-A2 (n = 12)

Dark-field micrographs illustrate the positive hybridization signal of c-*fos* mRNA in the MPAv and the OVLT 30 min after icv injection of aCSF, RLN3 or RXFP3-A2 (*C*). **, $p < 0.01$, ***, $p < 0.001$: significantly different from the aCSF-injected rats. #, $p < 0.05$, ###, $p < 0.001$: significantly different from the RXFP3-A2-injected rats. aca, anterior part of the anterior commissure; 3V, third ventricle. Scale bar, 500 µm.

2.6. Discussion

This study investigated the effects of the central (icv) administration of the selective RXFP3 agonist, RXFP3-A2, and the native RLN3 peptide, which activates RXFP1 and RXFP3 receptors, on food and water intake and on hypothalamic c-*fos* mRNA expression. In line with earlier reports (McGowan et al., 2005b, Bathgate et al., 2006, Otsubo et al., 2010, Smith et al., 2011b), this study has demonstrated that the icv injection of RLN3 induced a significant increase in food and water intake. Conversely, the administration of RXFP3-A2 strongly stimulated food intake, but did not affect water intake. The observed increase in food intake after the stimulation of RXFP3 is in agreement with the orexigenic effects of RLN3 and RXFP3-A2 that we and others reported earlier (McGowan et al., 2007c, Shabanpoor et al., 2012b, Calvez et al., 2015a, Lenglos et al., 2014). However, a direct comparison of the effects of the central administration of equimolar amounts of RLN3 and RXFP3-A2 revealed a significantly higher increase in cumulative food intake in response to RXFP3-A2 at 30 and 60 but not 120 min post-injection than to RLN3.

Differential effects of RLN3 and RXFP3-A2 may depend on a possible difference in tissue penetration for these peptides. However, the absence of differences at 120 min after injections may rather depend on a “ceiling effect” or a reaching of a maximum in food intake capacity. Similarly, different concentrations of RLN3 produced differential effects on cumulative food intake at 30 and 60 min

after injections, but not at 120 min or later (McGowan et al., 2005b, Lenglos et al., 2014).

Significant differences in food intake produced by RLN3 and RXFP3-A2 might be rather depend on the binding of RLN3 to its cognate receptor RXFP3 *and* to RXFP1, the native receptor for relaxin, which interferes with or offsets the effects of “pure” RXFP3 activation. In fact, icv administration of relaxin has been reported to reduce food intake in male rats (McGowan et al., 2010). This anorexigenic effect of relaxin may be mediated *via* actions within the PVN because the administration of relaxin into this region decreased food intake in the early dark phase (McGowan et al., 2010), and there is a high density of RXFP1 in the rat PVN (Ma et al., 2006b). The present results support the potential involvement of RXFP1 in the activation of the PVN because an increase in c-*fos* mRNA expression was observed in the parvocellular and magnocellular parts of PVN after the icv administration of RLN3 but not RXFP3-A2, which might be associated with the activation of both RXFP1 and RXFP3 rather than the activation of RXFP3 alone. In addition, induction of expression of *c-fos* mRNA in the magnocellular neurons may be triggered *via* activation of RXFP1 in the OVLT (Sunn et al., 2002).

RXFP1 and RXFP3 are G-protein-coupled receptors that induce different intracellular responses (Bathgate et al., 2013c). The activation of RXFP1 induces an increase in intracellular cAMP, while the activation of RXFP3 induces an inhibition of intracellular cAMP accumulation, suggesting an excitatory and inhibitory impact on target cells, respectively (Liu et al., 2003a, Bathgate et al., 2013c). Recent neurophysiological recordings revealed an inhibition of oxytocin and vasopressin PVN neurons in response to both RLN3 and RXFP3-A2; however, when RXFP3 was blocked by the specific RXFP3 antagonist R3 B1-22R, the addition of RLN3 caused an increase in action potential firing (Kania et al., 2017). RXFP3-induced inhibition was dependent on an outward calcium-dependent potassium current, and the blockade of $G_{i/o}$-proteins in PVN neurons prevented the inhibitory action of RXFP3-A2 (Kania et al., 2017).

In the current study, the icv administration of RXFP3-A2 did not induce the expression of c-*fos* mRNA in the PVN, consistent with the inhibition of PVN neurons after the activation of RXFP3. Conversely, the increase in c-*fos* mRNA expression in the PVN in response to RLN3 may be induced by the binding of this peptide to RXFP1 in the OVLT (Sunn et al., 2002). Parvocellular PVN neurons may affect food intake and energy expenditure due to the involvement of one or more of its several neuron populations, including CRF, oxytocin, and vasopressin neurons (Aoyagi et al., 2009, Richard and Timofeeva, 2009, Olszewski et al., 2010).

Activation of CRF neurons in the PVN is associated with decreases in food intake (Pelleymounter et al., 2000, Richard and Timofeeva, 2009). Similarly, oxytocin acts as an anorexigenic signal in both animals and humans (Olszewski et al., 2010, Sabatier et al., 2013, Kim et al., 2015, Klockars et al., 2015). Vasopressin is also considered an anorexigenic factor (Aoyagi et al., 2009), and icv or intraperitoneal (ip) injections of vasopressin lead to a reduction in food intake (Meyer et al., 1989, Ikemura et al., 2004). Administration of RLN3 activates the CRF neurons and increases the levels of CRF mRNA in the parvocellular PVN in rats (Watanabe et al., 2011). Conversely, the production of a selective RXFP3 agonist, R3/I5, by an adeno-associated virus within the PVN significantly reduced the expression of oxytocin and vasopressin mRNA (Ganella et al., 2013). Therefore, the induction of c-*fos* mRNA expression in the PVN in response to RLN3 administration may depend on the RXFP1-related activation of the CRF neurons (Watanabe et al., 2011) that would partially blunt orexigenic effects of RLN3. Conversely, the effects of RXFP3-A2 may depend on the activation of the RXFP3 inhibitory pathway via $G_{i/o}$-protein coupling (Liu et al., 2003a) in oxytocin and vasopressin neurons, leading to a higher increase in food intake.

The present results indicates a stronger potential of RXFP3-A2 than RLN3 to decrease plasma oxytocin levels. The levels of vasopressin were not significantly altered by either RLN3 or RXFP3-A2. The lack of significant effects may due to the blood sampling during the early light phase when the plasma levels of vasopressin are at their nadir (Greeley et al., 1982). Future estimation of the

effects of RLN3 and RXFP3-A2 on the levels of plasma vasopressin should be performed during the late light phase or the beginning of the dark phase, when plasma vasopressin increases and stabilizes (Greeley et al., 1982).

In addition to their effects on food intake, the CRF neurons in the parvocellular PVN are involved in the regulation of the HPA axis. This axis includes the hypophysiotropic parvocellular CRF neurons that release CRF from axonal terminals at the level of median eminence from where CRF is transported to the pituitary corticotropic cells, producing adrenocorticotropic hormone (ACTH), which stimulates adrenal glucocorticoid (cortisol in humans and corticosterone in rats) synthesis and release (Richard et al., 2002, Richard and Timofeeva, 2009). In addition to the activation of c-*fos* mRNA expression in the parvocellular PVN, RLN3 injections significantly increased the levels of plasma corticosterone.

These results are in agreement with the reported activation of the HPA axis activity in male rats in response to the intracranial administration of RLN3 (Watanabe et al., 2011a, McGowan et al., 2014, Lenglos et al., 2014). The present data suggest that the activation of the HPA axis in response to RLN3 depends on its binding to the RXFP1 because the activation of RXFP3 by its selective agonist RXFP3-A2 did not increase corticosterone plasma levels or c-*fos* mRNA expression in the PVN. This conclusion is supported by an earlier study that reported an increase in plasma corticosterone levels after the icv administration of relaxin, the cognate ligand of RXFP1 (McGowan et al., 2014).

Although activation of the NI was associated with heightened arousal and locomotion (Ma et al., 2017a), there is no direct evidence that RLN3 changes the activity of the autonomic nervous system. Even though intra-PVN administration of RLN3 significantly decreased the plasma levels of thyroid stimulating hormone (McGowan et al., 2006), oxygen consumption and respiratory exchange ratio were not affected by intra-PVN RLN3 (McGowan et al., 2006). Similarly, the current study did not detect significant changes in plasma epinephrine and norepinephrine levels after icv administration of RLN3 and RXFP3-A2.

In agreement with previous reports (Bathgate et al., 2006, Otsubo et al., 2010), a significant increase in water intake after the icv administration of RLN3 was observed. However, the present study suggests a particular involvement of RXFP1 in water intake. In fact, injection of RLN3 that activates RXFP1 and RXFP3 led to a strong dipsogenic effect. Conversely, the selective stimulation of RXFP3, but not RXFP1, by RXFP3-A2 did not change the water intake relative to the aCSF-injected control group. Notably, relaxin stimulates water intake but not food intake (McGowan et al., 2010).

The administration of both relaxin and RLN3 increased the expression of c-*fos* mRNA in the magnocellular neurons of SON and PVN (McKinley et al., 1997, Sunn et al., 2002, Otsubo et al., 2010). The magnocellular neurons produce vasopressin and oxytocin, which are directly involved in fluid regulation (Stricker and Sved, 2002, McKinley et al., 2004, Olszewski et al., 2010). There is evidence that activation of magnocellular neurons in the SON and PVN in response to relaxin and its dipsogenic effects depend on activation of the circumventricular organs such as the OVLT and subfornical organ (SFO), the small brain areas with disrupted blood-brain barrier (McKinley et al., 2001). In fact, ablation of the SFO prevented dipsogenic effects of intravenous injection of relaxin, while ablation of the OVLT significantly decreased c-Fos expression in magnocellular neurons (Sunn et al., 2002).

The present results do not exclude a possibility that the magnocellular regions were activated by RLN3 *via* the OVLT because the OVLT displayed a significant induction of c-*fos* mRNA expression in response to icv administration of RLN3 but not RXFP3-A2. The OVLT directly projects to the SON and magnocellular PVN and integrity of these connections are important for the activity of magnocellular neurons (Timofeeva et al., 2005). Strong activation of c-*fos* mRNA in the magnocellular PVN and SON and in the OVLT in response to RLN3 but not RXFP3-A2 suggests the dipsogenic effects are related to RXFP1, but not RXFP3, activation. This conclusion is supported by observations that icv administration of relaxin had comparable (Otsubo et al., 2010) or stronger

(McGowan et al., 2005) effects on water intake stimulation than produced by equimolar RLN3 in rats.

The central administration of RLN3 and RXFP3-A2 may stimulate food intake due to actions within the LHA, the brain region strongly involved in appetite regulation. The electrical stimulation of LHA, traditionally called the “hunger center,” increases appetite, while the inactivation of LHA produces anorexigenic effects (Hoebel and Teitelbaum, 1962, Teitelbaum and Epstein, 1962). The LHA neurons express orexin and melanin-concentrating hormone (MCH), which are neuropeptides with strong orexigenic effects (Bittencourt et al., 1992, Peyron et al., 1998). The LHA contains RXFP3 but not RXFP1 binding sites (Ganella et al., 2013a) that may limit the effects of RLN3 to RXFP3 in this region.

In the present study, c-*fos* mRNA expression was significantly increased in the perifornical but not in the lateral subdivision of LHA after icv administration of both RLN3 and RXFP3-A2. Both perifornical and lateral LHA contain orexin and MCH neurons (Calvez et al., 2015), but there is evidence of a particular role of the LHA subregions in eating behaviors. Thus, hyperphagic obesity-prone rats have more orexin-positive neurons in the perifornical than in the lateral LHA (Wortley et al., 2003). Moreover, obesity induced by a high-fat diet significantly increased the number of orexin neurons in the perifornical but not the lateral LHA (Wortley et al., 2003). In addition, an increased activity of orexin neurons in the perifornical but not the lateral LHA was detected in rats during the dark active circadian phase when their eating behavior increases (Estabrooke et al., 2001). Accordingly, rats that overeat a high-fat diet displayed elevated behavioral arousal and an increased expression of orexin mRNA in the perifornical but not the lateral LHA (Morganstern et al., 2010). The direct administration of RLN3 into the LHA did not affect food intake (McGowan et al., 2007), but these injections were aimed at the lateral LHA, where a significant effect of RLN3 or RXFP3-A2 administration on feeding activity was not observed. It is also possible that induction of c-*fos* mRNA expression in the LHA in response to RLN3 and RXFP2-A2 may depend on multisynaptic circuitry. The present results particularly implicate the perifornical LHA, which is

strongly interconnected with the brain structures of the gustatory and metabolic networks including the infralimbic and agranular insular areas, the central nucleus of the amygdala, the bed nucleus of the stria terminalis, the substantia innominata and the parabrachial nucleus (Hahn and Swanson, 2010).

In a previous study, the icv administration of 800 pmol RLN3 in male rats increased c-*fos* mRNA expression in the perifornical LHA by 14% compared to the control (Calvez et al., 2015); however, this increase was not statistically significant. In the present study, a higher (1.1 nmol) amount of RLN3 significantly increased c-*fos* mRNA expression in the perifornical LHA by 47%, which was statistically significant compared to the control aCSF-injected group. The same dose (1.1 nmol) of RXFP3-A2 also significantly increased c-*fos* mRNA expression in the perifornical LHA. The activation by RXFP3-A2 of perifornical LHA neurons, which contain the orexigenic neuropeptides orexin and MCH, and no activation of the PVN, which contains anorexigenic CRF, oxytocin, and vasopressin neurons, may reinforce its orexigenic effects. Conversely, a coinciding activation of the PVN and the LHA by RLN3 may counteract its orexigenic effects, which were lower than those of RXFP3-A2.

RLN3 has also a potential role in reproductive function. The icv administration of RLN3 stimulates the HPG axis by increasing the LH (McGowan et al., 2008) and FSH (Calvez et al., 2016a) plasma levels in male rats. These effects likely depend on the stimulation of GnRH neurons because the administration of RLN3 increases GnRH release from hypothalamic explants, and an increase in plasma LH and testosterone induced by the icv injection of RLN3 was blocked by pre-treatment with a peripheral GnRH antagonist (McGowan et al., 2008). GnRH is expressed by neurons in the MPA, and in this region, only a low number of GnRH-immunoreactive neurons (about 3%) co-express RXFP3 mRNA (Calvez et al., 2016a). In addition to RXFP3, MPA neurons express RXFP1 mRNA (Osheroff and Phillips, 1991, Ganella et al., 2013a). The induction of c-*fos* mRNA expression in the MPA in response to the icv administration of RLN3 but not RXFP3-A2 suggests that RXFP1 may be involved in MPA activation. The activation of the MPA by

RLN3 was accompanied by a strong increase in plasma testosterone. Conversely, the icv administration of an equimolar dose of RXFP3-A2 did not affect plasma levels of testosterone. Although the present data suggest that RXFP1 plays a role in the activation of the HPG axis by RLN3, the icv administration of relaxin, which selectively activates RXFP1, produced a consistent but statistically non-significant increase in LH and testosterone plasma levels (McGowan et al., 2008), suggesting that a synergistic effect of RXFP1 and RXFP3 may be required for the effects of RLN3 within the MPA on the HPG axis.

2.7. Conclusions

In summary, this study has further implicated RXFP1 in the regulation of water intake and activation of the HPA and HPG axes by RLN3. A strong stimulation of food intake produced by RXFP3-A2 may involve the activation of the perifornical LHA but not the brain regions that drive anorexigenic effects. The reduced stimulation of food intake produced by RLN3 compared to RXFP3-A2 may depend on the broader neuronal activation produced by RLN3, which involves the neuronal groups that produce orexigenic and anorexigenic effects.

2.8. Acknowledgements

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General discussion, perspectives and conclusion

Discussion

The experiments described in this thesis give continuity to previous studies conducted at our laboratory. We have characterized the physiological and neurobiological basis of diet and sex-related differences in hyperphagia susceptibility, demonstrating an important role of relaxin-3 in mechanisms of stress regulation and food intake.

The past studies used two approaches in order to trigger an increase of RLN3 mRNA levels in the brain: pharmacological, where icv RLN3 injections were used to mimic non-pathological (acute) and pathological (chronic) scenarios; and stress-induced, where rats were submitted to unpredictable stressful episodes, triggering RLN3 mRNA expression. Multiple feeding behaviors, c-*fos* mRNA brain activity, and peptides' levels were measured, shedding light on the putative role of the RLN3/RXFP3 system in stress-induced hyperphagia (Lenglos et al., 2014, Calvez et al., 2015, Calvez et al., 2016c).

Just like continuous RLN3 secretion, the lack of RLN3 could also represent a pathological state. In this regard, it has been shown that silencing the RLN3-positive neurons in the NI of adult male rats did not affect food intake, body weight, or anxiety (Callander et al., 2015), but the effect in females was still unknown and was addressed in the first study described in this thesis.

In this context, "Effects of silencing relaxin-3 production in *nucleus incertus* neurons on food intake, body weight, anxiety-like behaviour and limbic brain activity in female rats" has revealed that depletion of RLN3 mRNA/peptide expression in NI neurons of female rats produced a decrease in body weight, an imbalance in food intake, and an increase in levels of anxiety-like behavior in a paradigm with no escape. Furthermore, the depletion of NI RLN3 disrupted corticosterone regulation and levels of *c-fos*, OT, and AVP mRNA in brain regions involved in stress and food intake regulation, reinforcing the important role of

RLN3-positive neurons in the NI in fine-tuning stress responses and food intake regulation.

Importantly, while we have been studying RLN3 effects in several behaviors, there was a lack of understanding regarding the involvement or contribution of RXFP1 in these effects. Recently, a selective agonist of human RLN3 has been developed which can specifically activate RXFP3 over RXFP1, and is named analogue 2 (A2) (Shabanpoor et al., 2012). This molecule induces an increase in food intake after icv administration in satiated rats.

The second study, “Differential effects of relaxin-3 and a selective relaxin-3 receptor agonist on food and water intake and hypothalamic neuronal activity in rats,” clarifies the role of RXFP3 in food intake, water intake, and the HPA and HPG axes in males. The significance of this work was in differentiating relaxin-3 effects when coupling to RXFP3 and RXFP1.

Our recent studies revealed that the icv injections of a non-specific agonist of RXFP3 in male rats induced an increase in food and water intake, while the injections of a specific agonist induced an increase only in food intake. The data confirmed the orexigenic effect of RLN3 already described in the literature (McGowan et al., 2007a, Lenglos et al., 2014b, Calvez et al., 2015b), but also established that this response is dependent on the RXFP3 receptor because the orexigenic effects were stronger after the injections of A2, the specific agonist of RXFP3. The differential effects on food intake observed between RLN3- and A2-injected rats can be explained by the binding of RLN3 to another receptor, RXFP1. Our study also showed that RXFP1 receptor was specifically implicated in water intake, as there is no response after A2 injection.

Finally, RLN3 also plays a role in reproductive functions. An icv administration of RLN3 stimulates the release of GnRH from hypothalamic explants in male rats (McGowan et al., 2008a). GnRH neurons are located in the MPA, where our experiments showed an increase in c-Fos mRNA expression in male rats after icv injection of RLN3, as well as an increase in plasma testosterone

levels. RLN3 may also have a direct effect on neurons in the MPA, as data in the literature described sparse RLN3 fibers in this brain region (Ma et al., 2007). However, GnRH neurons have interactions with the PVN (Dobson et al., 2003), and intraparaventricular injection of RLN3 stimulated the HPG axis in male rats (McGowan et al., 2008).

In summary, the second study shows that involvement of RLN3 in stress-induced hyperphagia in male rats might depend on its interaction with RXFP1 and RXFP3, as RXFP3 alone did not affect the HPA axis (Fig. 3.1.). Finally, it is important to note that these findings are based on exogenous RLN3 injections, and that whether endogenous RLN3 binds to RXFP1 is still unknown. Additionally, future research using females would be beneficial in order to address sex-specific differences.

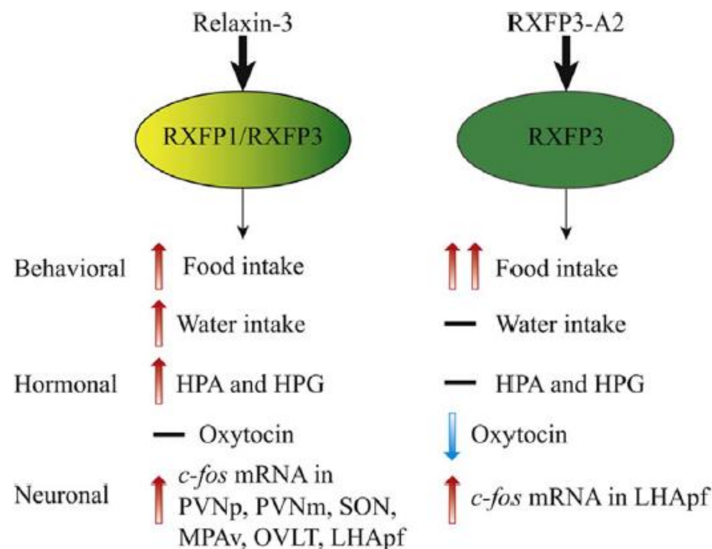

Figure 3.1. Summary of “Differential effects of relaxin-3 and a selective relaxin-3 receptor agonist on food and water intake and hypothalamic neuronal activity in rats”

Importantly, the methodological approaches chosen for these studies were based on availability and reliability. For example, we used in situ hybridization to detect mRNA and c-*fos* expression in the areas of interest. c-*fos* is one of many products of the immediate early gene family that have been reliably used as functional an anatomical mapping marker of activated brain regions (Sagar et al., 1988). *c-fos* mRNA is expressed within minutes, whereas the peak in c-fos protein occurs between 1-3 hours following stimulus delivery, providing a clear temporal boundary between their sampling (Kovacs, 1998).

The decision to use a knock-down model in chapter one rather than a knock-out model was made in order to avoid adaptation during the developmental phase. Additionally, it is important to emphasize that the relaxin-3 field is still considered a new one despite having been discovered in 2002, since relatively little is still known. The RLN3 knock-down model was first developed in 2012, when the effects of silencing RLN3-positive neurons in the NI persisted for fourteen weeks. (Callander et al. 2012).

Enzyme-linked immunosorbent assay (ELISA) has been adopted to detect peripheral levels of glucocorticoids, hormones and peptides. All ELISA kits were previously validated in our earlier studies and cited in the previous chapters.

Perspectives

Because of its sex-specific effect on HPA axis regulation and food intake, RLN3 would represent an ideal therapeutic approach. Indeed, BEDs are generally associated with body weight gain, anxiety, and mood disorders. It has been shown that the RLN3/RXFP3 system plays an important role in regulating these physiological responses. Since the increase in RLN3 expression is mediated by stress (Tanaka et al., 2005), the production of RXFP3-specific antagonists could limit the episodes of stress-induced hyperphagia episodes often found in bulimia. However, unlike other peptides such as leptin or insulin, RLN3 does not have a transporter, and whether it passes the blood-brain barrier is still unknown (Ganella et al., 2012). The minimization of RXFP3-specific antagonists while maintaining the same potency and lifespan of RLN3 remains a challenge to be met in order to plan clinical trials in humans. In addition, since RXFP3-specific agonists and antagonists induce a different effect on food intake between mice and rats, studies on the effect of these agonists in humans would allow a more detailed evaluation of their therapeutic potential. Until now, the available specific agonists and antagonists were neuropeptides which have been injected directly into the brain. Future development of small molecules targeting the RXFP3 receptor would be of great interest, as a non-invasive technique would allow the expansion of pre-clinical research to clinical trials.

Conclusion

These discoveries regarding the role of the RLN3/RXFP3 system in rats open up particularly interesting therapeutic prospects in populations at risk of developing stress-induced eating disorders. Synthesis of RXFP3 antagonists capable of crossing the blood-brain barrier could allow us to transition into clinical trials in humans, as part of the ongoing effort to combat stress-induced binge eating.

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