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OFFSPRING PROGRAMMING OF CENTRAL AND PERIPHERAL NUTRIENT-SENSING PATHWAYS BY THE EXPOSURE TO PERINATAL OBESOGENIC ENVIRONMENTS

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular orientada pelo/a Professor Doutor Paulo Nuno Centeio Matafome e pelo Professor Doutor Carlos Manuel Marques Palmeira apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, Departamento de Ciências da Vida

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Master in Cellular and Molecular Biology

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Diana Sousa: *Obesity – Offspring programming of central and peripheral nutrient-sensing pathways by the exposure to perinatal obesogenic environments*

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Oral Communication

1. **Sousa D**., Amaro A., Júnior M. F., Pereira S., Rocha M., Barra C., Mello-Gomes R., Oliveira P., Matafome P. *Exposure to obesogenic environments during perinatal development modulates offspring nutrient-sensing pathways in adipose tissue.*
56th Annual Scientific Meeting of the European Society for Clinical Investigation, Bari, Italy, 8th – 10th June 2022.

2. **Sousa D**., Amaro A., Júnior M. F., Pereira S., Rocha M., Barra C., Mello-Gomes R., Oliveira P., Matafome P. *Perinatal development in obesogenic environments causes changes in nutrient sensitivity pathways.*
18th Portuguese Diabetes Congress, Vilamoura, Portugal 10th – 12th March, 2022

3. Amaro A., Rocha M., **Sousa D**., Júnior M. F., Barra C., Monteiro T., Mello-Gomes R., Baptista F., Matafome P. *Neurometabolic and behavioural alterations in the adolescent offspring upon maternal glycation.*
56th Annual Scientific Meeting of the European Society for Clinical Investigation, Bari, Italy, 8th – 10th June 2022.

4. Amaro A., Rocha M., **Sousa D**., Júnior M. F., Barra C., Monteiro T., Mello-Gomes R., Baptista F., Matafome P. *Maternal glycation accelerates neurodevelopment in offspring, inducing metabolic changes and less anxious behavior in adolescence.*
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5. Rocha M., Amaro A., Júnior M. F., Pereira S., **Sousa D**., Júnior M. F., Barra C., Mello-Gomes R., Oliveira P., Matafome P. *Hypercaloric maternal diet during pregnancy and lactation and development of insulin resistance: role of dietary glycotoxins.*
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This work was also selected for a short oral communication to the 58th European Association for the Study of Diabetes Annual Meeting which will take place in Stockholm, Sweden, 19th – 23rd September 2022.

Data of this thesis will be included in the following manuscripts:

Marcos Divino Ferreira-Junior; Leandro Rodrigues Borelli; Daniela Rosendo-Silva; Keilah Valéria Naves Cavalcante; **Diana Sousa**; Carlos Henrique Xavier; Paulo Matafome, Rodrigo Mello Gomes. ***The role of Ghrelin Signalling in metabolic programming.*** In preparation.

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Abstract

In the last years, there was a marked global rise in the prevalence of obesity and type 2 diabetes. Metabolic diseases are associated with dysregulation of central and peripheral nutrient-sensing pathways. Obesogenic environments such as maternal obesity, postnatal overfeeding and maternal westernized diets can impair the gut-brain axis of offspring during embryonic development and lactation, disturbing energy balance by reducing energy storage and promoting food intake. This contributes to the predisposition for the development of metabolic diseases in adulthood. Westernized diets are usually rich in fats and sugars which are the main source of advanced glycation end-products (AGEs). These compounds are highly associated with type 2 diabetes and its complications. Thus, we hypothesized that the exposure to obesogenic environments such as maternal obesity, neonatal hyperphagia and maternal glycation during the perinatal period programs energy balance mechanisms in young animals.

The aim of this work was to understand the putative alterations in the nutrient-sensing pathways in peripheral tissues and in the hypothalamus in animals exposed to different obesogenic environments, revealing the role of breastmilk composition alterations induced by unhealthy maternal status/diets and the impact of neonatal hyperphagia.

For that, animals of four models were studied: 1) 42 days old male and female offspring from females fed a high-caloric diet during pregnancy and lactation (maternal obesity model); 2) 45 days old male newborns submitted to litter reduction in order to induce hyperphagia during lactation (early-life obesity model); 3) 45 days old male and female offspring of dams treated with S-p-bromobenzylglutathione cyclopentyl diester (BBGC), an inhibitor of glyoxalase system, during the first 6 days of breast-feeding (maternal glycation model). DMSO was used as a vehicle; 4) Model in which neonatal hyperphagia was combined with maternal glycation. On the last day, insulin tolerance tests (ITT) were performed. Lipid and glycemic profiles were analysed in breastmilk and offspring plasma samples. Neuropeptide Y (NPY), ghrelin, and dopamine (DA) pathways were studied in white visceral (WAT) and brown (BAT) adipose tissues, in the liver, and in the hypothalamus of offspring.

The male offspring submitted to a maternal obesogenic diet presented higher WAT levels of lipogenic [NPY receptor-1 (NPY1R), NPY receptor-2 (NPY2R) and ghrelin receptor (GHS-R1α)], but also lipolytic and catabolic mechanisms [dopamine-1 receptor (D1R)]. In females, GHS-R1α levels in were also increased but the NPY1R content was reduced. The hyperphagic animals exhibited higher WAT levels of NPY2R while the stimulatory receptor of food intake in the hypothalamus (NPY1R) was decreased in these animals. Maternal glycation provoked lower food intake in the lean male

descendants which is in accordance with the observed decrease in NPY1R hypothalamic levels. On the other hand, exposure to maternal glycation only induced changes in WAT of animals from small litters (SL), decreasing D1R and NPY2R levels. The hyperphagic animals exposed to glycotoxins during lactation did not become obese and presented a reduction in NPY2R at the hypothalamic level. Regarding the liver, both NPY1R and D1R levels were decreased in all animal models.

Early life obesity induced by maternal HFHS diet and by postnatal overfeeding induce compensatory mechanisms in WAT, increasing at the same time signals related to lipid storage and oxidation. On the other hand, such mechanisms may be disrupted by simultaneous exposure to glycotoxins during lactation, contributing to a greater predisposition for metabolic diseases later in life. Moreover, all the obesogenic environments studied reduced dopaminergic signalling in the liver, impairing lipid mobilization. At the central level, the combination of hyperphagia and maternal glycation also disrupts the compensatory mechanisms developed in each condition individually.

Key words: metabolic diseases; energy balance; metabolic programming; AGEs; adipose tissue.

Resumo

Nos últimos anos, tem crescido abruptamente a prevalência de doenças metabólicas nomeadamente diabetes tipo 2 e obesidade que estão intimamente associadas à desregulação de mecanismos de balanco energético. Obesidade materna, hiperfagia neonatal e dietas ocidentais são considerados ambientes obesogénicos que durante o desenvolvimento embrionário e a lactação desregulam o controlo do balanco energético feito pelo eixo entre o intestino e o cérebro. O armazenamento energético é reduzido enquanto há maior ingestão alimentar, contribuindo assim para a predisposição de desenvolvimentos de doenças metabólicas na vida adulta. As dietas típicas do Oeste são a maior fonte de produtos finais de glicação avançada (AGEs) que estão altamente associados a diabetes tipo 2. Assim, a exposição a ambientes obesogénicos durante o período perinatal programa os mecanismos responsáveis pelo balanco energético nos recém-nascidos.

Com este trabalho pretende-se compreender as possíveis alterações nas vias da sensibilidade aos nutrientes nos tecidos periféricos e no hipotálamo em animais expostos a ambientes obesogénicos, compreendendo o efeito da composição do leite que pode ser alterada pelas dietas maternas ou pelo estado metabólico e o impacto da hiperfagia neonatal.

Para tal, quatro modelos animais foram estudados: 1) descendentes machos e fêmeas com 42 dias de idade provenientes de fêmeas alimentadas com dieta hipercalórica durante a gestação e lactação (modelo de obesidade materna); 2) recém-nascidos do sexo masculino com 45 dias de idade submetidos à redução da ninhada de modo a induzir hiperfagia durante a lactação (modelo de obesidade infantil); 3) descendência masculina e feminina de 45 dias de idade de fêmeas que durante os primeiros 6 dias de amamentação foram tratadas com S-p-bromobenzilglutationa ciclopentil diéster (BBGC), um inibidor do sistema da glioxalase (modelo de glicação materna). O DMSO foi usado como veículo; 4) Modelo em que a hiperfagia neonatal foi combinada com glicação materna. No último dia realizaram-se testes de tolerância à insulina (ITT). Os perfis lipídicos e glicêmicos foram analisados em amostras de leite materno e no plasma dos recém-nascidos. As vias do neuropeptídeo Y (NPY), da grelina e da dopamina (DA) foram estudadas nos tecidos adiposos castanho (BAT) e branco (WAT), no fígado e no hipotálamo da descendência.

Os machos submetidos à dieta materna obesogénica exibiram maiores níveis de mecanismos lipogénicos [NPY receptor-1 (NPY1R), NPY receptor-2 (NPY2R) e recetor de grelina (GHS-R1α)] no WAT, mas também de mecanismos lipolíticos e catabólicos [dopamina-1] recetor (D1R)]. Nas fêmeas, os níveis de GHS-R1α também aumentaram, contrariamente ao NPY1R que reduziram. Os recém-nascidos hiperfágicos apresentaram níveis mais elevados de NPY2R no WAT enquanto o recetor responsável

pela estimulação da ingestão alimentar no hipotálamo (NPY1R) estava diminuído nestes animais. A glicação materna provocou menor consumo de alimentos na descendência masculina magra, o que está de acordo com a diminuição observada nos níveis hipotalâmicos de NPY1R. Por outro lado, a exposição à glicação materna apenas induziu alterações no WAT dos animais de ninhadas reduzidas, tendo provocado uma diminuição dos níveis de D1R e de NPY2R. Os recém-nascidos hiperfágicos expostos a glicotoxinas durante a lactação não se tornaram obesos e apresentaram um decréscimo do NPY2R a nível hipotalâmico. Em relação ao fígado, os níveis de NPY1R e D1R estavam diminuídos em todos os modelos animais.

A obesidade infantil induzida pela dieta materna hipercalórica e pela hiperfagia neonatal induzem mecanismos de compensação no WAT, aumentando ao mesmo tempo o armazenamento de lipídios e a sua oxidação. Por outro lado, a exposição a glicotoxinas em obesos durante a lactação destrói estes mecanismos adaptativos, contribuindo para uma maior predisposição para doenças metabólicas mais tarde na vida. Além disto, todos os ambientes obesogénicos estudados reduziram a sinalização dopaminérgica no fígado, afetando a mobilização de lipídios. A nível central, a combinação de hiperfagia e glicação materna também perturba os mecanismos compensatórios desenvolvidos em cada condição individualmente.

Palavras-chaves: doenças metabólicas, balanço energético, programação metabólica, AGEs; tecido adiposo

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List of Acronyms and Abbreviations

α-MSH – α-Melanocyte-stimulating hormone

ACC – Acetyl-CoA carboxylase

ACL – ATP citrate lyase

AGEs – Advanced glycation end-products

AgRP – Agouti-related peptide

AMP – Adenosine monophosphate

AMPK – 5' AMP-activated protein kinase

ap2 – Apetala 2

ARC – Arcuate nucleus of the hypothalamus

AT – Adipose tissue

BAT – Brown adipose tissue

BBGC – S-p-bromobenzylglutathione cyclopentyl diester

BCA – Bicinchoninic acid assay

BMI – Body mass index

BSA – Bovine serum albumin

cAMP – Cyclic adenosine monophosphate

CCK – Cholecystokinin

CG1-58 – Comparative gene identification-58

CNS – Central nervous system

CPT1 – Carnitina palmitoil transferase 1

C/EBP – Ccaat-enhancer-binding proteins

D1R – Dopamine receptor 1

D2R – Dopamine receptor 2

D3R – Dopamine receptor 3

D4R – Dopamine receptor 4

D5R – Dopamine receptor 5

DA – Dopamine

DGAT1 – Diacylglycerol o-acyltransferase 1

DMSO – Dimethyl sulfoxide

DRs – Dopamine receptors

DVC – Dorsal vagal complex

ECM – Extracellular matrix

EDTA – Ethylenediaminetetraacetic acid

EGTA – Ethylene glycol tetraacetic acid

FFA – Free fatty acids

GABA – Gamma-aminobutyric acid

GHS-R1α – Growth hormone secretagogue receptor 1 α

Gi – inhibitory G

GIP – Gastric inhibitory peptide

GIT – Gastrointestinal tract

GLO – Glyoxalase

GLO1 – Glyoxalase 1

GLP-1 – Glucagon-like peptide-1

GOAT – Ghrelin O-acyltransferase

GPCR – G protein-coupled receptor

Gs – stimulatory G

HF – High fat

HFHS – High fat high sucrose

HSL - Hormone-sensitive lipase

IML – Intermediolateral nucleus

IP – Intraperitoneal

IR – Insulin receptor

ITT – Insulin tolerance test

kITT – decay of the glucose rate during the insulin tolerance test

LCFAs – Long-chain fatty acids

LH – Lateral hypothalamus

LPL – Lipoprotein lipase

MC4R – Melanocortin 4 receptor

MetS – Metabolic syndrome

MG – Methylglyoxal

mRNA – messenger ribonucleic acid

mWAT – mesenteric white adipose tissue

Na3VO4 – Sodium orthovanadate

NAc – nucleus accumbens

NaF – Sodium fluoride

NAFLD – Non-alcoholic fatty liver disease

NPCs – neuronal progenitor cells

NPY – Neuropeptide Y

NPY1R – Neuropeptide Y receptor 1

NPY2R – Neuropeptide Y receptor 2

NPY5R – Neuropeptide Y receptor 5

NPYRs – Neuropeptide Y receptors

NTS – Nucleus of the solitary tract

PPARγ – Peroxisome proliferator-activated receptors γ

PBS – Phosphate buffered saline

PMSF – phenylmethylsulfonyl fluoride

PND – Postnatal day

POMC – pro-opiomelanocortin

PVDF – Polyvinylidene difluoride

PVH – Paraventricular nucleus of the hypothalamus

PYY – Peptide YY

RAGE – Receptor for advanced glycation end-product

ROS – Reactive oxygen species

RT – Room temperature

SAT – subcutaneous adipose tissue

SCD1 – Stearoyl-CoA desaturase 1

SDS – Sodium dodecyl sulfate

SEM – Standard error of the mean

SL – Small litter

SNS – Sympathetic nervous system

SST – Somatostatin

T2D – Type 2 diabetes

TBS-T – Tris-buffered saline-tween

UCP-1 – Uncoupling protein 1

UCP-3 – Uncoupling protein 3

VAT – Visceral adipose tissue

VMH – Ventromedial nucleus of the hypothalamus

VTA – Ventral tegmental area

WAT – White adipose tissue

Chapter 1 **INTRODUCTION**

I. Obesity and type 2 diabetes

In the last years, the incidence and prevalence of obesity and type 2 diabetes (T2D) are increasing worldwide [1]. The high consumption of lipids and sugars is associated with obesity, cardiovascular diseases and T2D. Furthermore, sugars are the main source of advanced glycation end-products (AGEs), which are associated with the development of these metabolic diseases and their complications [2]. Obesity is classified according to body mass index (BMI) which corresponds to the ratio between the weight in kg and the square of height in meters. Individuals with BMI over 25 Kg/m^2 are overweight and BMI higher than 30 Kg/m^2 is considered obese [3]. Obesity is characterized by an abnormal fat accumulation in the body, which can promote the development of other diseases such as type 2 diabetes, some types of cancer and cardiovascular diseases [1]. Overfeeding and an imbalance between energy expenditure and food intake are among the main factors for the development of obesity [4].

The strong interaction between obesity and T2D originates the expression “diabesity” [1]. T2D is characterized by the ineffective use of insulin caused by pancreatic β-cells dysfunction and insulin resistance in target organs [5]. The global rise in sedentary lifestyles and obesity contribute to the increased prevalence of T2D [5]. In 2021, 537 million adults are diabetic and this condition was responsible for 6.7 million deaths [6]. Insulin resistance in peripheral organs causes hyperinsulinemia due to the continued release of insulin by pancreatic β-cells leading to its exhaustion [5], [7], [8]. Furthermore, oxidative stress is also associated with β-cells dysfunction [9]. In addition to this dysfunction and insulin resistance in peripheral organs, several factors favor the development of T2D such as reduced response to incretins, inflammatory environment, alterations in the gut microbiota colonization and weaker satiety response [5]. Moreover, in fat accumulation conditions, the increasing levels of free fatty acids contribute to insulin resistance, lipotoxicity [10] and dysregulation of adiponectin levels, a hormone released by adipose tissue [1]. This peptide plays an important role in insulin sensitivity and also preserves pancreatic β-cells. However, in obese people the levels of this hormone are lower, contributing to hyperlipidemia, insulin resistance and T2D [1].

a. The expansion of westernized diets: AGEs and glyoxalase system

The massive increase of obesity in the last years is a consequence of the escalation of westernized diets, which are rich in saturated and unsaturated fats, simple carbohydrates and poor in fibers [11], [12]. Furthermore, these diets are a major source of advanced glycation end-products (AGEs), which can be absorbed and accumulated in the liver, kidney, adipose

tissue, vessels and heart [13],[14]. Glycotoxins such as methylglyoxal (MG) can react with and modify proteins and nucleic acids forming AGEs. This glycation process can occur in plasma, nucleus, cytoplasm and even in the extracellular matrix (ECM) [14]. AGEs can be present in early-stage diabetes, being involved in insulin resistance and β-cells damage (figure 1) [2]. AGEs formation impairs antioxidant defenses and detoxifying mechanisms, causing a dysregulation of lipid and glucose metabolism [15]. Moreover, AGEs promote oxidative stress triggered by RAGE (AGEs receptor) activation and by mitochondria dysfunction due to glycation of mitochondrial proteins and ROS production [16]. These events continue to occur even when glycemic levels are stabilized, indicating that there is a "metabolic memory" of AGEs and ROS formation [14]. Indeed, the system responsible for detoxifying AGEs, the glyoxalase (GLO) system, can also be dysregulated in pathologic conditions such as obesity and diabetes [17].

The maintenance of a healthy diet and lifestyle is crucial to prevent both AGEs accumulation and oxidative stress and therefore insulin resistance and diabetes complications.

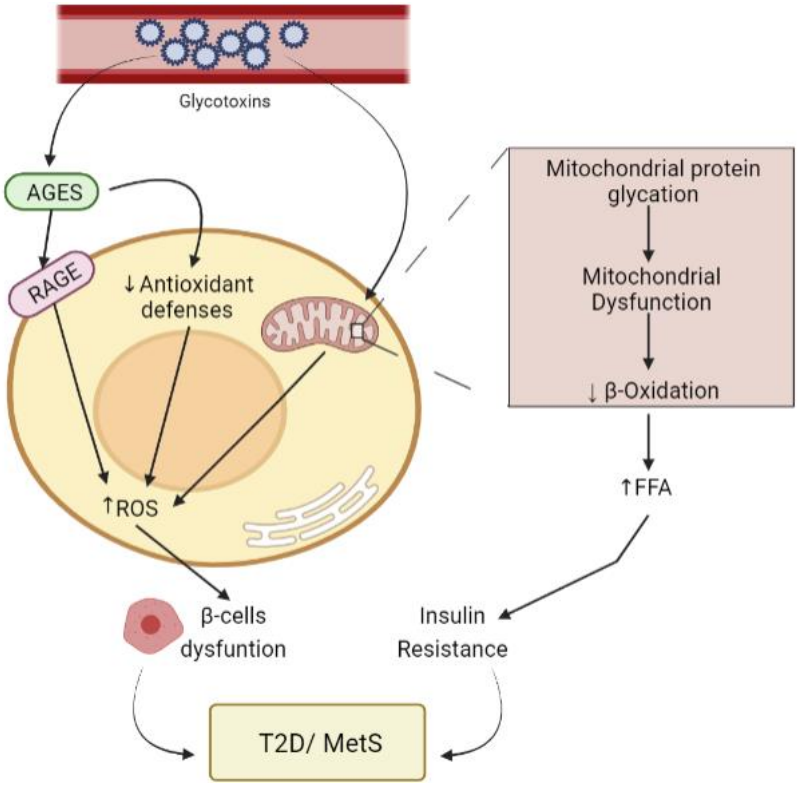

Figure 1 – Glycotoxins contribute to de development of metabolic diseases. *Glycotoxins react with and modify proteins and nucleic acids forming AGEs which actives RAGE. Glycotoxins promote oxidative stress by mitochondria dysfunction and by forming AGEs that impair antioxidant defenses and active RAGE, a receptor that triggers ROS formation. An oxidative environment leads to β-cells damage, one of the causes of T2D and MetS development. Mitochondrial dysfunction induced by glycotoxins also increases FFA, contributing to insulin resistance. Created with BioRender.com.*

II. Satiety and energy balance regulation

Obesity, T2D, and metabolic syndrome (MetS) are all associated with an imbalance between sympathetic and parasympathetic drive activity. The autonomic nervous system regulates anabolic and catabolic processes, controlling energy balance [18]–[20]. Satiety plays an important role in metabolic control and energy balance regulation, occurring after the eating process, in order to prevent the return of hunger for a few hours [21]. In postprandial situations, satiety is one of the mechanisms that inhibit food intake due to the release of several hormones that stimulate energy expenditure and suppress food intake [21]. Consequently, a weaker satiety response contributes to overfeeding, promoting metabolic diseases [22]. The regulation of satiety hormones occurs due to the sensing of nutrients in the gastrointestinal tract (GIT) [21]. Some foods may promote satiety more effectively than others, the duration of this psycho-biological mechanism depends on the nutrients consumed, weight, volume and energy content [21]. For instance, satiety is enhanced by meals with high levels of proteins and fibers [21].

a. Satiety-regulating hormones

The gut-brain axis is crucial for the regulation of energy balance, corresponding to bidirectional signalling between the GIT and some brain areas, namely the nucleus of the solitary tract (NTS) at the brainstem and the arcuate nucleus of the hypothalamus (ARC) [23]. The metabolic factors involved in the whole satiety process are known as satiety peptides. These hormones can be secreted by the GIT such as glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), peptide YY (PYY), gastric inhibitory peptide (GIP) and ghrelin or by adipose tissue such as leptin [21]. Some neurotransmitters such as neuropeptide Y (NPY) and dopamine (DA) are also energy balance regulators [21]. Moreover, the hormone insulin released by pancreatic β-cells also acts on the brain promoting satiety [21].

The ability to sense nutrients begins on the tongue and continues throughout all the digestive system, namely in the gut, which is constituted by three cell types - enterocytes, brush cells, and enteroendocrine cells (D cells, L cells, K cells, I cells, M cells and S cells) [24], [25]. Nowadays it is believed that nutrient-sensing is mediated by G protein-coupled receptor (GPCR), some of them taste receptors, promoting the release of gut hormones responsible for regulating hunger, satiety, metabolism and gut motility [24]. As mentioned above, satiety is a nutrient content-dependent process, and meals rich in proteins maximize satiety response. On

the contrary, the high-fat (HF) diet has less satiety effect [21]. The different effects promoted by these macronutrients are associated with the hormonal response, for instance, the drop in total ghrelin levels, an appetite-stimulating hormone, is higher upon ingestion of meals with high carbohydrates levels when compared to HF diets [21]. An unhealthy diet can disturb the taste response and modify food preference due to the "metabolic memory", dysregulating the expression of gut hormones [26]. Furthermore, the density of enteroendocrine cells responsible for the release of these hormones can also be altered by imbalanced diets [25]. Indeed, HF diets promote L and I cells density decreased, reducing the levels of GLP-1, PYY and CCK, satiety-promoting hormones [25].

1. Ghrelin

Ghrelin is an orexigenic hormone released in the preprandial state, promoting food intake and adiposity [27]. Ghrelin acts on the hypothalamus, in neurons that express NPY and Agouti-related peptide (AgRP) through growth hormone secretagogue receptor 1a (GHS-R1a) activation (figure 2) [28]. These two neuropeptides both known for stimulating food intake are released upon GSH-R1a activation [28]. AgRP/NPY neurons and the anorexigenic pro-opiomelanocortin (POMC) neurons are present in ARC, a brain region well known for controlling feeding behavior [29]. The pre-proghrelin, produced by P/D1 cells in humans, is converted into mature ghrelin which has two forms: acyl-ghrelin which can bind to GHS-R1a [27], [28]; and des-acyl ghrelin which is the most common form found in circulation [27]. Ghrelin O Acyl Transferase (GOAT) is responsible for ghrelin acylation upon meal consumption using fatty acids available from the diet [30]–[33], while the release of acyl-ghrelin occurs during fasting periods inducing food intake and reducing energy expenditure [31], [34]. However, prolonged fasting (more than 24 hours) declines acylated ghrelin levels possibly due to reduced availability of gut-derived medium-chain fatty acids [35].

Ghrelin plays a role in glucose metabolism at the peripheral level, reducing insulin secretion and decreasing glucose uptake in skeletal muscle and white adipose tissue (WAT) [36]. The action of ghrelin in insulin regulation depends on heterodimerization of GHS-R1a and somatostatin receptor 5, forming a dimer linked to a Gi protein which triggers the inhibition of insulin release [37], [38]. In low glucose levels conditions, acyl-ghrelin levels are high, providing conditions for dimerization of the 2 receptors [37], [38]. Moreover, acyl-ghrelin also exerts actions in lipogeneses through sympathetic innervation to the WAT (figure 3) [39]. Acyl-ghrelin stimulates the expression of genes involved in lipid storage such as lipoprotein lipase (LPL),

Acetyl-CoA carboxylase (ACC) α and Stearoyl-CoA desaturase-1 (SCD1), and also impairs fatty acid oxidation, decreasing Carnitine palmitoyltransferase 1 (CPT1) α expression [39], [40]. Directly on fat cells, ghrelin binds to its receptor, increasing peroxisome proliferator-activated receptors γ 2 (PPARγ2) expression and therefore playing a crucial role in the differentiation of preadipocytes into mature cells and triglyceride storage [41]. Furthermore, acylated ghrelin decreases brown adipose tissue thermogenesis reducing mitochondrial uncoupling proteins 1 and 3 (UCP 1 and 3, respectively) [39].

On the other hand, in postprandial situations, ghrelin levels decrease due to increased levels of ghrelin-inhibiting hormones (insulin and CKK) and also due to the sensing of nutrients by taste receptors (glucose, amino acid and long-chain fatty acid (LCFAs)) [27], [42]. For many years, des-acyl ghrelin was thought of as a hormone without metabolic activity. However, it has been suggested that during fasting this form may play a role as an acyl ghrelin antagonist regarding insulin secretion and also be capable of promoting pancreatic β-cells survival [28], [43]. The studies carried out so far have shown that the des-acyl form does not exert action by itself upon its administration [43]. However, when co-administrated with acylated ghrelin, reduces the effect of acyl ghrelin on glucose metabolism and food intake [43]. In WAT, overexpression of des-acyl ghrelin enhances insulin sensitivity and also protects against HF diet-induced obesity [43].

Ghrelin plays an important role in energy balance homeostasis, however, in metabolic dysfunction conditions such as obesity and diabetes, ghrelin levels are reduced in circulation, being more accentuated when these two pathologies are combined [40], [44] suggesting a less protective response of deacylated ghrelin against its acylated form. Indeed, in obese people with and without MetS the levels of total ghrelin and des-acyl ghrelin are decreased [44]. Counteracting this reduction, the levels of acylated ghrelin doubles in obesity [44], contributing to lipogenesis and the impairment of glucose homeostasis. The disruption in ghrelin signalling may contribute to the development and escalation of unhealthy metabolic states.

2. NPY

NPY is the most powerful neuropeptide in controlling appetite at the central level [45]. As already mentioned, NPY and AgRP neurons are activated by ghrelin as well as by fasting. However, the mechanism behind the activation of these orexigenic neurons by fasting is still unknown [46]. Currently, it is known that there are six receptors of NPY, all coupled to a Gi-protein [47]. Two of these receptors promote anabolic effects: NPY receptors 1 and 5 (NPY1R

and NPY5R, respectively) [45]. On the other hand, the effect of NPY receptor 2 (NPY2R) depends on the tissue [47]. The activation of NPY neurons occurs in low glucose levels conditions, releasing NPY to promote food intake and suppress energy expenditure [48]. On the other hand, during postprandial situations, the glucose levels are elevated, inducing AMPK inhibition, and leading to a decline in the activity of these anabolic neurons since free fatty acids are the main source of their energy [48]–[50].

NPY neurons have projections to both hypothalamic and extrahypothalamic areas, such as the paraventricular nucleus of the hypothalamus (PVH), dorsal vagal complex (DVC), intermediolateral cell column (IML) and the ventromedial nucleus of the hypothalamus (VMH) [51]. Moreover, it has been demonstrated that NPY neurons activation induces food craving. This motivation depends on anticipatory stimuli for food, suggesting a relation between these neurons and the reward system [52], [53]. The NPY effect on the hypothalamus depends on the receptor type. The response mediated by NPY1R in PVH is anabolic only when the levels of this neuropeptide are high. The deletion of this receptor in the hypothalamus does not exert effects on food intake or body weight [54]. However, the deletion of the NPY5R induces hyperphagia in mice [54]. On the other hand, the most expressed form in the hypothalamus and throughout the central nervous system, NPY2R, has the opposite role, reducing the expression of NPY (figure 2) [54]. NPY2R in the hypothalamus acts as an inhibitory autoreceptor, suppressing NPY expression and release from NPY neurons in the ARC [47], [55]. The loss of NPY2R in the hypothalamus induces hyperphagia in mice and they are more prone to gain weight [56].

In addition to its anabolic functions, NPY also inhibits the neurons responsible for inducing satiety. The relation between NPY and POMC neurons is well known. These two types of neurons inhibit each other, controlling energy expenditure and energy consumption (figure 2). The *pomc* gene expresses a precursor peptide that originates several peptides such as α-Melanocyte-stimulating hormone (α-MSH), which actives its receptor, melanocortin 4 receptor (MC4R), inducing food intake inhibition and increasing energy expenditure [51], [57]. Two subpopulations of POMC neurons are known: 1) one of them promotes food intake inhibition independent of energy expenditure through MC4R activation in PVH; 2) the other subpopulation stimulates energy expenditure by stimulating MC4R activity in DCV and IML [29], [51]. In PVH, NPY suppresses MC4R expression, encouraging food intake [51]. Furthermore, GABA released by the NPY/AgRP neurons to the POMC neurons inhibits anorexigenic peptides secretion, contributing to the reduction of energy expenditure and satiety in the preprandial state [29], [51]. Counteracting NPY action, PYY plays an antagonistic

role to NPY [58]. This 36-amino-acid peptide is produced by the gut in L cells and released after meals, maintaining high levels for 6 hours [58]. PYY binds to all NPYRs but presents a higher affinity for NPY2R, having an anorexigenic effect on the hypothalamus, inhibiting NPY neurons activity and stimulating POMC neurons [58]. Being a peptide regulated by taste receptors, the type of nutrients consumed can alter PYY secretion. Indeed, as mentioned above, L cells quantity decrease with HF diets, disturbing PYY release. On the other hand, diets rich in protein enhance the secretion of this peptide [58].

NPY released by neurons present in ARC can also regulate peripheral actions. NPY decreases insulin sensitivity and glucose metabolism in peripheral tissues such as liver, brown adipose tissue, heart and skeletal muscle by VMH inhibition [59], [60]. VMH is known as a satiety center, being the center of the sympathetic nervous system, controlling positively energy expenditure [61]–[63]. So, NPY inhibits this satiety center, reducing energy expenditure regulated by sympathetic innervation. Nevertheless, NPY receptors are also present in other tissues besides the nervous system. In the liver, few studies have been published on the function of NPY and its receptors. It is known that NPY1R and NPY2R are expressed in this tissue, however, the mechanisms triggered by NPY in the liver are still unknown [54]. Contrastingly, the role of NPY in the pancreas is well established. NPY acts as a growth factor in the development of Langerhans islets [64]. During fetal development, pancreatic endocrine cells synthesize NPY, namely insulin-producing β-cells [64], [65]. However, in adulthood, these cells lose the ability to express NPY [64]. Some studies have proven that islet immature cells can produce NPY [66]. In fact, HF diet and diabetes type 1 and 2 induce changes in the islet cells composition, increasing the number of cells expressing NPY [66]. Furthermore, NPY1R activation on β-cells suppresses insulin secretion [67], contributing to the high glucose levels in circulation. Interestingly, in contrast to the increased expression of NPY in the pancreas, mice HF-fed demonstrated a reduction of NPY1R in β-cells [68]. This may be a compensatory mechanism to reduce the inhibition of insulin release, offsetting for the effects of HF diet. Moreover, the NPY release by the ARC also controls insulin levels through NPY1R in the vagal nerve inhibiting glucose-induced insulin secretion [65], [67]. On the other hand, central NPY also acts on sympathetic innervation, stimulating the release of insulin from pancreatic β-cells [68].

NPY also exerts effects on both white and brown adipose tissues (figure 3). The neuropeptide produced by the brain reaches WAT through the sympathetic enervation and it is also produced in adipose tissue [69]. Kos showed that human adipocytes in culture release NPY to the medium indicating that this neuropeptide is also biosynthesized in WAT [69], [70].

The adipocytes and their precursor cells express both NPY1R and NPY2R [54]. Activation of NPY1R on WAT promotes the proliferation of preadipocytes and contributes to the development of insulin resistance [69], [71]. Moreover, the block of this receptor in WAT enhances lipolysis [71], and it was shown that the absence of this neuropeptide enhances catabolic processes (lipolysis) and reduces PPARγ levels, impairing anabolic processes such as lipogenesis and adipogenesis[72]. In obese adult rats, the levels of NPY are increased, corroborating the obesogenic effect of NPY1R activation in WAT [69]. In opposition to what occurs in the hypothalamus, NPY2R activation leads to an anabolic response, stimulating tissue growth [54]. The study performed by Yan-Chuan Shi et. al (2011) shows that the peripheral deletion of NPY2R protects the mice against obesogenic diets, reducing adiposity, and weight gain and improving glucose tolerance [73]. Regarding brown adipose tissue (BAT), NPY triggers a reduction of glucose uptake [59] and regulates thermogenesis through sympathetic innervation [74]–[76]. The activation of NPY1R in PVN inhibits UCP-1 expression on BAT, decreasing thermogenesis [74]. Although BAT expresses NPY receptors (NPY1R and 5), Kohei Shimada proved that NPY does not exert any effect on BAT thermogenesis by activation of them, indicating that energy expenditure in BAT is exclusively regulated by the sympathetic nervous system (SNS) [75]. The direct role of NPY in BAT needs to be studied since its receptors are present in this tissue.

3. Dopamine

DA is a neurotransmitter involved in neuronal pathways responsible for decision making, executive function, working memory and food craving [77], [78]. The dopaminergic neurons in the ventral tegmental area (VTA) send projections to the nucleus accumbens (NAc) forming the mesolimbic pathway [79]. This pathway, known as the reward system, requires food and cues that predict food to be activated, regulating food intake [80]. Binge-eating is a term to define compulsive food consumption and this behavior is associated with the reward system [81]. Moreover, dopaminergic neurons are also present in the ARC, as mentioned above, involved in energy balance [79]. DA synthesis takes place in the cell bodies of dopaminergic neurons of the VTA, substantia nigra, and ARC [79]. However, DA is also present in peripheral organs [82]. Indeed, DA levels in the periphery are superior when compared to the Central Nervous System (CNS) [82]. DA production is not exclusively of sympathetic neurons, non-neuronal cells such as intestinal cells and gut microorganisms can also synthesize DA, modulating the gut microbiota composition [82].

Nowadays, five receptors of DA (DRs) are known, divided into two subfamilies: D1-like receptors type which are coupled to stimulatory G (Gs) proteins – DA 1 and 5 receptors (D1R and DR5, respectively); D2-like receptors type which are coupled to inhibitory G (Gi) proteins, belonging to this family DA 2, 3 and 4 receptors (D2R, D3R and D4R, respectively) [83]. The Gs proteins trigger cAMP activation, increasing AMP levels and, consequently, protein kinase is activated, regulating several pathways. On the other hand, Gi proteins contradict this action, inhibiting cAMP and therefore protein kinase activity [84]. Recently, several studies have shown heterodimerization between the DA receptors such as the D1R-DR2 heterodimer [82]. Furthermore, DA receptors can also form heterodimers with adenosine receptors [82]. The signalling activated upon DA binding is dependent on heterodimer pharmacological properties, having distinct physiological roles [82]. In the CNS, the two most abundant forms are D1R and D2R [83]. Moreover, both receptors are present in pancreatic β-cells, although D2R expression is much higher [85], [86]. On the other hand, in WAT, D1R expression is greater than D2R in mature adipocytes [87].

i) Dopamine in brain

In the hypothalamus, D2R is the most expressed form whereas D1R expression is low, although the expression of D1R increases in obesity [88]. DA on the hypothalamus can modulate leptin signalling in POMC neurons (figure 3). Leptin is an anorexigenic hormone produced by the adipose tissue and triggers POMC neurons activity [89], [90]. DA secreted by the hypothalamic nuclei actives D2R in the hypothalamus inhibiting leptin signalling and therefore the release of anorexigenic neuropeptides [86],[91],[92]. On the other hand, the NPY neurons also express DRs. The activation of D1R in these orexigenic neurons promotes the expression of food-intake stimulatory neuropeptides (figure 2) [88]. Each DRs requires a different amount of DA to exert activity [88]. In the beginning, DA activates D2R, suppressing food-intake inhibitory neuropeptides followed by the D1R activation in NPY neurons [88]. Interestingly, upon meal consumption, DA is released in VMH and this event is more pronounced in obese people [93]. Furthermore, HF diets and obesity are associated with D2R upregulation in POMC neurons, increasing the inhibition of leptin signalling in the hypothalamus and consequently the energy balance [91],[92]. Taking this into account, DA, D2R and D1R levels are higher in obesity, contributing to an increase in food intake by inducing NPY release as well as by reducing leptin and other food-intake inhibitory neuropeptides signalling.

As mentioned above, DA also increases food craving through the reward system. In the mesolimbic pathway, feeding behavior is controlled by DR2, whereas D1R manages motor behavior [80]. Upon ingestion of palatable food, DA is released by VTA dopaminergic neurons into NAc, stimulating food intake [80], [94]. Nevertheless, continuous exposure to reward food provokes alterations in dopaminergic signalling promoting excessive food craving [94]. Regions belonging to the reward system have more neuronal activity in obese people subjected to pictures of high-calorie food [95]. Indeed, HF diets and obesity are correlated with downregulation of D2R in neurons belonging to this pathway and with higher levels of DA, suggesting that in these conditions the food craving is higher [80]. In the reward system, D2R has an inhibitory role regarding DA release [94], [96], so the dysregulation of DA levels observed in obesity may be a consequence of the D2R decreased levels, contributing to food craving stimulation through the mesolimbic pathway.

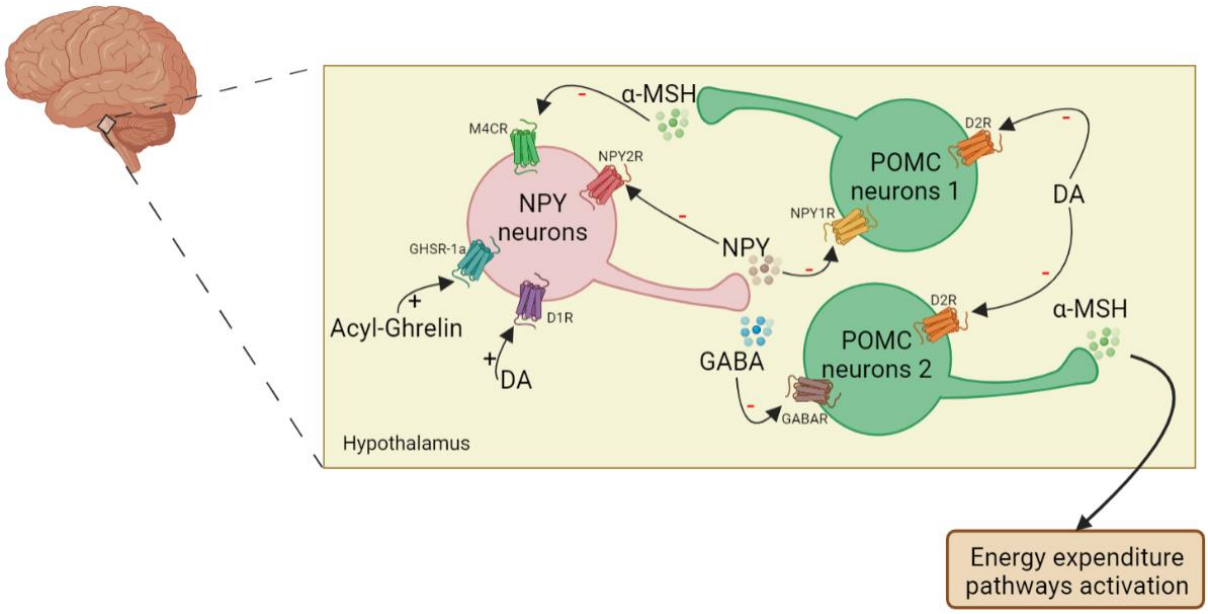

Figure 2 – Orexigenic and anorexigenic actions on the hypothalamus. *Ghrelin binding to GHS-R1α and DA binding to D1R stimulate NPY neurons that release NPY and GABA. NPY binding to NPY1R and D2R activation inhibit POMC neurons subpopulation 1 that mediates inhibition of NPY neurons by the release of α-MSH. The subpopulation 2 of POMC neurons that is suppressed by GABAR and D2R activation, releases α-MSH, stimulating the activation of energy expenditure pathways. Created with BioRender.com.*

ii) Dopamine in peripheral organs

DA is also synthesized in the gut, pancreatic β-cells and mesenteric WAT (mWAT) [83], [84], [86]. More than 40 years ago, it was proven that DA injection leads to hyperglycemia, indicating an inhibitory effect on insulin release and, consequently, a decrease in glucose

uptake [97]. In the postprandial state, simultaneously with insulin, DA is released and binds to D2R, inhibiting the glucose-stimulated insulin secretion, an opposite effect to incretins [98]. Contrastingly, when DA binds to D1R present in pancreatic β-cells, insulin release is stimulated [83]. However, the expression of D1R is much higher than D2R expression, suggesting an autocrine stimulatory effect of DA regarding insulin release in β-cells [85]. This contradicts the inhibitory effect of DA on insulin secretion observed over the years, so this outcome may be a repercussion of the paracrine role of DA in insulin secretion. DA acts on δ-cells activating D2R leading to suppression of somatostatin (STT) release, which in turn conducts to the disinhibition of SST-induced insulin release [83]. These mechanisms are highly complex and further studies are needed to understand which dopaminergic receptors are involved in the regulation of insulin release.

DA also acts on WAT via sympathetic nerves, circulation, or by infiltration of immune cells (macrophages and lymphocytes) [87]. In mWAT, all the DRs were found except D3R [84]. D1R is the most expressed form and it is involved in the release of adipokine whereas D2R modulates lipid processes [87]. Activation of DR1 induces lipolysis and inhibits leptin release and, consequently, the satiety role played by this hormone is decreased [84], [87]. On the other hand, a D2R agonist modulating DA signalling in WAT improves the metabolic profile in diabetic patients [84]. DA binding to D2R leads to inhibition of lipolysis and stimulation of adipogenesis (figure 3) [87]. The blocking of this receptor increases the phosphorylation of proteins such as AMPK, HSL and ACL which promotes a higher lipid oxidation rate while inhibiting ACC, an enzyme involved in fatty acid synthesis [84]. Regarding the insulin pathway in WAT, D2R potentiates insulin-mediated glucose uptake, but its inhibition increases IR phosphorylation suggesting that this receptor controls other insulin receptor-mediated functions than glucose uptake [84]. In addition to WAT, DA acts on the BAT. Interestingly, despite the improvement in glucose uptake in BAT, no alterations were observed in insulin receptor and AMPK activation [84]. Thus, it has been suggested that this alteration on BAT occurs in response to changes in afferent signalling from other tissues, such as WAT or liver, modulating DA in the parasympathetic vagus nerve that mediates sympathetic innervation of BAT [84].

In the skeletal muscle, DA enhances glucose uptake mostly mediated by D1R whereas in the liver D2R is responsible for this function independent of insulin signalling [84]. In skeletal muscle, this event is observed even without insulin action, suggesting that DA alone modulates glucose metabolism in the major organ involved in glucose homeostasis [84]. On the other hand, D2R may act also in glucose homeostasis by AMPK activation in both liver and skeletal muscle [84]. These results of peripheral dopaminergic effects differ between in vivo and ex

vivo, probably due to systemic innervation. More studies are needed to understand the DA pathways and how dopaminergic signalling is modulated by western diets and metabolic disorders.

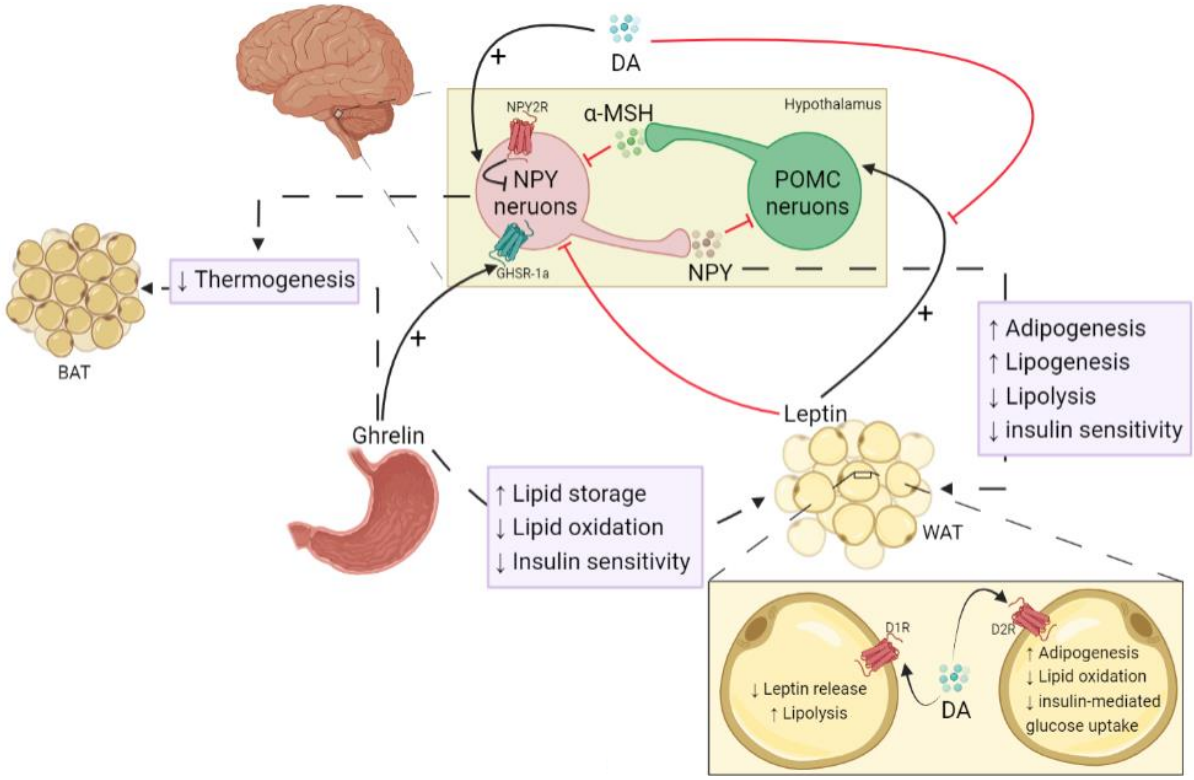

Figure 3 – An integrated view on the gut-brain-adipose tissue crosstalk. *Ghrelin which is released by the stomach and DA stimulate NPY neurons that release NPY. In the hypothalamus, NPY binds to NPY1R inhibiting POMC neurons that release α-MSH. NPY2R activation and α-MSH suppress NPY neurons activity. NPY and ghrelin reduce insulin sensitivity and modulate lipid processes in WAT, while in BAT these two orexigenic factors decrease thermogenesis. The hormone released by WAT, leptin, inhibits NPY neurons whereas stimulates POMC neurons, an action repressed by DA. On WAT, D1R activation reduces leptin release and promotes catabolic processes contrary to D2R which also promotes WAT enlargement. Insulin action regarding glucose uptake is downregulated by D2R. Created with BioRender.com.*

III. The crosstalk between gut hormones and neuroendocrine mechanisms

The link between ghrelin and NPY is well established in the brain. Ghrelin is produced by the stomach and acts on NPY neurons in ARC, inducing the release of food-intake stimulatory neuropeptides such as NPY and AgRP. Nevertheless, it has been suggested a crosstalk between dopamine and NPY signalling. Extrahypothalamic regions also express NPY and its receptors, inclusively in centers responsible for the hedonic eating such as the reward system [99]–[101]. I.e, NPY can stimulate food consumption and can also promote food motivation. For instance, the motivation for sugar mediated by NPY on VTA is dependent on Nac dopaminergic signalling

(mesolimbic pathway). In the lateral hypothalamus (LH), NPY injection also triggers sugar intake without DA action [102]. Several studies showed the role of NPY5R in DA release in Nac, although this may not be a direct consequence of NPY signalling but by the inhibition of GABAergic activity in this area [103]–[105]. On the other hand, NPY effect on dopamine release in the striatum is mediated by the NPY2R [106]. However, the NPY effect on VTA dopaminergic neurons is still unclear, and several studies have shown opposite effects. One possibility for this discrepancy could be the existence of different subpopulations of VTA dopaminergic neurons and the activation of different types of NPYRs [107]. Interestingly, ghrelin may also induce food motivation, despite this action being dependent on NPY signalling [108]. Nevertheless, the mechanism involved is still unclear, and it could be by LH-VTA connection or by some pathway that leads to NPY release in Nac, activating dopaminergic signalling [108].

The interaction between NPY and DA is bidirectional, the levels of this neuropeptide are regulated by DA and, therefore, DA indirectly regulates food intake, which may be involved in hedonic feeding. In ARC, pro-pre-NPY mRNA levels decrease when D2R is blocked, whereas D1R inhibition leads to an increase in pro-pre-NPY mRNA [109]. On the other hand, Xiaobing Zhang et. al (2016) demonstrated that DA stimulates AgRP/NPY neurons activity via D1R [110]. In obesity, the role of NPY is attenuated upon D1R/D2R agonist administration, reducing food consumption, and normalizing the lipidemic profile and the bodyweight [111]. Overall, DA regulation of NYP orexigenic effect appears to depend on the receptor involved and possibly the brain region.

The relationship between neuroendocrine and gut hormones is a world to discover, especially at the peripheral level. Nowadays, it is known that there is a link at the central level, as discussed above, although it is imprecise. However, research in peripheral organs is scarce. Our group has observed that DA can also modulate the signalling of gut hormones such as GLP-1 in WAT, but we also observed that the reverse does not occur (data not published). Moreover, our preliminary data also indicates that D2R agonist increases NPYRs and GHS-R1α in WAT. In addition, studies in preadipocytes and mature adipocytes show that the relationship between NPY and ghrelin signalling may depend on the stage of differentiation of the cell. For instance, NPY2R is modulated by ghrelin in preadipocytes but not in mature adipose cells (data not published). Therefore, metabolic pathologies can be associated with the dysregulation of gut hormones and neuroendocrine mechanisms crosstalk which requires further study.

IV. Metabolic programming

During the life course, each organism is programmed to respond to an insult at different levels. Some life periods such as preconception and perinatal (gestational and lactation phases) are more prone to this programming in a consequence of the high cell proliferation and differentiation rate [112]. Metabolic programming consists in the modulation of several metabolic factors in offspring in response to maternal stress factors during early life, including maternal lifestyle and nutritional state [113]. These alterations might endure over the life course, which may manifest at any period of their life, influencing energy expenditure pathways and food intake behavior, contributing to the development of metabolic disorders [114]. Maternal nutrition impacts on organ development, gene expression and epigenome, altering offspring metabolism and cellular function [115]. Worldwide, studies have proven that there is higher risk of obesity development when the organism is exposed to maternal obesity and overnutrition [116]–[118]. In addition, several studies have shown that maternal diabetes increases birthweight in offspring, which remains for life [119]. An unhealthy maternal state may also alter the expression of neuropeptides responsible for the regulation of food intake and energy expenditure in the newborn. For instance, maternal obesity increases the hypothalamic expression of NPY and AgRP and it also leads to the downregulation of POMC in offspring [115], [120]. Thus, it may lead to dysregulation of energy balance, contributing to a higher risk of metabolic diseases in offspring.

a. Gestation and lactation phase

As mentioned above, the perinatal period plays an important role in organism development, therefore, the intrauterine environment and breast milk are the two main factors influencing metabolic programming [113]. The impact of maternal dietary patterns or metabolic state may differ in pregnancy and lactation. It has been described that offspring metabolism is altered in consequence of insults in both of these programming windows, although it is still unclear which of these periods is more critical.

During fetal development, both maternal undernutrition and maternal overnutrition can provoke alterations in the uterine environment [121]. Indeed, obesity during pregnancy is correlated with the dysregulation of intrauterine metabolism [122]. Focusing on maternal overnutrition, maternal diets with high carbohydrate and energy content during pregnancy are associated with a higher adiposity in offspring [119]. On the other hand, elevated protein intake during gestation promotes lower body fat mass in offspring at birth [119].

Milk composition can further affect energy balance regulation due to the exposure to maternal nutrients in the offspring´s gut (figure 4). Genetic factors, obesity and mainly maternal diet and metabolic state affect milk composition. Breast milk influences the CNS and peripheral organs involved in energy expenditure and food intake such as adipose tissue, liver and pancreatic β-cells, as well as gut microbiota [114]. For instance, a high-caloric maternal diet impairs the lipid and vitamin milk profile leading to higher adiposity, insulin resistance, changes in gut microbiota composition and alterations in gut hormones secretion in offspring [114], [123]–[126]. On the contrary, maternal protein restriction during breastfeeding induces a lean phenotype in offspring and it also prevents the development of obesity in adulthood [114]. Colonization of gut microbiota depends on breast milk and undergoes changes throughout life [127]. Furthermore, the growth of certain bacteria is favored by some food products that act as prebiotics and, consequently, there is fermentation and formation of byproducts. These compounds reduce the number of stem cells, impairing the differentiation of endocrine cells responsible for hormones secretion [127]. Hence, it is possible that the gut microbiota may regulate gut hormones release and thus several other processes such as gut motility, nutrient absorption, and food intake [127]. Besides, the gut microbiota also influences the gut-brain axis, which is the main regulator of energy balance [128].

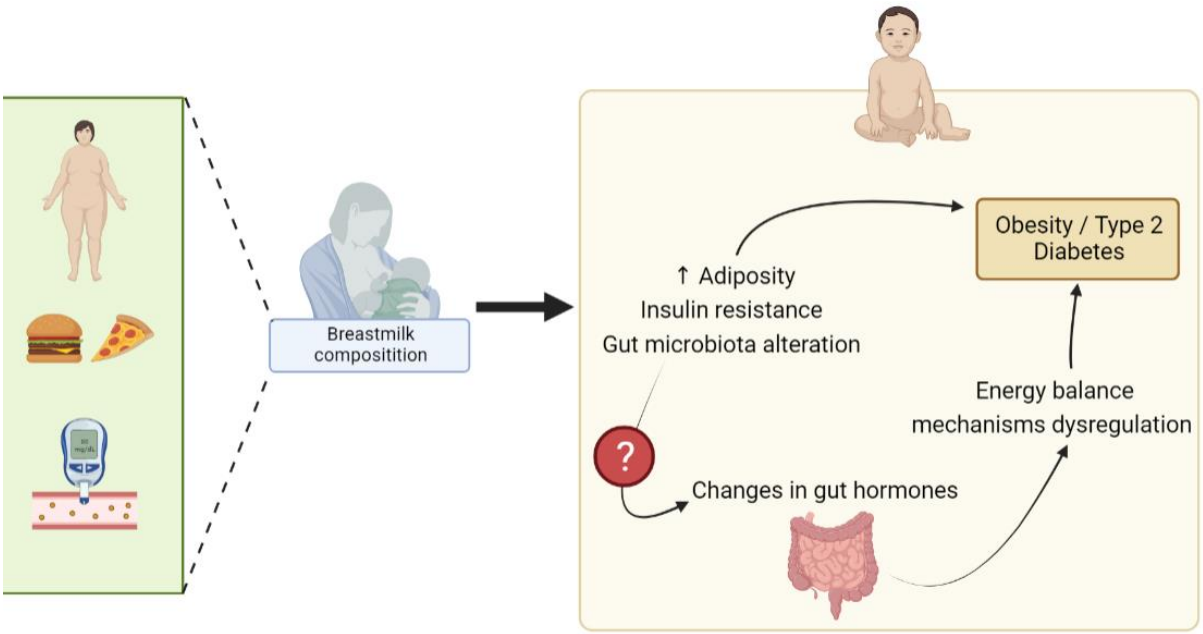

Figure 4 – The effect of obesogenic environments during lactation. *Obesity, westernized diets and diabetes change breastmilk composition, altering mechanisms in offspring. These obesogenic environments increase adiposity, promote insulin resistance and alter gut microbiota on offspring, predisposing the development of obesity and T2D. Created with BioRender.com.*

b. The impact of maternal metabolic state and westernized diets in offspring

Diverse studies have shown that, not only the maternal metabolic state, but also the type of maternal diet can alter the regulatory hormones of food intake and energy expenditure. It has been proven that maternal obesogenic diets, such as westernized diets, can induce obesity and contribute to metabolic complications during lactation [129], [130].

The effects of maternal factors in the offspring can be observed at the central and peripheral levels. For instance, Desai *et al.* (2020) found that a maternal HF diet induces a greater differentiation of hypothalamic neural progenitor cells (NPCs) into NPY neurons in offspring, unsettling energy balance [131]. In another study, it was observed that maternal gestational diabetes or metabolic syndrome is associated with a reduction of GLP-1 levels in offspring, which can diminish the duration of satiety and impair insulin release [114]. Several studies have found that insulin resistance during the gestation period impairs both peripheral and brain insulin function in offspring [132].

Another important discovery was the alteration of sweet taste receptors observed in taste buds of offspring from obese mothers [133]. These receptors are also present in the gut [24], [134]. Hence, it is possible that the maternal diet can induce modifications in gut hormones-secreting cells, altering taste receptors and, consequently, the levels of these hormones. Moreover, a study with Yucatan minipig demonstrated that westernized diets during pregnancy and lactation even without inducing maternal obesity, affect the gut microbiota in offspring. Furthermore, the study also proved that energy balance is not restored when the offspring are submitted to a standard diet, evidencing the importance of metabolic programming in offspring health [135]. In other words, maternal western diets can turn the newborn more prone to develop metabolic disorders in part due to alteration of the nutrient-sensing pathways in the offspring, promoting overfeeding and impairing energy expenditure. However, the factors involved in such regulation are unknown. Changes in nutrient supply during gestation or in breastmilk may affect central nutrient-sensing but gut factors may also be involved, such as changes in gut microbiota and gut hormones release. The nutritional composition of breastmilk and other factors like AGEs may affect the function of enteroendocrine cells, although the mechanisms involved are also unknown.

These findings indicate that somehow the maternal state and dietary patterns during the perinatal period program the nutrient-sensing pathways of newborns, decreasing satiety and increasing food intake, contributing to the global rise in obesity, T2D and metabolic syndrome.

c. Hyperphagia-induced obesity in early life

Hyperphagia, the strong sensation of hunger, during lactation can also promote alterations in nutrient-sensing pathways changing satiety hormones levels.

High consumption of breast milk induces both hyperphagia and overweight [136]. It was observed that this high ingestion of milk increases food intake and reduces energy expenditure. Accordingly, under these conditions, the number of neurons responding to NPY in the VMH is higher, indicating lower activity by this satiety center [136]. Besides that, leptin levels are also higher due to increased fat mass, even in adulthood, demonstrating that hyperphagia in early life has repercussions that remain for life [136], [137]. Moreover, the study also reports insulin and leptin resistance, impairing the effect of these hormones on food intake and energy expenditure [136]. Furthermore, Argente-Arizón et. al (2016) have found that in animals from small litter (SL), an animal model of hyperphagia due to the high breast milk consumption, the levels of acyl ghrelin mRNA are upregulated in the stomach [138], [139]. These results corroborate the observed increase of food intake in animals that were induced with hyperphagia during lactation.

Chapter 2 SCIENTIFIC FRAMEWORK AND OBJECTIVES

SCIENTIFIC FRAMEWORK

The topic of metabolic programming has been growing in recent years, demonstrating its importance in the newborn's life. Likewise, it has been shown that dysregulation in the energy balance brings metabolic consequences to the organism, contributing to the development of pathologies. For instance, exposure to maternal obesity and glycotoxins is highly associated with insulin resistance, lipotoxicity and low energy balance. Moreover, differences in the number of cells that produce NPY have also been observed at the central level in newborns exposed to maternal HF diet [131]. However, some questions remain to be discovered, for instance, how exposure to obesogenic environments during the perinatal period can lead to changes in energy balance. Furthermore, it is still unknown whether these changes occur at the central or peripheral levels or both. Thus, it would be interesting to understand if insulin-sensitive tissues such as the liver and adipose tissue undergo alterations in the pathways that regulate energy expenditure and storage and if this exposure to obesogenic environments predisposes the offspring to a higher food consumption rate at the central level. For instance, some groups have shown that obesity alters the synthesis and levels of some peripheral hormones such as ghrelin [138], [139].

It would also be interesting to understand whether milk nutrient composition has a critical role in such alterations and if hyperphagia by itself modulates energy balance regulatory pathways.

Also, given that westernized diets are often rich not only in lipids but also in sugars, it is important to disclose whether glycotoxins like AGEs are involved in the modulation of central and peripheral energy balance mechanisms, namely their involvement in the loss of sensitivity to nutrients in critical programming windows.

Thus, considering the importance of DA, NPY and ghrelin in the control of energy balance, it would be important to study their signalling pathways in the major central regulator of energy balance, the hypothalamus, and peripherally in insulin-sensitive tissues from newborns exposed to hypercaloric maternal diets and glycotoxins during perinatal periods. Importantly, our group has shown impaired dopaminergic signalling in the adipose tissue of insulin-resistant patients, which was observed to be modulated by a D2R agonist in obese T2D rats [140]. Preliminary data from our group show that NPY receptors are also unbalanced in such patients, being correlated with impaired energy balance in the tissue (storage/oxidation), supporting a crucial metabolic function of NPY in the adipose tissue.

The wistar animals used in this work were shared for the realization of the thesis of Andreia Amaro and Mariana Rocha. Thus, the results referring to the metabolic parameters of these animals are the same in all dissertations. The Sprague-Dawley rats were bred at the University of Porto and later I had access to the tissues to perform western blots.

MAIN OBJECTIVE

The main objective of this work was to study the morphology of the putative alterations in the nutrient-sensing pathways in peripheral tissues and in the hypothalamus in animals exposed to different obesogenic environments, disclosing the role of maternal diet composition, hyperphagia and milk quality (figure 5).

SPECIFIC OBJECTIVES

i. To understand the effect of maternal obesity induced by a HF diet in the mechanisms of energy balance (ghrelin, NPY and DA) in the liver and adipose tissue of the offspring.

ii. To disclose the role of neonatal hyperphagia-induced obesity in regulating peripheral and central mechanisms of energy balance.

iii. To address the impact of maternal glycotoxins in nutrient-sensing pathways at both peripheral and hypothalamic levels in normal and hyperphagic newborns.

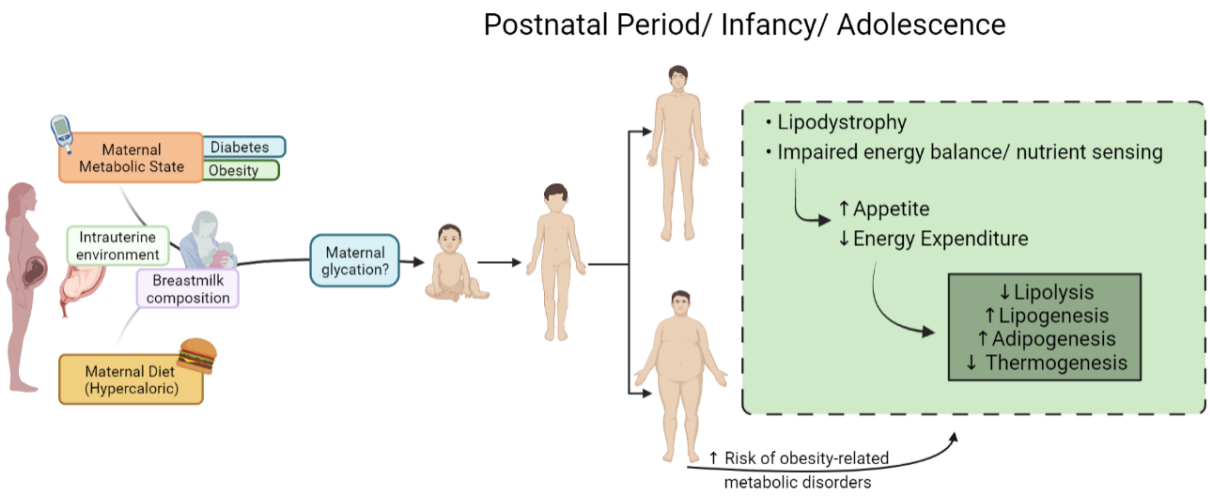

Figure 5 – Schematic representation of our hypothesis: The effect of obesogenic environments during the perinatal period on offspring energy balance regulation. *The maternal metabolic condition and unhealthy lifestyle may promote adipose tissue dysfunction, impair energy expenditure mechanisms (lipolysis and thermogenesis) and contribute to greater energy storage, increasing lipogenetic and adipogenic processes. Created with BioRender.com*

Chapter 3 MATERIALS AND METHODS

Reagents

Unless stated elsewhere, all reagents and common products were bought from Sigma – Merck (USA) and Fisher Scientific - Thermo Fisher Scientific (USA).

Antibodies

Calnexin (AB0037, Sicgen, Portugal), D1R, GHS-R1α (ab81296, ab85104, Abcam, Cambridge, United Kingdom), NPY1R (6732-0150, Bio-Rad, California, EUA), NPY2R (AB0328-20, Sicgen, Portugal).

Table 1 – Primary antibodies used in Western Blotting.

Antibody	Molecular weight (kDa)	Dilution	Manufacturer	Secondary Antibody
Anti-Calnexin	83 kDa	1:1000	Sicgen	Anti-Goat
Anti-D1R	≈ 48 kDa	1:1000	Abcam	Anti-Rabbit
Anti-GHSR1α	≈ 42 kDa	1:500	Abcam	Anti-Rabbit
Anti-NPY1R	≈ 52 kDa	1:1000	Bio-Rad	Anti-Sheep
Anti-NPY2R	≈ 52 kDa	1:1000	Sicgen	Anti-Goat

Methods

In vivo models of metabolic programming

Maternal diet-induced obesity during gestation and lactation

The procedures involving Sprague-Dawley rats were approved by The Ethical Committee of the Institute for Research and Innovation in Health – i3S, University of Porto, and National Government Authority (Direção Geral de Alimentação e Veterinánia – No.0421/000/000/2018) approved the experimental protocol, which followed the Guidelines for Care and Use of Laboratory Animals in Research advised by the Federation of European Laboratory Animal Science Associations (FELASA).

To study the effect of maternal obesity 42 days-old female Sprague-Dawley rats (150–200 g) were randomly divided into 2 diet groups: control and high-fat high-sucrose (HFHS) diet. On day 91 the dams were mated with male rats fed with a standard diet. The maternal obesity-inducing diet contained 42% metabolizable energy from fat (vs. 10% in standard), 27% from proteins (vs. 20% in standard), and 31% from carbohydrates (mainly sucrose, vs. 70% in standard with 1% sucrose), with crude fat of 23.1% (vs. 4.1% in standard), high cholesterol content and increased proportion of long-chain fatty acids. After delivering naturally on day 112, litter size was standardized, reducing to 3 male and 3 female pups to avoid competition for food. On day 141 (corresponding to offspring day 21 after the birth), the newborns were weaned and fed with chow. On the 42nd postnatal day (PND), offspring were

euthanized after overnight fasting and WAT and liver were collected for study (figure 6). More information of this animal model can be consulted on Jelena Stevanović-Silva et. al (2021) [141].

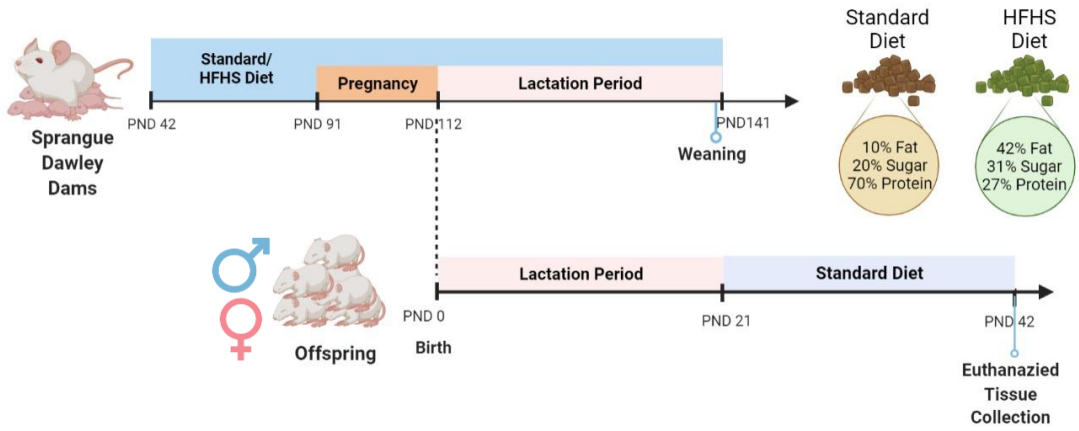

***Figure 6 – Maternal obesity experimental design**: Female Sprague-Dawley rats were fed a HFHF diet (contained 42% metabolizable energy from fat, 27% from proteins and 31% from carbohydrates) before pregnancy until lactation. At PND 42, male and female offspring were euthanized, peripheral tissues were collected for molecular and cellular analysis. Created with BioRender.com*

Exclusive metabolic programming during lactation

Wistar rats were used, and the procedures were approved by the Animal e Committee of the Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra. The animal experimentation was conducted in accordance with the European Community directive guidelines for the use of laboratory animals (2010/63/ EU), transposed into the Portuguese law in 2013 (Decreto-Lei 113/2013).

Wistar rats were housed under standard animal conditions (ventilation; 22°C temperature; 55% humidity; 12h/12h light/dark cycle) and ad libitum access to food and water. In this work, three animal models of newborns Wistar rats were used (Neonatal hyperphagia model, maternal glycation model and Neonatal Hyperphagia Exposed to Maternal Glycotoxins). After birth, body weight was monitored at PND 0, PND 4, PND 7, PND 14, PND 21, PND 35 and PND 45. On PND 21, male newborns were separated from their mother until PND 45 and fed with a standard diet. During this period, food consumption was monitored weekly. At PND 45, the levels of triglycerides were measured, and an insulin tolerance test was performed. After blood collection, animals were anesthetized with an IP injection of ketamine/chlorpromazine, euthanized by cervical displacement and the hypothalamus, visceral WAT, BAT and liver were collected for molecular analysis. The dams were anesthetized with an IP injection of ketamine/chlorpromazine, euthanized by cervical displacement at the 21 PND and their livers were collected for tissue morphology analyses.

Neonatal hyperphagia model

To study childhood obesity a model of neonatal hyperphagia was used. On the third day after the birth, a SL protocol was induced where the litter size was reduced to 3 pups per dam to induce postnatal hyperphagia (number of normal litters (NL)=10 number of SL=3;). After weaning, the newborns were fed a standard diet and euthanized by cervical displacement at PND 45 (figure 7).

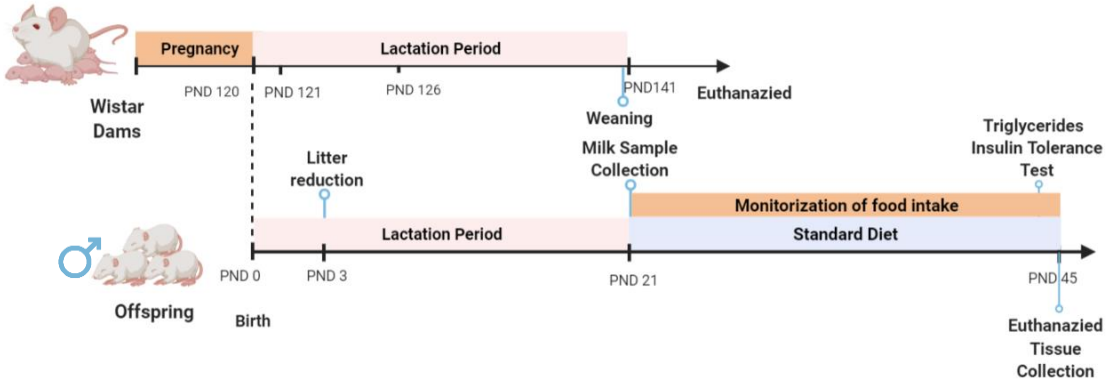

***Figure 7 – Neonatal hyperphagia-induced obesity experimental design**: Litters of male newborns Wistar rats were reduced to 3 pups per dams on the PND 3 to induce hyperphagia. At PND 45, the levels of triglycerides were measured, and an insulin tolerance test was performed. After that obese newborns were euthanized, peripheral tissues and hypothalamus were collected for molecular and cellular analysis. Created with BioRender.com.*

Maternal glycation model

To address the impact of maternal glycotoxins during lactation period, Wistar dams were injected via intraperitoneal (IP) with S-P-Bromobenzylgutathione cyclopentyl diester – BBGC (5 mg/kg) – a selective inhibitor of Glyoxalase 1 (GLO1), during the first six days post-partum, whereas control dams were injected with the vehicle dimethyl sulfoxide – DMSO (60µL) (number of NL = 10; number of DMSO = 5; number of BBGC = 5). After weaning, the newborns were fed a standard diet and euthanized by cervical displacement at PND 45 (figure 8).

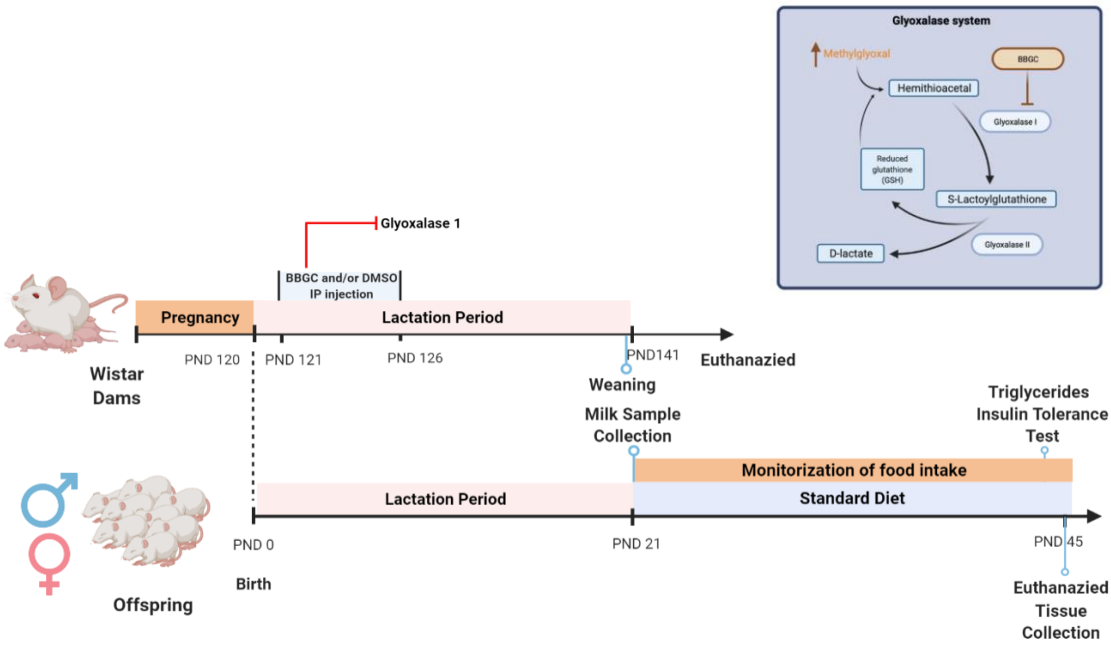

Figure 8 – Maternal glycation experimental design: *Wistar dams were treated with BBGC (5mg/kg), a selective inhibitor of GLO1, during the first six days post-partum. At PND 21 (weaning day), milk samples were collected, and dams euthanized by cervical displacement. At PND 45, the levels of triglycerides were measured, and an insulin tolerance test was performed. After that, male and female offspring were euthanized, peripheral tissues and hypothalamus were collected for molecular and cellular analysis. Created with BioRender.com.*

Neonatal hyperphagia exposed to maternal glycotoxins model

To study the exposure to maternal glycotoxins in hyperphagic newborns we combined the neonatal hyperphagia and glycation models (number of NL = 10; number of SL = 3; number of SL+BBGC = 3). After weaning, the newborns were fed a standard diet and euthanized by cervical displacement at PND 45 (figure 9).

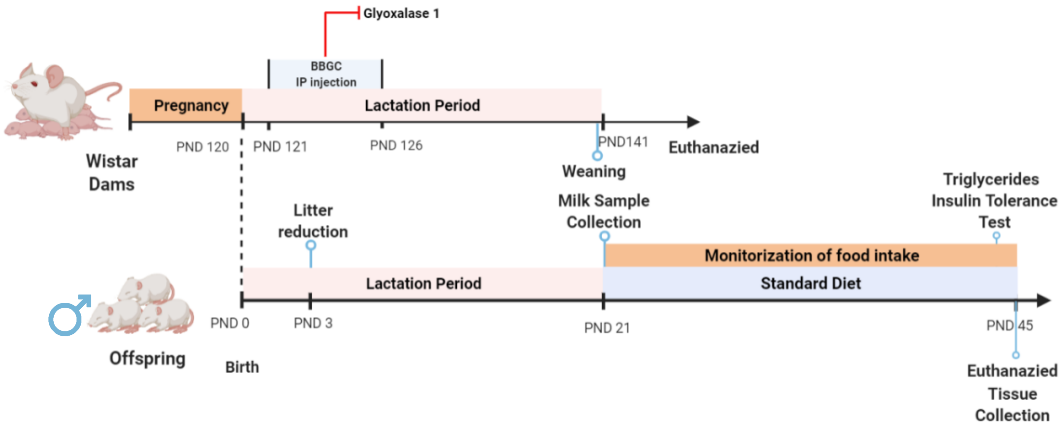

Figure 9 – Maternal glycation on obese newborns experimental design: *Wistar dams were treated with BBGC (5mg/kg), a selective inhibitor of GLO1, during the first six days post-partum. Litters of male Wistar rats were reduced to 3 pups per dams on the PND 3 to induce hyperphagia. At PND 21 (weaning day), milk samples were collected, and dams euthanized by cervical displacement. At PND 45, the levels of triglycerides were measured, and an insulin tolerance test was performed. After that, male offspring were euthanized, peripheral tissues and hypothalamus were collected for molecular and cellular analysis. Created with BioRender.com.*

Histological Colorimetric assay

Livers from dams were fixed in formalin solution (10%), dehydrated in an increasing series of alcohol concentrations (70% to 100%), cleared in xylene and then embedded in histological paraffin. The livers were sectioned in a microtome, on a non-serial section of 4 µm thickness (n=2/group) and subsequently dried overnight at RT. The paraffin-embedded liver sections were submitted to paraffin-removing processes, using xylol, progressive hydration (EtOH 100%/70%/30% 1x3'/ each and Milli-Q water 1x3' at RT) and stained with haematoxylin and eosin (H&E). Then, the liver sections were washed again, and coverslips were mounted using mounting medium (DAKO, JAPAN). Lastly, images (100x) were captured in a Zeiss microscope with incorporated camera (Germany).

Milk Sample Collection and determination of Total Antioxidant Capacity and Triglycerides

The Wistar female dams were anesthetized and injected with oxytocin (Facilpart) with a concentration of 2.5 UI/mL after 6h of fasting, on PND 21. Milk samples were collected, triglycerides of milk were measured, and milk total antioxidant capacity was assessed with an Assay Kit (ab65329) according to the manufacturer's instructions.

Determination of Insulin Levels

At PND45, newborns blood samples were collected from the tail vein to Vacuette K3EDTA tubes (Greiner Bio-one, Kremsmunster, Austria). Blood samples were immediately centrifuged (2200 × g, 4˚C, 15') and the plasma fraction was stored at -80˚C and used to perform the Rat Insulin ELISA Kit (Mercodia, Uppsala, Sweden), according to the manufacturer's instructions.

Western Blot Analysis

Tissues were collected (visceral WAT, BAT, liver and hypothalamus) washed with PBS and disrupted in lysis buffer (0.25 M Tris-HCl, 125 mM NaCl, 1% TritonX-100, 0.5% SDS, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 2 mM Na3VO4, 10 mM βglycerophosphate, 2.5 mM sodium pyrophosphate, 10 mM PMSF, 40 µL of protease inhibitor) using the TissueLyser system (Quiagen, Germany). The BCA Protein Assay Kit was carried out on the supernatant (14 000 rpm for 20 min at 4 ˚C, followed by the addition of Laemmeli buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue) [12]. Tissue samples (20 µg) were loaded into SDS-PAGE and electroblotted into PVDF membrane (Advansta, San Jose, CA, USA). TBS-T 0.01% and BSA 5% was used to block the membranes and then incubated with primary (overnight, 4˚C) and secondary antibodies (2h, room temperature), following the dilutions listed in Table 1. Membranes were detected using ECL substrate with LAS 500 system (GE-Healthcare). The obtained bands quantification was performed through Image Quant 5.0 software (Molecular Dynamics). The results were expressed as a percentage of control and normalized for the loading control (calnexin, 83 kDa).

Statistical Analysis

The results are presented as mean + standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software, Inc, San Diego, USA). The normality of the data was assessed with Shapiro-Wilk normality test. Accordingly, data with two conditions were analysed with nonpaired t-test or Mann-Whitney test and data with more than two conditions analysed with Kruskal-Wallis test or with one-way ANOVA followed by Tukey´s post-hoc test. Differences were significant for $p < 0.05$.

Chapter 4 RESULTS

Maternal diet-induced obesity during gestation and lactation

Maternal obesity modulates nutrient-sensing pathways in the visceral AT of male offspring

Maternal obesity increases NPY signalling at the hypothalamus [115], [120], thus some alterations in the periphery may be observed. Indeed, with this work, we demonstrated that maternal obesity induced by HFHS diet modulates NPY receptors system in male offspring WAT. Both NPY1R (figure 10A) and NPY2R (figure 10B) were significantly increased in young mice WAT ($p<0.001$ vs control and $p<0.05$ vs control, respectively). Moreover, maternal HFHS diet increased GHS-R1α (figure 10C) and D1R (figure 10D) levels in WAT compared to offspring from dams fed a standard diet ($p<0.05$ vs control).

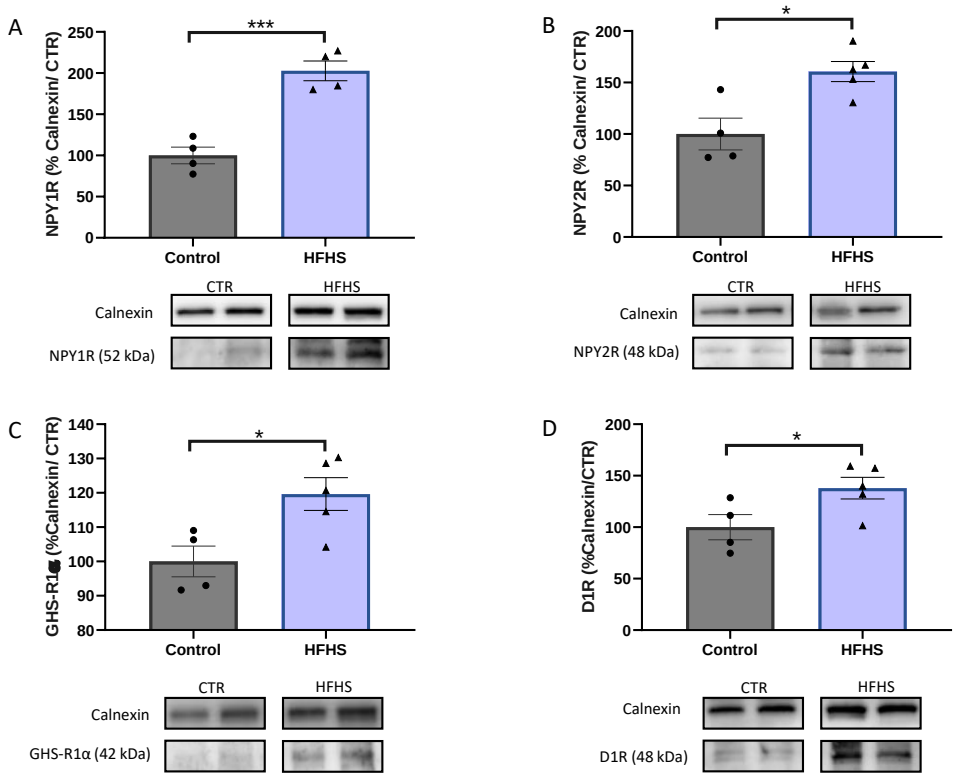

Figure 10 – The effect of maternal obesity on energy balance regulators in WAT from male offspring*. Both NPY receptors, ghrelin receptor and D1R levels are decreased in visceral AT of male offspring exposed to HFHS maternal diet. NPY1R (A), NPY2R (B), GHS-R1α (C) and D1R (D). Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 42-day-old Sprague-Dawley males from dams fed a standard diet; HFHS – male offspring with 42-day-old of dams Sprague-Dawley fed a HFHS diet. Bars represent mean ± SEM of 4 or 5 animals per group and unpaired t tests were conducted to compare among the groups. * p<0.05; *** p<0.001.*

Exposure to maternal HFHS diet impairs D1R signalling in the liver from male offspring

Regarding the liver, exposure to maternal HFHS diet induced a drastic reduction of D1R levels ($p<0.001$ vs control) without affecting GHS-R1a and NPY1R levels (figure 11).

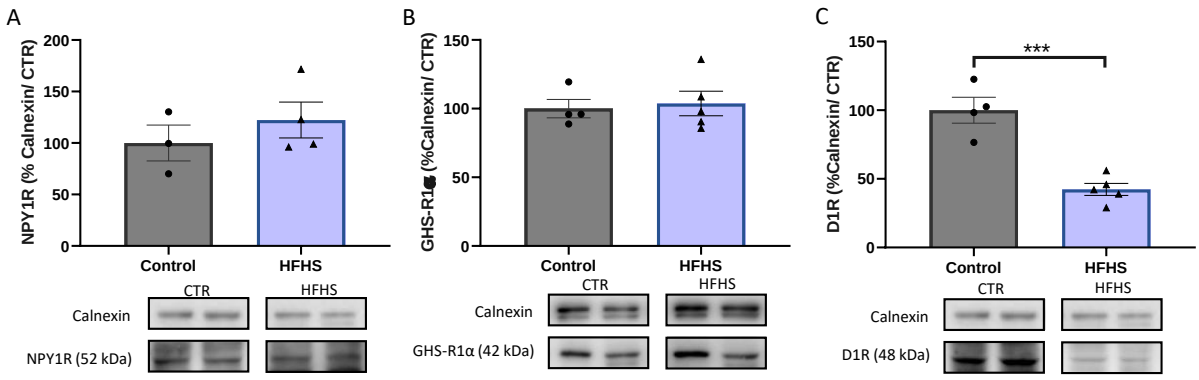

***Figure 11 – Maternal obesity during lactation impair dopaminergic signalling in the liver of male offspring**. D1R levels are reduced in the liver of young mice from obese dams (C). NPY1R (A) and GHS-R1α (B) levels remain similarity to the control. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 42-day-old Sprague-Dawley males from dams fed a standard diet; HFHS – male offspring with 42-day-old of dams Sprague-Dawley fed a HFHS diet. Bars represent mean ± SEM of 3, 4 or 5 animals per group and unpaired t tests were conducted to compare among the groups. *** p<0.001.*

Maternal obesity alters levels of energy balance-regulating receptors in the visceral AT of female offspring

The maternal metabolic state may influence offspring differently depending on sex. For instance, the increase in the NPY2R and D1R levels observed in male WAT were lost in the visceral AT of female offspring exposed to maternal obesity induced by HFHS diet (figure 12B and 12D, respectively). Surprisingly, the NPY1R levels are significantly decreased in the WAT of newborn females ($p<0.05$ vs control), demonstrating the crucial role of sex (figure 12A). In the case of ghrelin receptor, as well as in males, it was observed an increase in visceral AT from female offspring ($p<0.05$ vs control) (figure 12C).

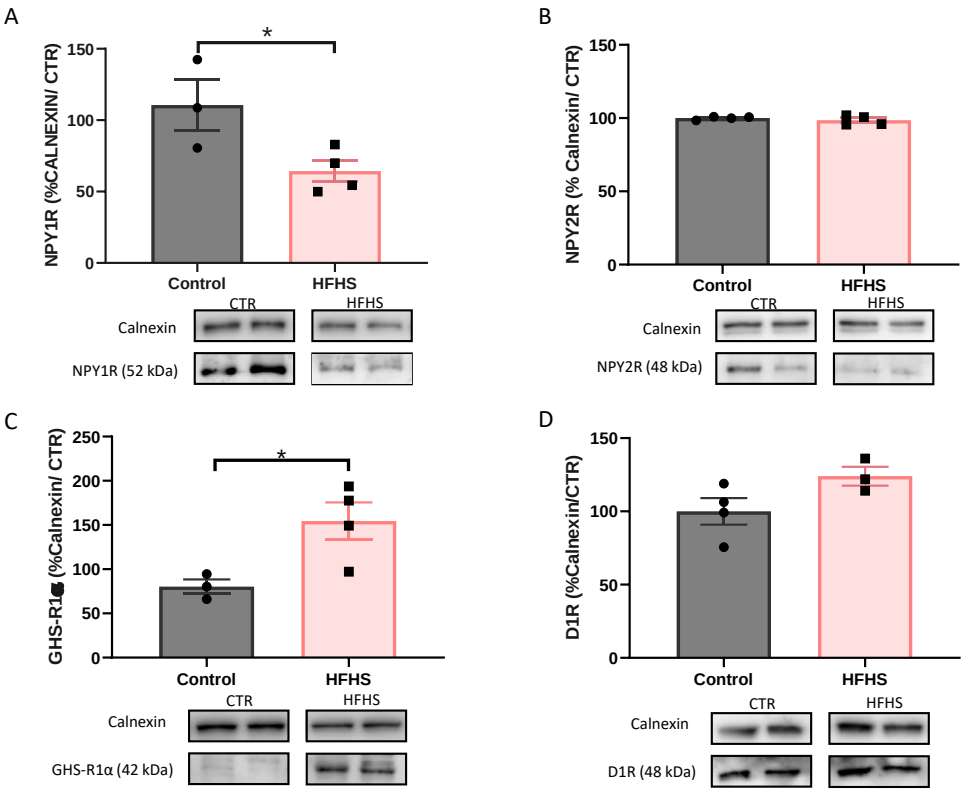

***Figure 12 – Maternal obesity reduces NPY1R levels while increasing GHS-R1α in WAT from female offspring**. NPY1R levels were reduced in females exposed to maternal obesity (A) while the quantity of its regulator (GHS-R1α) was higher in WAT of female offspring exposed to HFHS maternal diet (C). Both NPY2R (B) and D1R (D) levels were maintained. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 42-day-old Sprague-Dawley females from dams fed a standard diet; HFHS – female offspring with 42-day-old of dams Sprague-Dawley fed a HFHS diet. Bars represent mean ± SEM of 4 or 5 animals per group and unpaired t tests were conducted to compare among the groups. * p<0.05.*

Maternal HFHS diet induces the same response in dopaminergic signalling in the liver from both sex

Regarding the liver, the effect on D1R levels was the same in both sexes, a remarkable reduction in offspring exposed to HFHS diet during the perinatal period ($p<0.001$ vs control) (figure 13C). Furthermore, in female livers, the NPY1R levels were also decreased, showing another disparity between the sex ($p<0.05$ vs control) (figure 13A). As well in the male liver, there was no alterations observed in GHS-R1α levels (figure 13B).

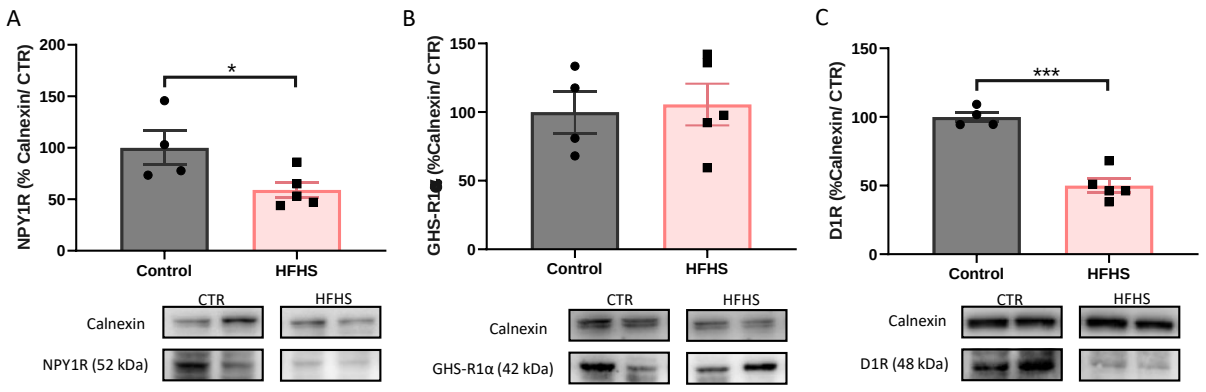

Figure 13 – Maternal HFHS diet reduces both NPY and dopamine 1 receptors in the liver of female offspring*. In the liver, NPY1R (A) and D1R (C) levels decreased when animals are exposed to maternal obesity, without affecting GHS-R1α levels (B). Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 42-day-old Sprague-Dawley females from dams fed a standard diet; HFHS – female offspring with 42-day-old of dams Sprague-Dawley fed a HFHS diet. Bars represent mean ± SEM of 3, 4 or 5 animals per group and unpaired t tests were conducted to compare among the groups. * p<0.05; *** p<0.001.*

Neonatal hyperphagia model

Neonatal hyperphagia-induced obesity increases triglycerides levels while reducing plasma insulin levels

Several studies have been demonstrating that neonatal hyperphagia induces obesity in early life [136], [142]. In this study, we have used male from SL as a model of hyperphagia without modification in maternal diet. Indeed, animals from SL have significantly higher body weight than the control group at the weaning day – day 21 ($p<0.001$ vs control) (figure 14A). This weight gain was maintained over time until the day of euthanasia – day 45 ($p<0.01$ vs control) (figure 14B). Despite the WAT mass per body weight was not altered in obese young mice (figure 14D) as well as the liver weight (figure 14C), the absolute value of WAT mass was increased in hyperphagic animals ($p<0.01$).

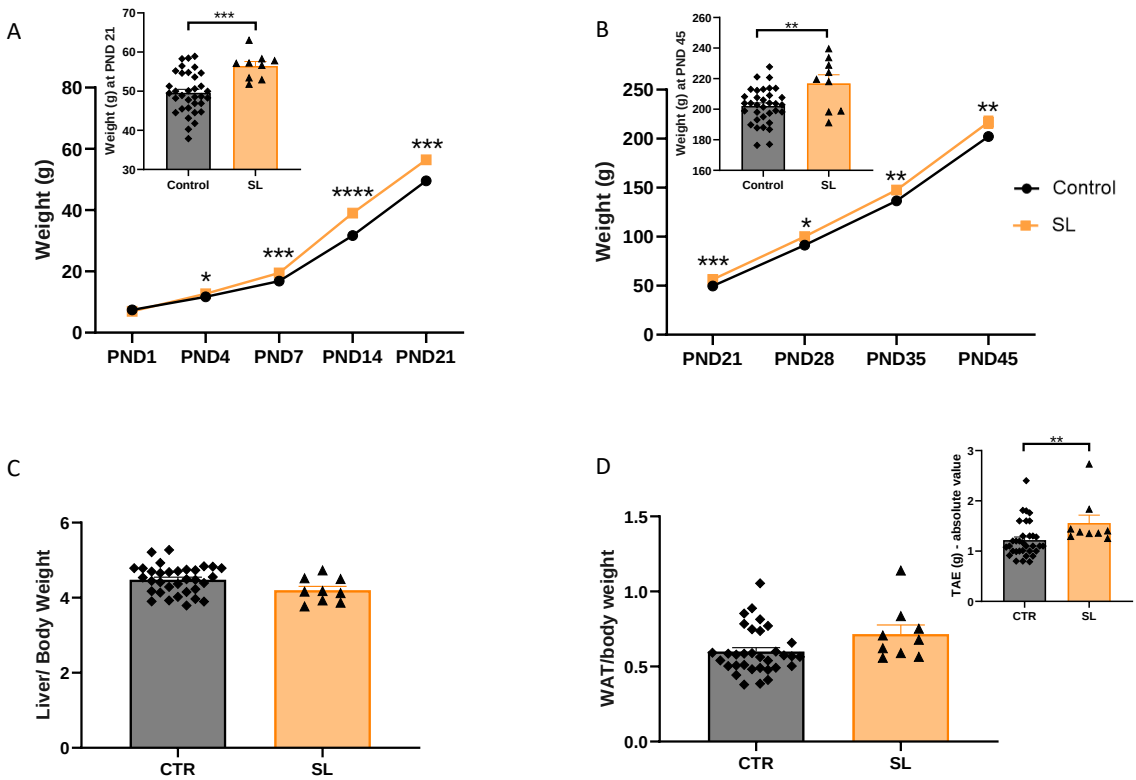

Figure 14 – Neonatal hyperphagia induces weight gain. *Weight gain curves during the first 21 PND (weaning day) (A). Weight gain curves between 21 PND to 45 PDN (period after weaning) (B). Liver weight per body weight (C) and WAT weight per body weight and absolute value of fat mass (D) at 45 PND. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters. Bars represent mean ± SEM of 35 animals in control and 9 in SL group and unpaired t tests were conducted to compare among the groups. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.*

Nevertheless, the insulin tolerance test performed at the 45th day reveals that insulin sensitivity was not affected in hyperphagic offspring (figure 15A). The decay of the glucose rate during the insulin tolerance test per minute (kITT) corroborated this result (figure 15B). On the other hand, insulin levels were decreased in plasma of obese young mice (p<0.001 vs control) (figure 15C), while the triglyceride levels in plasm of animals submitted to neonatal hyperphagia did not alter (figure 15D).

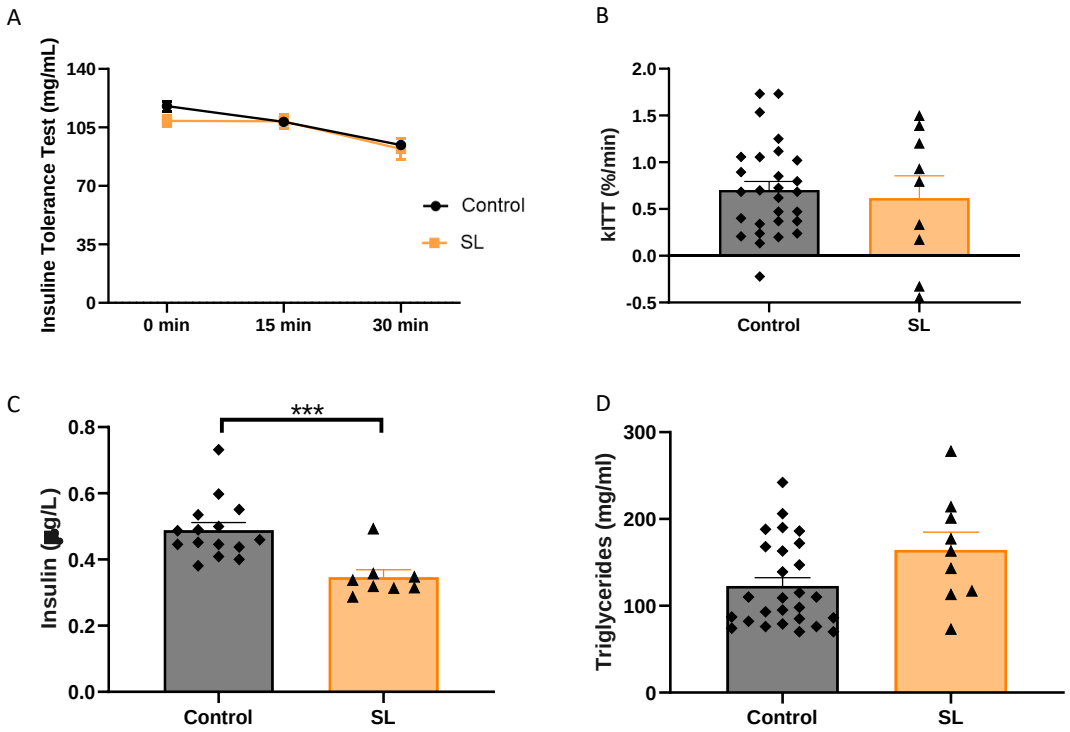

Figure 15 – Plasma insulin levels is reduced in obese animals. *Insulin tolerance test (ITT) curve during 30 minutes at 45 PND (A). Decay of the glucose rate during the insulin tolerance test per minute (kITT) (B). Hyperphagia-induced obesity reduces plasma insulin levels (C) and maintains plasma triglycerides (D). Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters. Bars represent mean ± SEM of 28 animals in control and 9 in SL group and unpaired t tests were conducted to compare among the groups. *** p<0.001.*

Early life obesity increases receptors levels associated with adiposity and WAT enlargement on male

Using this neonatal hyperphagia model of obesity, we studied possible alterations in WAT. Indeed, this model presented higher NPY2R levels ($p<0.05$ vs control) (figure 16B), although the levels of NPY1R, GHS-R1α and D1R in WAT were maintained (figure 16A, 16C and 16D, respectively).

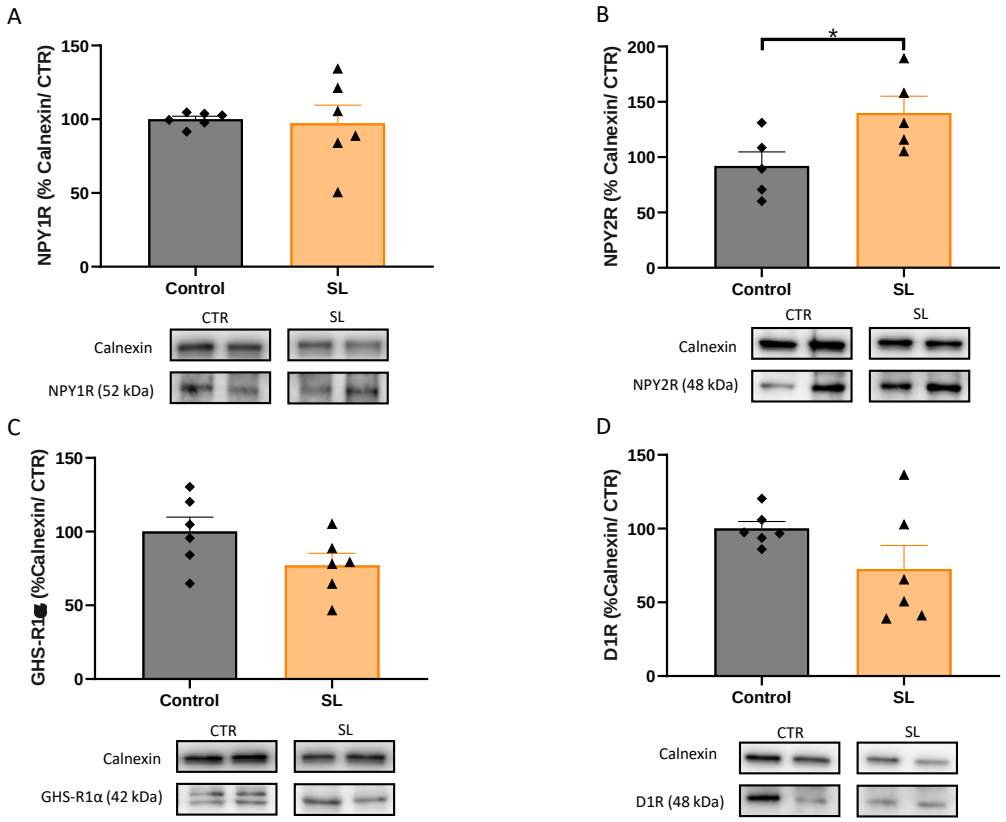

***Figure 16 – Early life obesity enhances signalling of NPY receptor associated with AT enlargement**. NPY2R levels were increased in visceral AT of neonatal hyperphagic male (B), without affecting NPY1R (A), GHS-R1α (C) and D1R (D) levels. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters. Bars represent mean ± SEM of 6 animals per group and unpaired t tests were conducted to compare among the groups. * p<0.05.*

Energy balance mechanisms in BAT remain unchanged in early life obesity induced by hyperphagia

The BAT plays an important role in energy balance, especially in early life. The NPY1R and NPY2R levels in this tissue did not significantly alter in obesity induced by neonatal hyperphagia (figure 17). The GHS-R1α levels were not detectable, possibly due to the low presence of this receptor in the tissue. However, more studies need to be performed to clarify this pathway in obesity conditions.

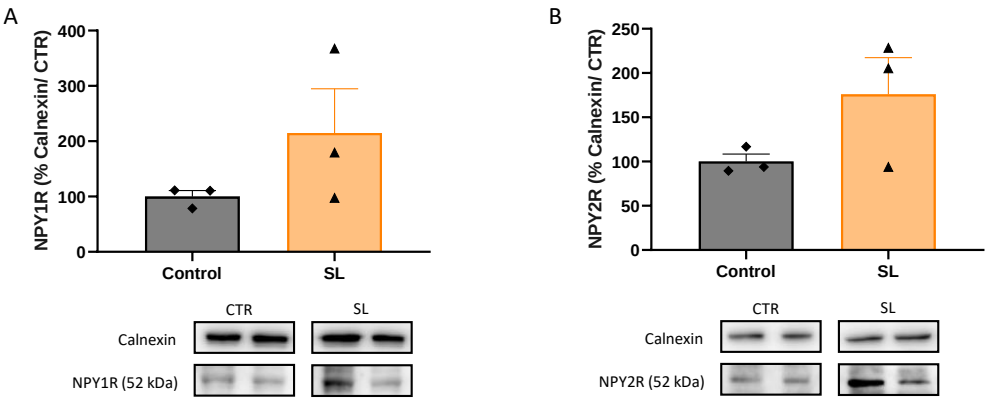

Figure 17 – Obesity induced by neonatal hyperphagia seems to not alter NPY signalling in BAT. *NPY1R (A) and NPY2R (B) levels in BAT. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters. Bars represent mean ± SEM of 3 animals per group and unpaired t tests were conducted to compare among the groups.*

Dopaminergic signalling in the liver was impaired in hyperphagic animals

The levels of NPY1R, GHS-R1α also did not alter when the animals are exposed to a higher consumption of breastmilk during the perinatal period (figure 18A and 18B, respectively). On the other hand, D1R levels were decreased ($p<0.05$) (figure 18C), in accordance with the observed in the hepatic tissue from the offspring with early-life obesity induced by maternal HFHS.

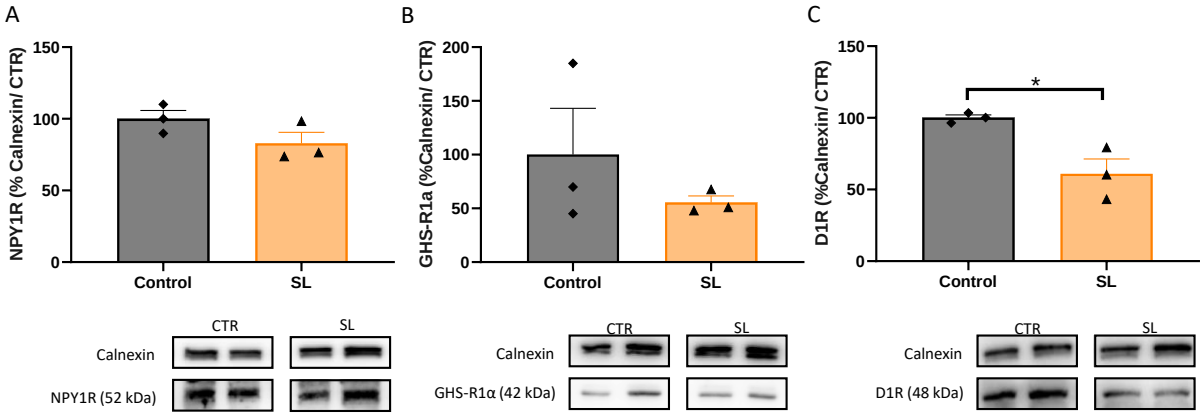

Figure 18 – Obesity in early life impairs dopaminergic signalling in the liver. *D1R levels were reduced in the liver of obese animals (C). NPY1R (A) and GHS-R1α (B) levels remained similar to the control. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters. Bars represent mean ± SEM of 3 animals per group and unpaired t tests were conducted to compare among the groups. * $p<0.05$*

Neonatal hyperphagia promotes a reduction in NPY action at the hypothalamic level

The hypothalamus, namely the arc, plays an important role in food intake regulation. Although these animals are considered an early-life hyperphagic model, no alteration on food consumption was observed after weaning comparatively to control (figure 19). However, in this model, some alterations may exist on pathways associated with food consumption and satiety. It was already proven that neonatal hyperphagia is associated with a higher number of NPY neurons [136]. Here, we demonstrated that in the hypothalamus of hyperphagic animals the NPY1R levels were decreased ($p<0.05$ vs control) (figure 20A). On the other hand, the autoinhibitory NPY receptor (NPY2R) levels did not alter (figure 20B). Moreover, the levels of other receptors that induce NPY neurons activity such GHS-R1a and D1R were maintained in obese animals induced by hyperphagia (figure 20C and 20D, respectively).

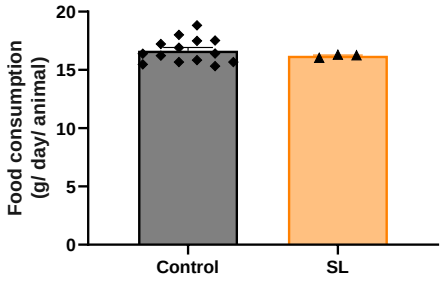

***Figure 19 – Neonatal hyperphagia did not alter food intake in obese animals after weaning**. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters. Bars represent mean ± SEM of 35 animals in control and 9 in SL group and unpaired t test was conducted to compare among the groups.*

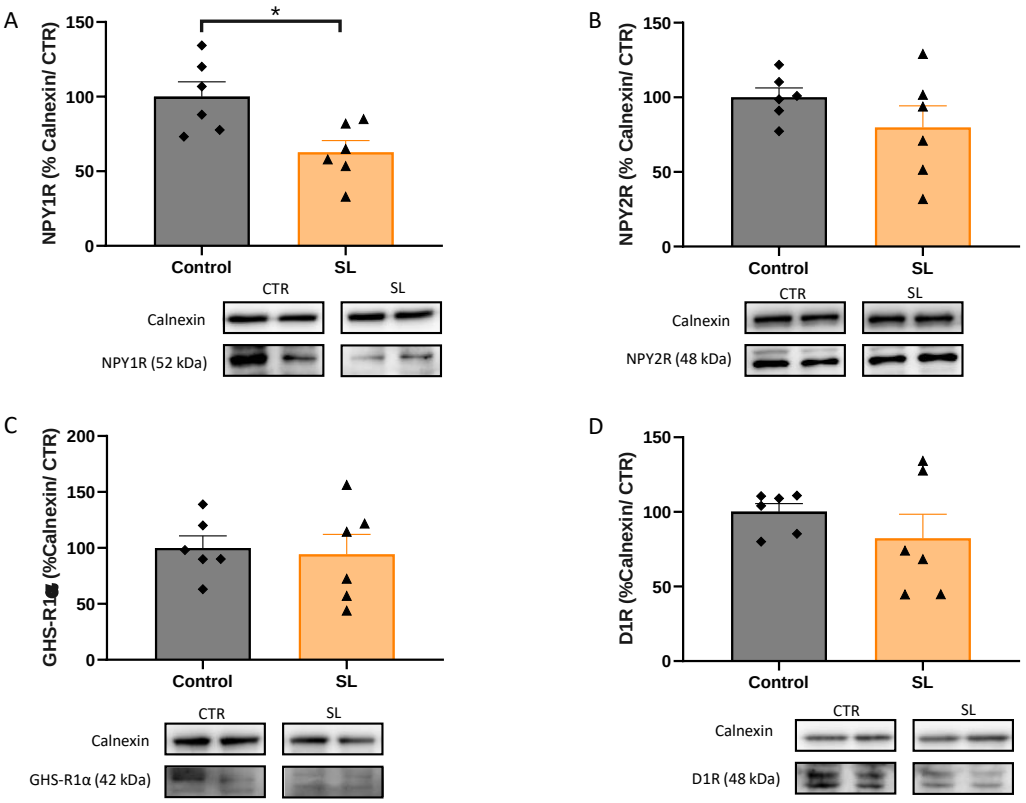

Figure 20 – Neonatal hyperphagia reduces the food intake stimulating receptor (NPY1R) in the hypothalamus. *NPY1R levels were decreased in early life obesity (A) whereas levels of NPY2R (B), GHS-R1α (C) and D1R (D did not suffer alteration. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters. Bars represent mean ± SEM of 6 animals per group and unpaired t tests were conducted to compare among the groups. * p<0.05*

Maternal glycation model

Glyoxalase 1 inhibition impairs the antioxidant capacity as well as decreases triglycerides levels in breastmilk

The glyoxalase 1 inhibition during 6 days did not change the body weight of dams (figure 21A). Moreover, we also observed that BBGC did not promote toxicity in the liver of dams (figure 21B). Nevertheless, the inhibition of this enzyme led to a decrease in triglycerides levels ($p<0.01$ vs control) (figure 21C) and a reduction of total antioxidant capacity ($p<0.05$) in milk from dams treated with BBGC collected on PND 21 (figure 21D).

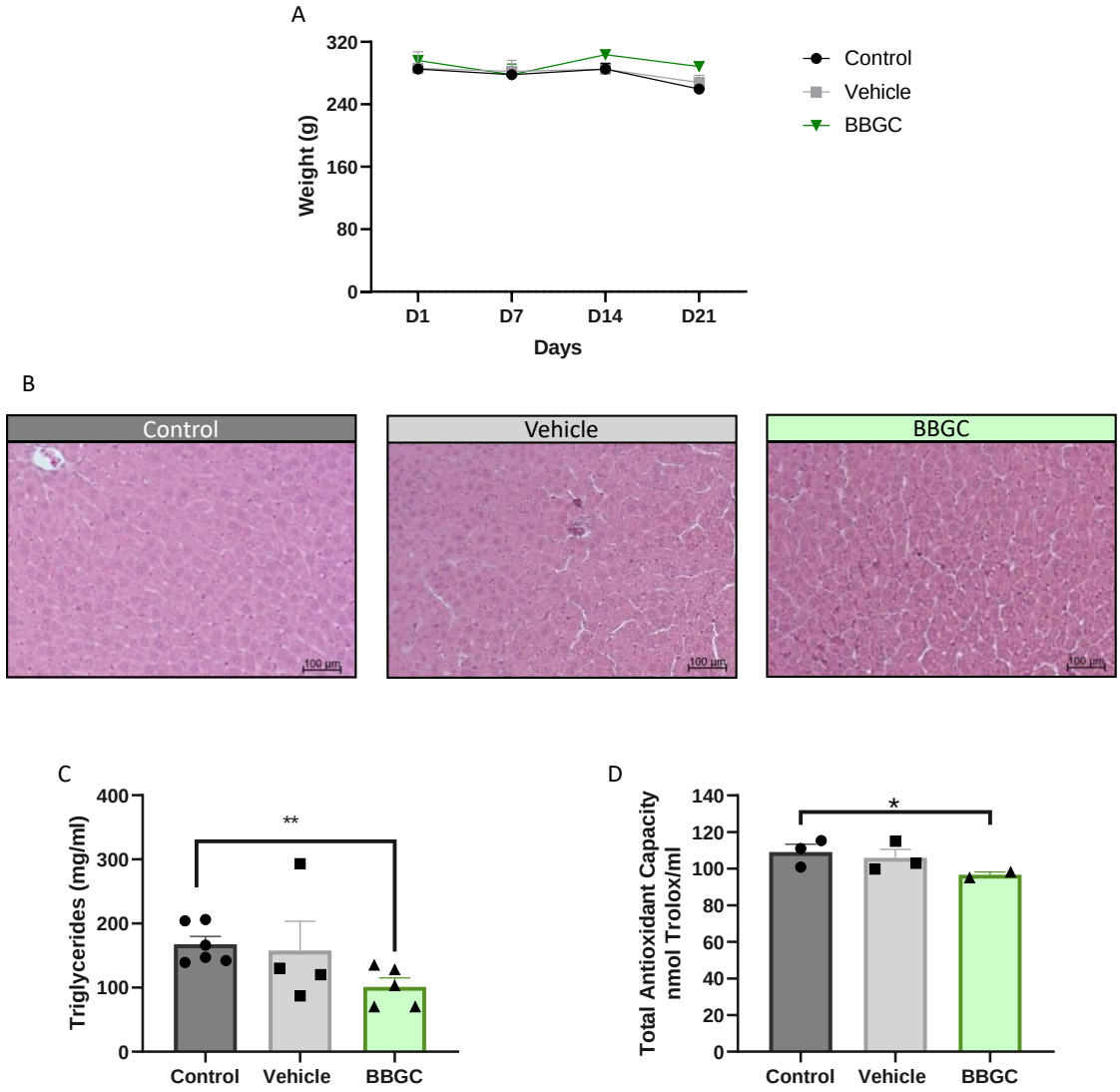

***Figure 21* – Glyoxalase 1 inhibitor reduces triglycerides levels and total antioxidant capacity in breastmilk.** Weight gain curves of dams treated with BBGC during the first 6 days post-partum (A). Representative histology of liver from control dams, dams treated with vehicle or BBGC (B). BBGC decreased triglycerides breastmilk levels (C) and impaired antioxidant capacity (D). Control – Wistar control dams; Vehicle – Wistar dams treated with DMSO; BBGC- Wistar dams treated with BBGC. Bars represent mean ± SEM of 2 – 5 animals per group and Kruskal-Wallis test or one-way ANOVA were conducted to compare among the groups. * $p<0.05$; ** $p<0.01$.

Exposure to glycotoxins during lactation does not affect body weight in male offspring

Obesogenic environments contribute to body weight alteration in early life, which is not often observed in lean experimental models of glycation. Here, we observed that maternal glycation did not affect the body weight of male young mice either during the breastfeeding period or after weaning (figure 22A and 22B). Furthermore, liver weight and fat mass were also not altered in offspring exposed to glycated products (figure 22C and 22D, respectively).

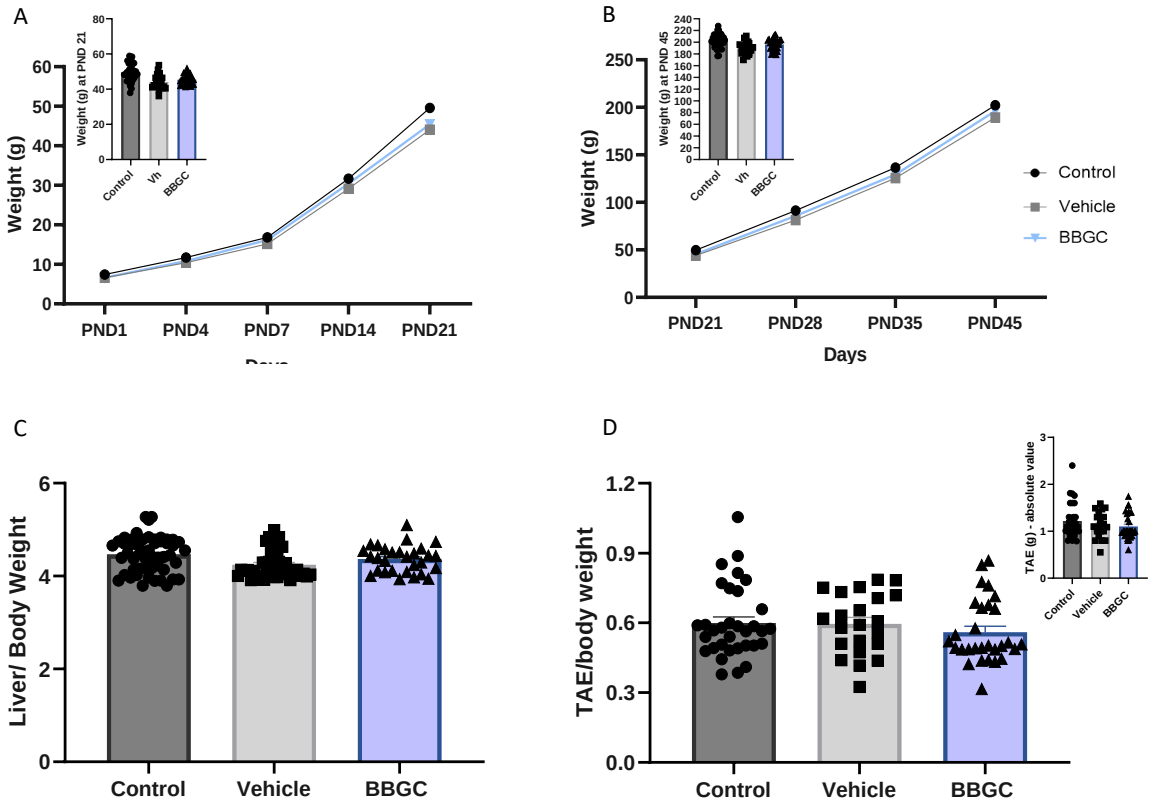

Figure 22 – Body weight is not affected by maternal glycation in lean phenotype. *Weight gain curves during the first 21 PND (weaning day) (A). Weight gain curves between 21 PND to 45 PDN (period after weaning) (B). Liver weight per body weight (C) and WAT weight per body weight and absolute value of fat mass (D) at 45 PND. Control – male offspring of control dams; Vehicle – male offspring of dams treated with DMSO; BBGC- Wistar male offspring of dams treated with BBGC. Bars represent mean ± SEM of 34 animals in control group, 22 animals in vehicle group and 29 animals in BBGC group and Kruskal-Wallis test or one-way ANOVA were conducted to compare among the groups.*

Maternal glycation impairs insulin-dependent glucose uptake without affecting the insulin levels on male offspring

In order to study the effect of exposure to glycated products during the lactation period, an ITT was performed. Through this procedure, it was not observed changes in insulin action regarding glucose uptake (figure 23A). However, the decay of the glucose rate during the ITT (kITT) was reduced in male offspring exposed to maternal glycation ($p<0.05$ vs control) (figure 23B). On the other hand, plasma insulin and triglycerides levels of male offspring from dams treated with glyoxalase 1 inhibitor also did not change (figure 23C and 23D, respectively).

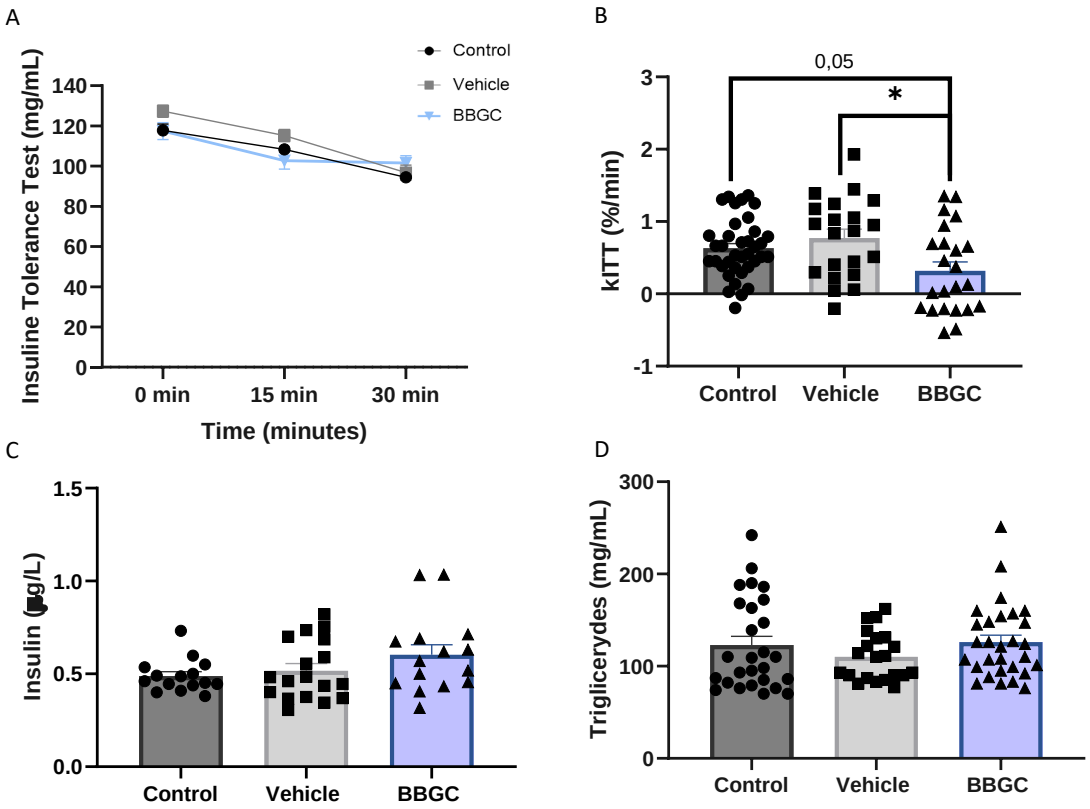

Figure 23 – Exposure to glycated products during lactation impaired insulin action during the ITT. *Insulin tolerance test (ITT) curve during 30 minutes at 45 PND (A). Maternal glycation reduced the decay of the glucose rate during the insulin tolerance test per minute (kITT) (B) without affecting plasma insulin levels (C). Plasma triglyceride levels do not change (D). Control – male offspring of control dams; Vehicle – male offspring of dams treated with DMSO; BBGC- Wistar male offspring of dams treated with BBGC. Bars represent mean ± SEM of 34 or 15 animals in control group, 22 or 18 animals in vehicle group and 29 or 15 animals in BBGC group and Kruskal-Wallis test or one-way ANOVA were conducted to compare among the groups. * p<0.05.*

Energy balance mechanisms in both AT (WAT and BAT) are not affected by maternal glycation exposure during lactation in lean male offspring

Adult models exposed to glycated products showed that glycation does not cause significant alterations in WAT function in the lean phenotype [15]. Here we demonstrated that male offspring from dams treated with glyoxalase 1 inhibitor did not present changes in energy expenditure and storage mechanisms in visceral AT. The receptors of NPY, ghrelin and dopamine evaluated (NPY1R, NPY2R, GHS-R1α and D1R, respectively) maintained their levels when compared with the vehicle and with the control (figure 24).

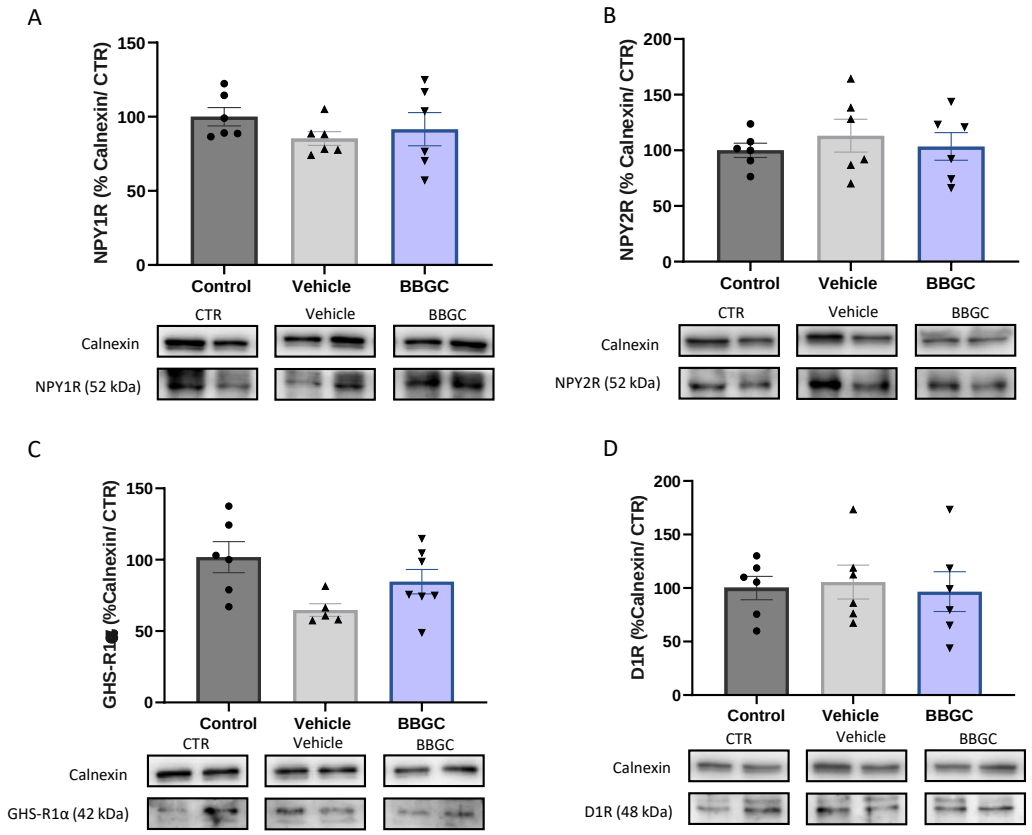

Figure 24 – Mechanisms of WAT energy storage and expenditure were not altered in male offspring exposed to maternal glycation. *NPY1R (A), NPY2R (B), GHS-R1α (C) and D1R (D). Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – male offspring of control dams; Vehicle – male offspring of dams treated with DMSO; BBGC- Wistar male offspring of dams treated with BBGC. Bars represent mean ± SEM of 6 animals per group and one-way ANOVAs were conducted to compare among the groups.*

The NPY1R levels in BAT remained unchanged when the male offspring are exposed to glycotoxins during lactation (figure 25). However, more studies should be performed to elucidate NPY pathway in this condition and its effects on NPY-regulated thermogenesis in BAT.

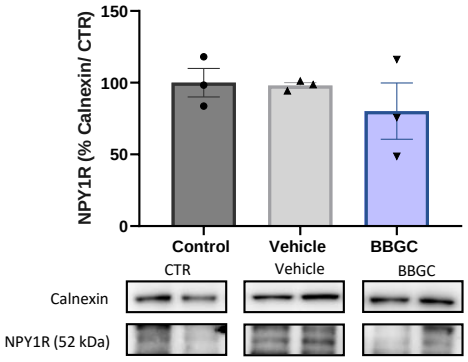

Figure 25 – Levels of NPY receptor responsible for inhibiting thermogenesis in BAT (NPY1R) do not change in male offspring exposed to glycotoxins during lactation*. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – male offspring of control dams; Vehicle – male offspring of dams treated with DMSO; BBGC-Wistar male offspring of dams treated with BBGC. Bars represent mean ± SEM of 3 animals per group and one-way ANOVAs were conducted to compare among the groups.*

Exposure to maternal glycated products reduces both NPY and Dopamine signalling in the liver from male offspring

In the liver, maternal glycation induced a reduction of D1R ($p<0.01$ vs control; $p<0.05$ vs vehicle) and NPY1R levels ($p<0.01$ vs control) without affecting ghrelin receptor levels (figure 26).

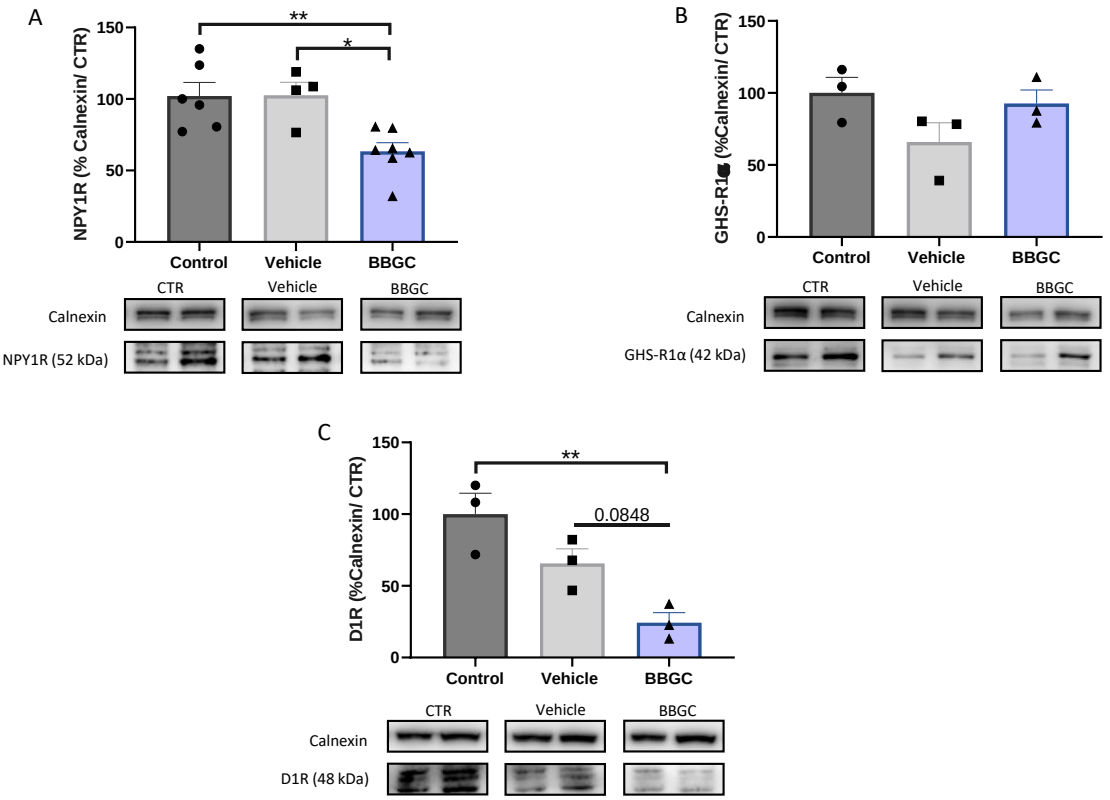

Figure 26 – The impairment of dopaminergic signalling in the liver is accompanied by NPY1R levels decrease in conditions of maternal glycation on male offspring. *Newborns of dams treated with glyoxalase 1 inhibitor presented lower levels of NPY1R (A) and D1R (C), but GHS-R1α levels (B) were maintained. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – male offspring of control dams; Vehicle – male offspring of dams treated with DMSO; BBGC- Wistar male offspring of dams treated with BBGC. Bars represent mean ± SEM of 3 or 6 animals per group and one-way ANOVAs were conducted to compare among the groups. * p<0.05; ** p<0.01.*

Maternal glycation diminishes food consumption whereas promoting hypothalamic alteration on energy balance receptors levels in male offspring

As has been mentioned, food intake is regulated by hypothalamic areas. In here, we observed a reduction on food consumption in male animals exposed to glycated products during lactation ($p<0.01$ vs control) (figure 27).

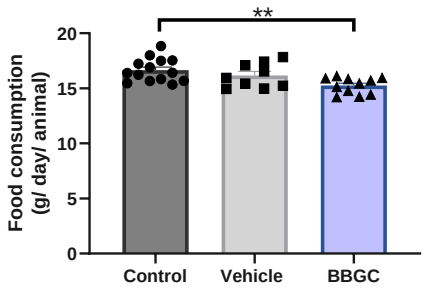

Figure 27 – Maternal glycation reduces food consumption in male offspring*. Control – male offspring of control dams; Vehicle – male offspring of dams treated with DMSO; BBGC- Wistar male offspring of dams treated with BBGC. Bars represent mean ± SEM of 34 animals in control group,22 animals in vehicle group and 29 animals in BBGC group and one-way ANOVA was conducted to compare among the groups. ** p<0.01.*

Accordingly, the NPY receptor that triggers food intake in the hypothalamus was also reduced on this group of animals ($p<0.05$ vs control) (figure 28A). On the other hand, the D1R signalling has a drastic increase in the hypothalamic area ($p<0.001$ vs control; $p<0.001$ vs vehicle) (figure 28D). NPY2R and GHS-R1α levels were maintained (figure 28B and 28C, respectively).

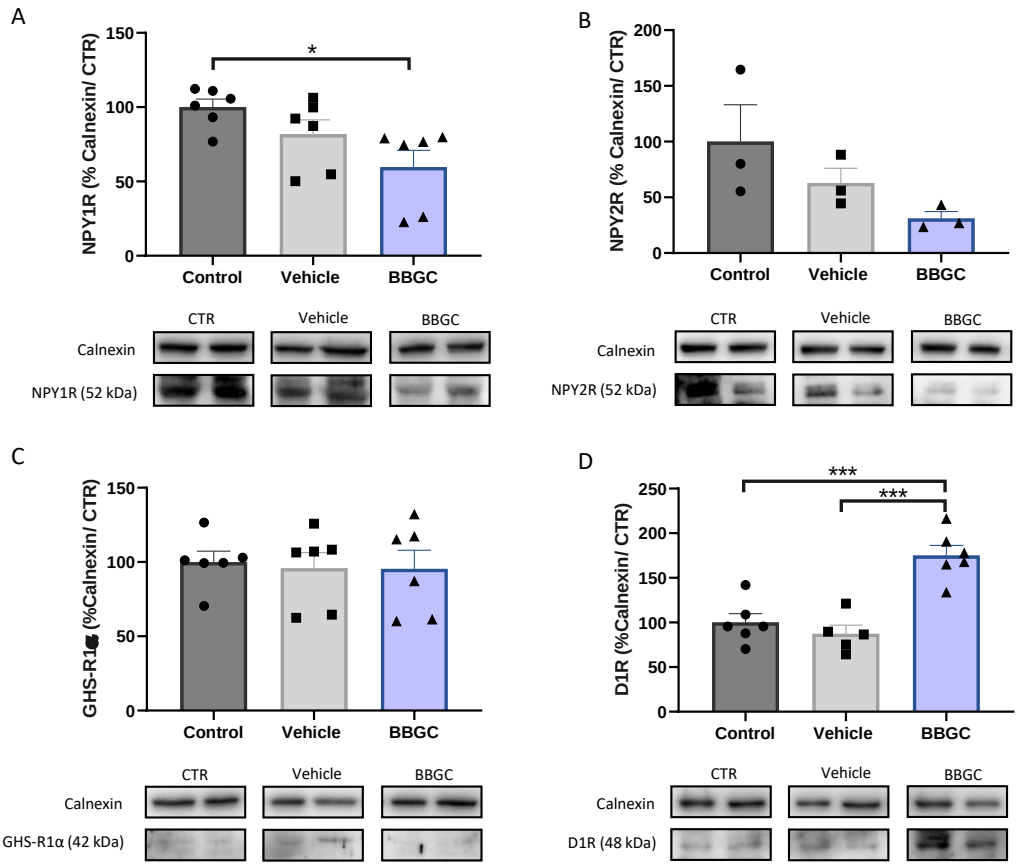

Figure 28 – Exposure to glycotoxins during lactation dysregulated regulatory receptors of food intake and energy expenditure at hypothalamic level*. NPY1R decreased in the hypothalamus of male offspring from dams treated with glyoxalase 1 inhibitor (A) without affecting NPY2R (B) and GHS-R1α (C) levels. D1R levels were drastic increased in this condition (D). Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – male offspring of control dams; Vehicle – male offspring of dams treated with DMSO; BBGC- Wistar male offspring of dams treated with BBGC. Bars represent mean ± SEM of 3 or 6 animals per group and Kruskal-Wallis test or one-way ANOVA were conducted to compare among the groups. * p<0.05; ** p<0.001*

BBGC-induced maternal glycation does not affect insulin action on glucose uptake nor triglyceride levels in female offspring

As well as in the male offspring, during the lactation period and after weaning no alteration in body weight of females exposed to maternal glycation was observed (figure 29A and 29B). Moreover, liver weight and fat mass were also not modified in female offspring (figure 29C and 29D, respectively).

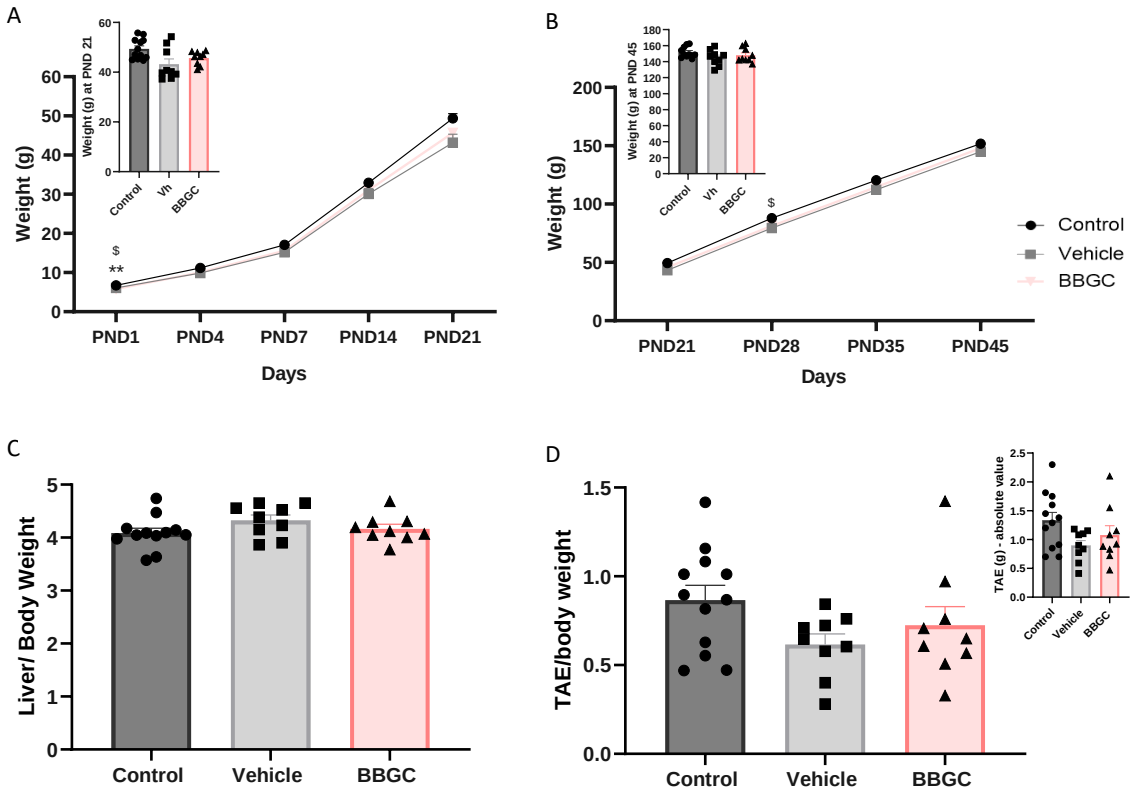

Figure 29 – Body and tissue weight in female offspring submitted to maternal glycation were not affected. *Weight gain curves during the first 21 PND (weaning day) (A). Weight gain curves between 21 PND to 45 PDN (period after weaning) (B). Liver weight per body weight (C) and WAT weight per body weight and absolute value of fat mass (D) at 45 PND. Control – female offspring of control dams; Vehicle – female offspring of dams treated with DMSO; BBGC- Wistar female offspring of dams treated with BBGC. Bars represent mean ± SEM of 12 animals in control group, 9 animals in vehicle group and 9 animals in BBGC group and Kruskal-Wallis test or one-way ANOVA were conducted to compare among the groups.*

Additionally, no changes were observed for the ITT and kITT on female offspring exposed to glycated products during lactation (figure 30A and 30B). Although plasma insulin levels from female offspring were decreased ($p<0.01$ vs control), this was also observed in the vehicle group ($p<0.01$ vs control) (figure 30C). Triglyceride levels were also unaltered between groups (figure 30D).

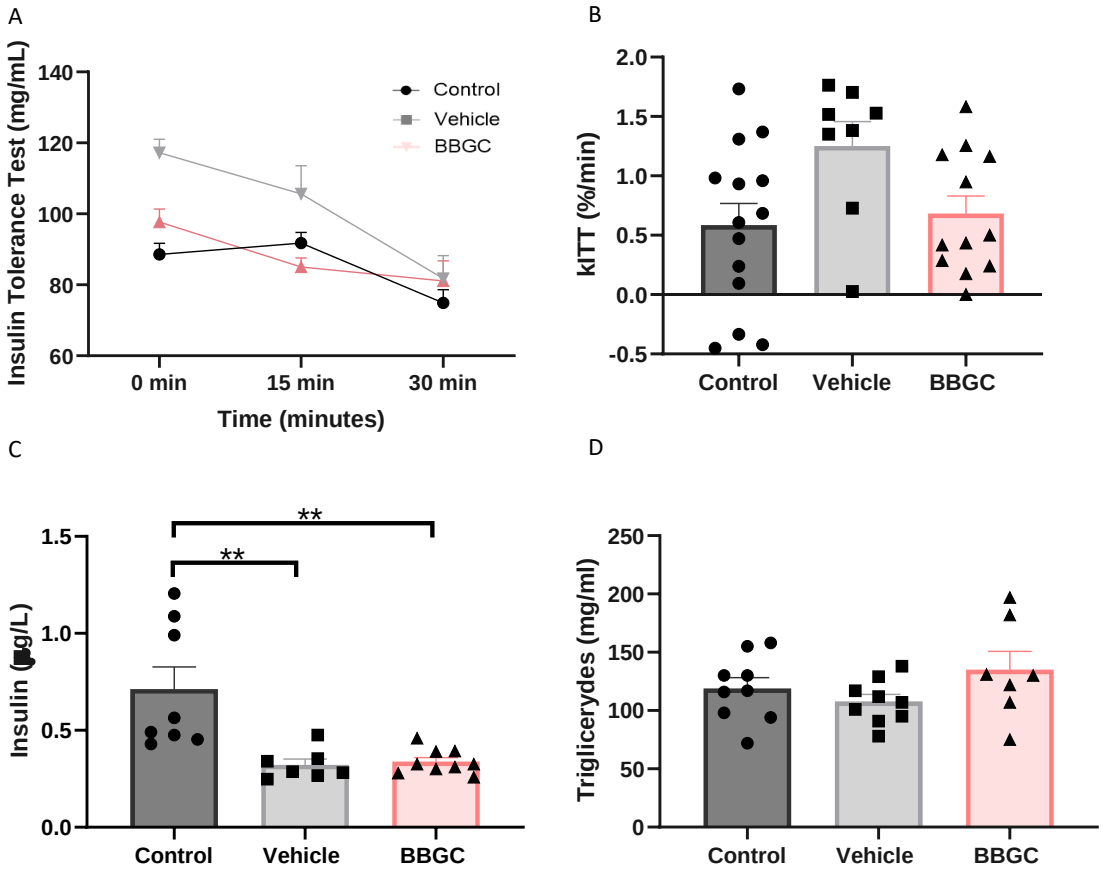

Figure 30 – Maternal glycation did not change insulin action regarding glucose uptake in female offspring. *Insulin tolerance test (ITT) curve during 30 minutes at 45 PND (A). The decay of the glucose rate during the insulin tolerance test per minute (kITT) (B). Both vehicle and BBGC injection in the dams affects plasma insulin levels female offspring (C). Plasma triglyceride levels do not change (D). Control – female offspring of control dams; Vehicle – female offspring of dams treated with DMSO; BBGC- Wistar female offspring of dams treated with BBGC. Bars represent mean ± SEM of 12 animals in control group, 9 animals in vehicle group and 9 animals in BBGC group and Kruskal-Wallis test or one-way ANOVA were conducted to compare among the groups. ** p<0.01.*

Energy balance mechanisms are not affected in visceral AT of female offspring

Has mentioned above, sex may be an important factor on the modulation of energy balance mechanisms. Here we demonstrate that the exposure to glycotoxins during lactation did not change the receptors associated with energy expenditure and storage in WAT (NPY1R, NPY2R, GHS-R1α and D1R) in female offspring (figure 31).

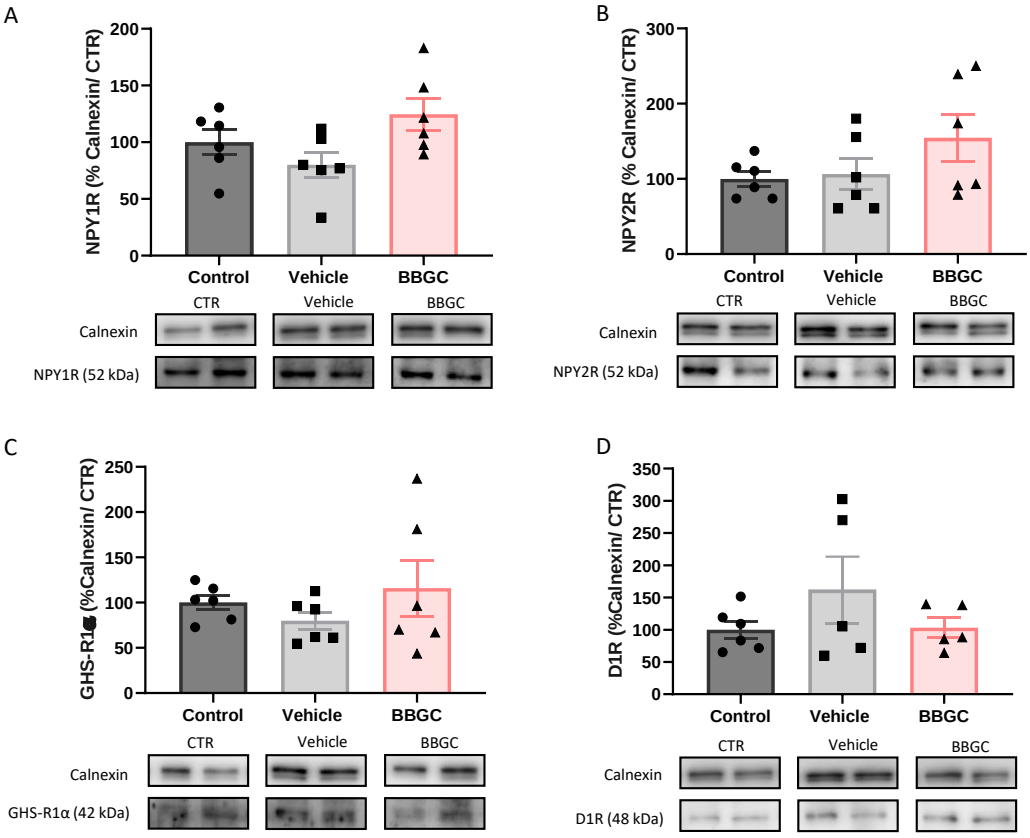

Figure 31 – Mechanisms of energy balance were not altered in WAT of female offspring exposed to maternal glycation. *NPY1R (A), NPY2R (B), GHS-R1α (C) and D1R (D). Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – female offspring of control dams; Vehicle – female offspring of dams treated with DMSO; BBGC- Wistar female offspring of dams treated with BBGC. Bars represent mean ± SEM of 6 animals per group and one-way ANOVAs were conducted to compare among the groups.*

Dopaminergic signalling is affected in the liver from female offspring exposed to glycotoxins during lactation

The effects observed in the liver from female offspring were mostly attributed to the vehicle. All the receptors evaluated were altered in both vehicle and BBGC conditions. Regarding NPY1R and GHS-R1α, both levels were decreased in newborn females from dams treated with DMSO ($p<0.01$ vs control) and from dams submitted to glyoxalase 1 inhibition ($p<0.01$ vs control) (figure 32A and 32B, respectively). However, the effect on D1R observed in the vehicle group ($p<0.01$ vs control) seems to be partially independent of the reduction induced by BBGC ($p<0.001$ vs control; $p<0.05$ vs vehicle) (figure 32C), what is in accordance with the results observed in male offspring.

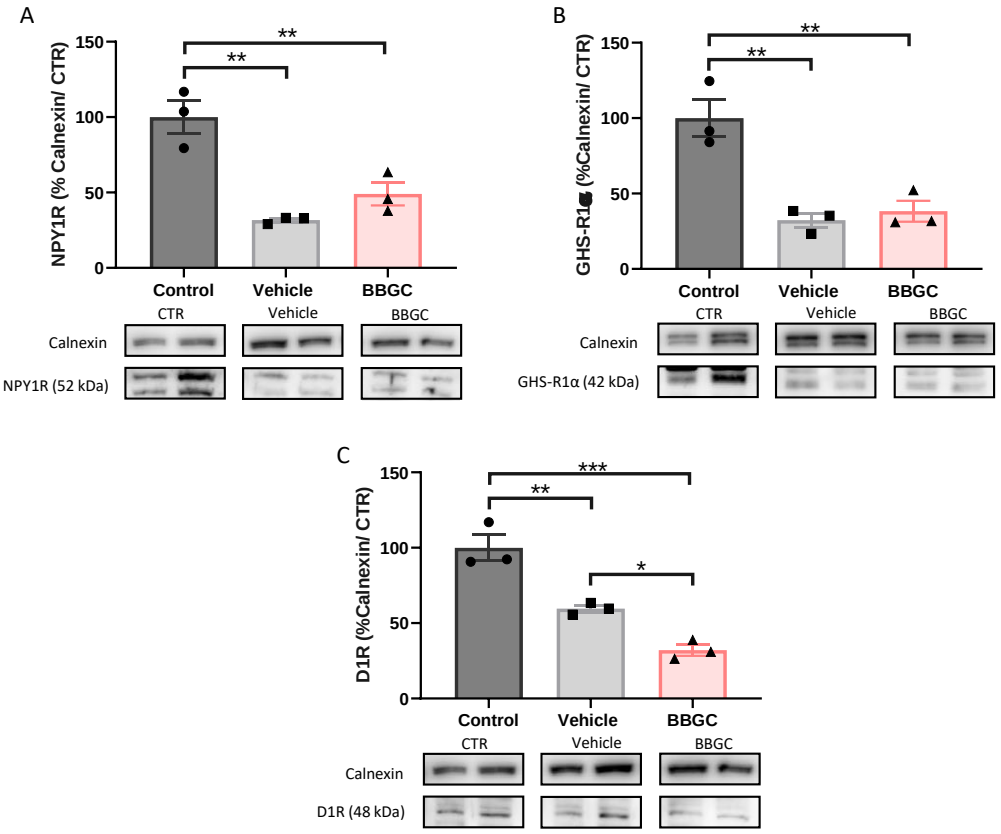

Figure 32 – Both vehicle and BBGC treatment in dams decreased NPY1R (A), GHS-R1α (B) and D1R (C) levels in liver of female offspring. *Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – female offspring of control dams; Vehicle – female offspring of dams treated with DMSO; BBGC- Wistar female offspring of dams treated with BBGC. Bars represent mean ± SEM of 3 animals per group and one-way ANOVAs were conducted to compare among the groups. * p<0.05; ** p<0.01; *** p<0.001.*

Maternal glycation does not induce major alterations in the hypothalamus in female offspring

Contrary to what was observed in food behavior in males, there was no alteration on food intake in female exposed to glycotoxins during lactation (figure 33). Another gender discrepancy observed was in NPY1R and D1R levels which were maintained through the different experimental groups in the hypothalamic area from females (figure 34A and 34D, respectively). In male offspring, NPY2R levels were maintained while in females there was an increase ($p<0.05$ vs control; $p<0.05$ vs vehicle) (figure 34B) while ghrelin receptor levels decreased ($p<0.05$ vs control) (figure 34C).

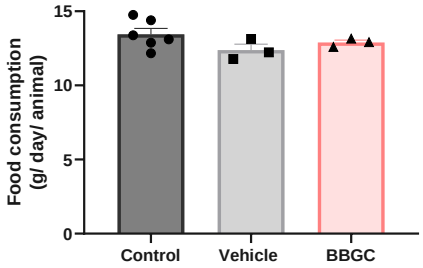

***Figure 33 – Food consumption of female offspring was unaltered upon maternal glycation exposure**. Control – female offspring of control dams; Vehicle – female offspring of dams treated with DMSO; BBGC- Wistar female offspring of dams treated with BBGC. Bars represent mean ± SEM of 12 animals in control group, 9 animals in vehicle group and in BBGC group and one-way ANOVA was conducted to compare among the group.*

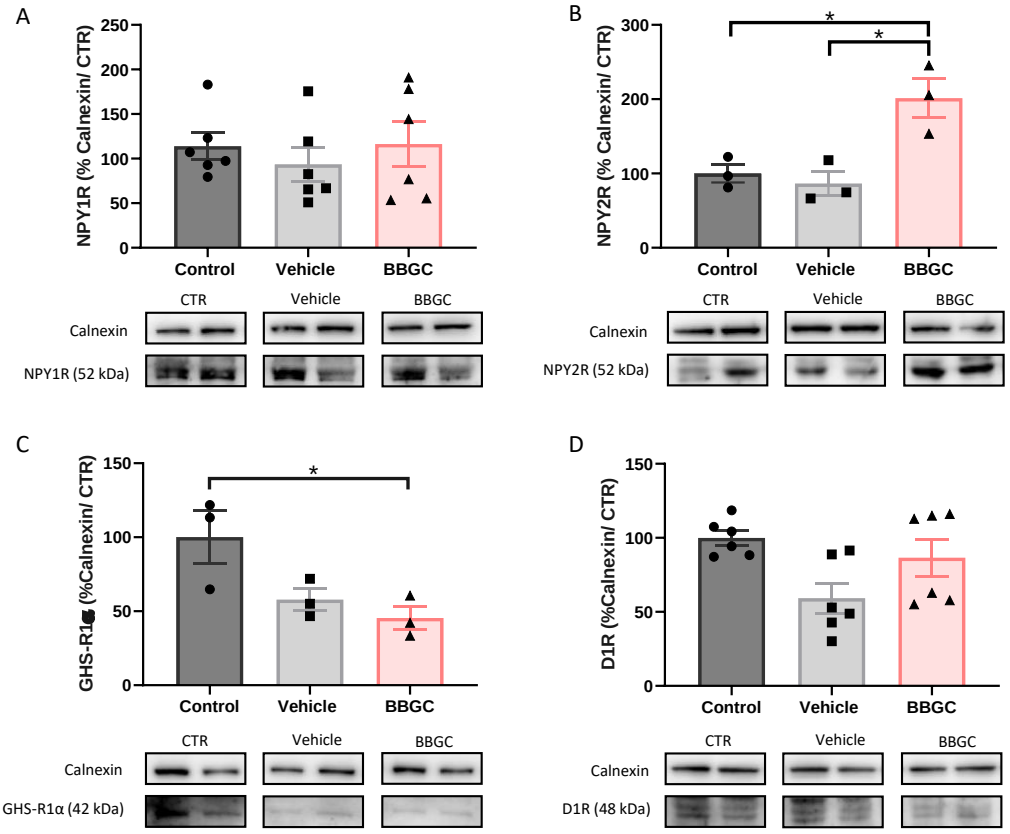

***Figure 34 – Exposure to maternal glycated products reduced NPY neurons activity at hypothalamic level in female offspring**. The autoinhibitory NPY receptor (NPY2R) levels were increased (B) while GHS-R1α levels were reduced (C) in the hypothalamus of female newborns exposed to maternal glycation, without affecting NPY1R (A) and D1R (D) levels. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – female offspring of control dams; Vehicle – female offspring of dams treated with DMSO; BBGC- Wistar female offspring of dams treated with BBGC. Bars represent mean ± SEM of 3 or 6 animals per group and Kruskal-Wallis test or one-way ANOVA were conducted to compare among the groups. * p<0.05*

Neonatal hyperphagia exposed to maternal glycotoxins model

Inhibition of the glyoxalase system on dams leads to a deficient antioxidant capacity in breastmilk also in conditions of litter reduction

As discussed, BBGC did not change dams body weight, which was also observed in those with litter reduction (figure 35A). The inhibition of glyoxalase 1 also did not promote hepatoxicity at the cytologic level on dams of small litters treated with BBGC (figure 35B).

Moreover, as observed in normal litter dams, BBGC caused a reduction of milk triglycerides levels, showing no influence of the litter reduction process ($p<0.05$ vs control) (figure 35C). Regarding breastmilk, antioxidant capacity, which was reduced in BBGC treated damns, was also decreased in the dams with the litter reduction process ($p<0.05$ vs control). Such decrease is maintained in dams summited to both procedures ($p<0.05$ vs control) (figure 35D).

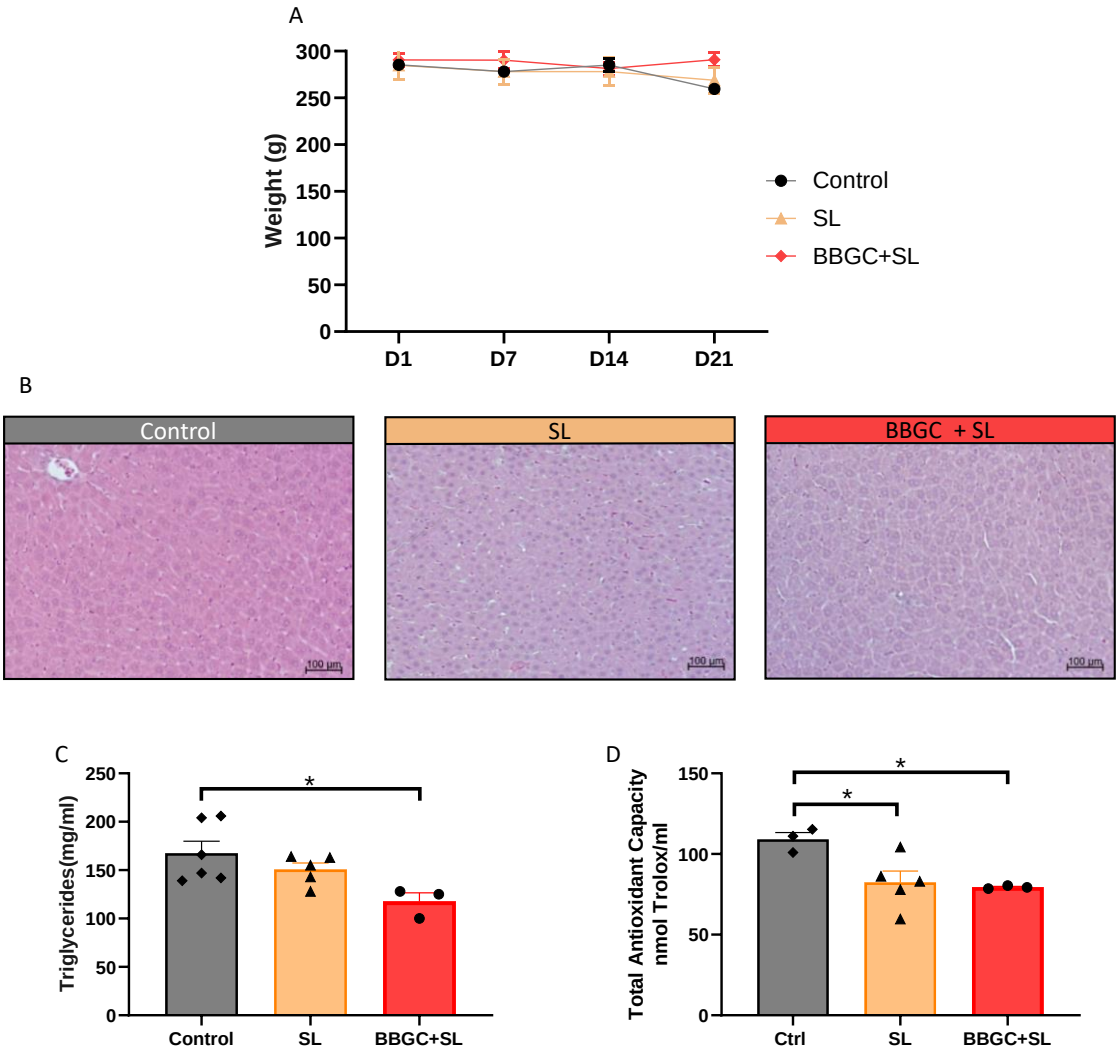

Figure 35 – Glyoxalase 1 inhibitor reduces triglycerides levels and total antioxidant capacity in breastmilk. *Weight gain curves of dams treated with BBGC and submitted to a small litter reduction of their pups (A). Representative histology of liver from control dams, dams of SL and dams of SL treated with BBGC (B). The triglycerides levels were decreased in breastmilk of BBGC dams (C). The total antioxidant capacity in breastmilk was affected by both SL process and BBGC treatment (D). Control – Wistar control dams; SL – Wistar dams in with their litters were reduced; BBGC + SL- Wistar dams treated with BBGC in with their litters were reduced. Bars represent mean ± SEM of 3 – 5 animals per group and Kruskal-Wallis test or one-way ANOVA were conducted to compare among the groups. * p<0.05;*

Maternal glycation inhibits SL-induced weight gain on male

As mentioned above, neonatal hyperphagia induces early life overweight. However, when SL animals are exposed to maternal glycotoxins during lactation the excessive weight gain is prevented from PND14 to the last day - PND45 ($p<0.05$ vs SL) (figure 36A and 36B). Fat mass was higher in animals from

SL exposed to glycated products (figure 36D) (p<0.05 vs control), while no alterations were observed in liver weight (figure 36C).

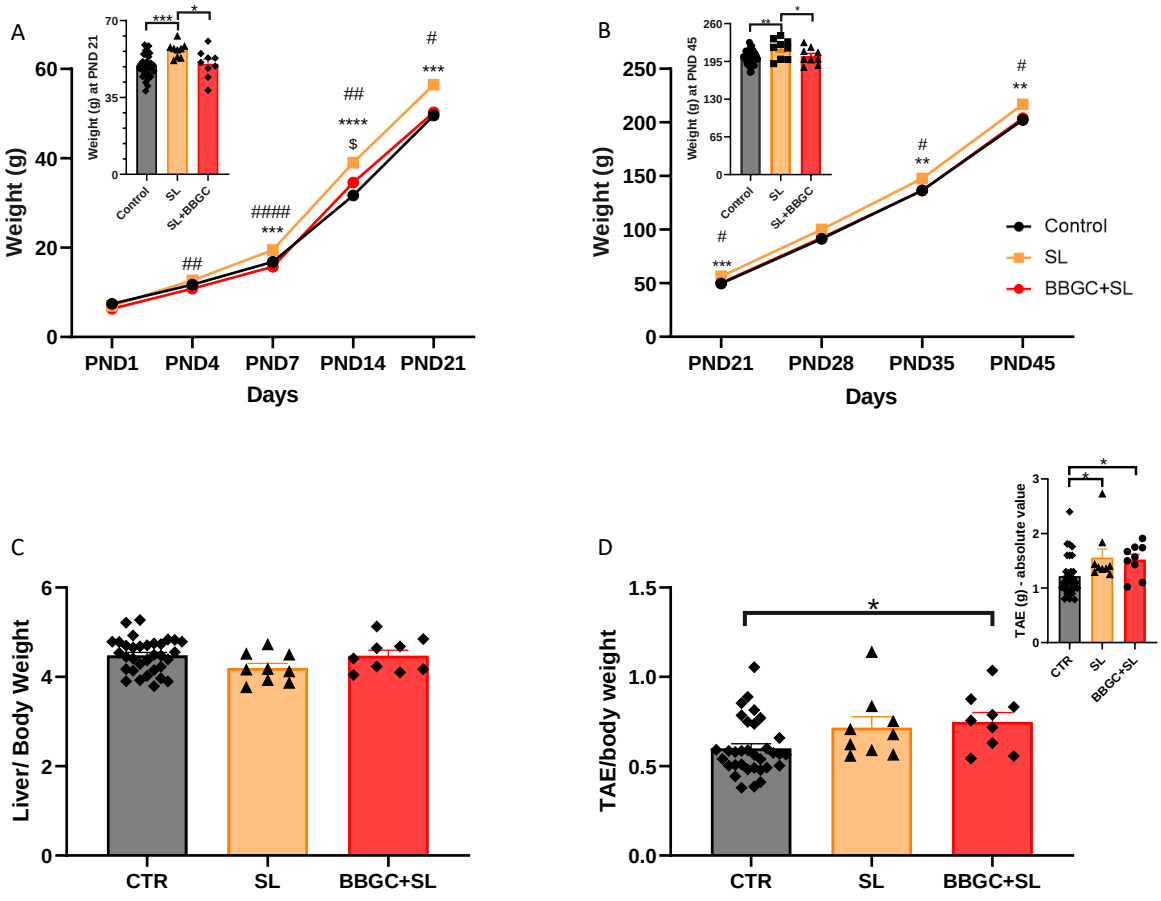

Figure 36 – The increase in fat mass is still observed despite the reverse in weight gain of obese animals exposed to maternal glycation during lactation. *Weight gain curves during the first 21 PND (weaning day) (A). Weight gain curves between 21 PND to 45 PDN (period after weaning) (B). Liver weight per body (C) and WAT weight per body and absolute value of fat mass (D) at 45 PND. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters; BBGC +SL – 45-days-old male obese offspring of dams treated with BBGC. Bars represent mean ± SEM of 35 animals in control, 9 animals in SL group and 9 animals in the BBGC+SL group and Kruskal-Wallis test or one-way ANOVA were conducted to compare among the groups. * control vs SL; $ control vs BBGC+ SL; # SL vs BBGC + SL. 1 symbol p<0.05; 2 symbols p<0.01; 3 symbols p<0.001; 4 symbols p<0.0001.*

The reduction of plasma insulin levels is maintained in obese animals exposed to maternal glycation

No alterations were observed on ITT and kITT in the BBGC+SL group (figure 37A and 37B). On the other hand, the plasma levels of this hormone, which were decreased in SL offspring, were not affected by exposure to glyoxalase 1 inhibitor in this model (p<0.01 vs control) (figure 37C). Interestingly, plasma

triglyceride levels were increased by exposure to glycated products in hyperphagic rats (p<0.01 vs control) (figure 37D).

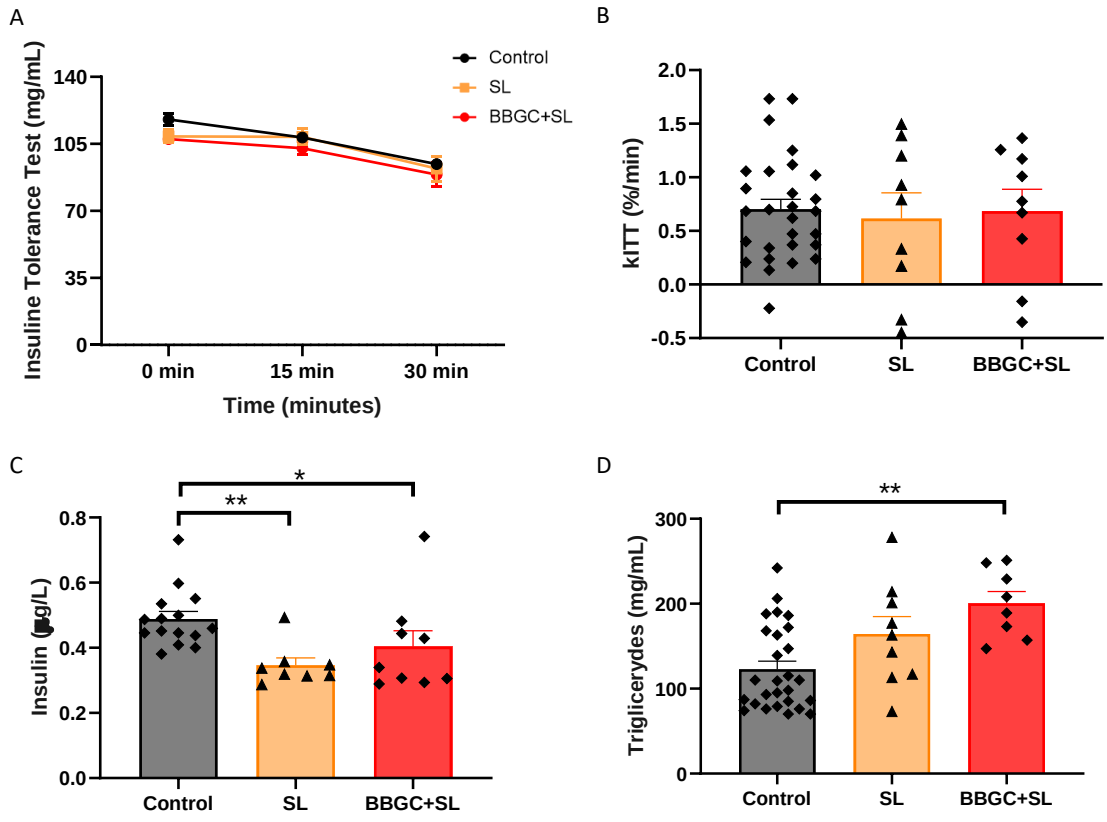

Figure 37 – Plasma insulin levels are reduced in obese newborn exposed to maternal glycation. *Insulin tolerance test (ITT) curve during 30 minutes at 45 PND (A). Decay of the glucose rate during the insulin tolerance test per minute (kITT) (B). Exposure to glycotoxins during lactation in obesity condition reduced plasma insulin levels (C) and increases plasma triglycerides levels (D). Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters; BBGC +SL – 45-days-old male obese offspring of dams treated with BBGC. Bars represent mean ± SEM of 35 animals in control, 9 animals in SL group and 9 animals in the BBGC+SL group and Kruskal-Wallis test or one-way ANOVA were conducted to compare among the groups. ** p<0.01.*

Energy balance pathways in WAT from obese animals are affected by maternal glycation

As already mentioned, the mechanisms of energy storage and expenditure in WAT were not affected by maternal glycation in the lean phenotype. On the other hand, the exposure to glycated products during lactation induces WAT energy balance dysregulation in obese offspring. Indeed, the increase in NPY2R levels in WAT from obese animals was lost when they were exposed to maternal glycation (p<0.05 vs SL) (figure 38B). The NPY1R and GHS-R1α did not change in visceral AT from obese offspring from dams treated with BBGC (figure 38A and 38C, respectively). Moreover, the dopaminergic

signalling in WAT from SL exposed to glycotoxins during lactation was impaired ($p<0.05$ vs control) further aggravating the decrease observed in SL animals (figure 38D).

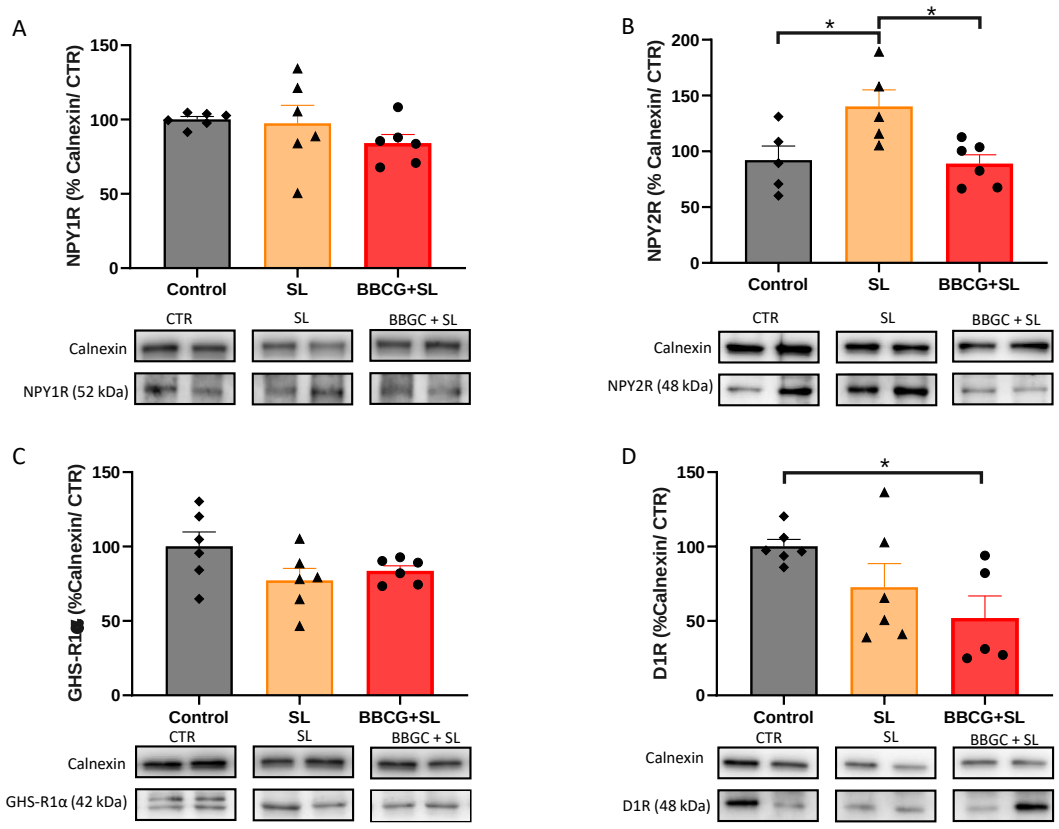

Figure 38 – Maternal glycation impairs WAT dopaminergic signalling in a small litter model. *Exposure to glycotoxins during lactation reversed the increase in NPY2R levels provoked by early life litter reduction (B) without affecting NPY1R (A) GHS-R1α (C) levels. D1R levels decreased in WAT of obese offspring from dams treated with glyoxalase 1 inhibitor (D). Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters; BBGC +SL – 45-days-old male obese offspring of dams treated with BBGC. Bars represent mean ± SEM of 6 animals per group and one-way ANOVAs were conducted to compare among the groups. * p<0.05*

Exposure to glycotoxins through breastmilk does not affect BAT in obese offspring

The NPY signalling in BAT is not affected in hyperphagia-induced obesity as mentioned before. Furthermore, in conditions of maternal glycation, the NPY1R and NPY2R levels remained unaltered (figure 39).

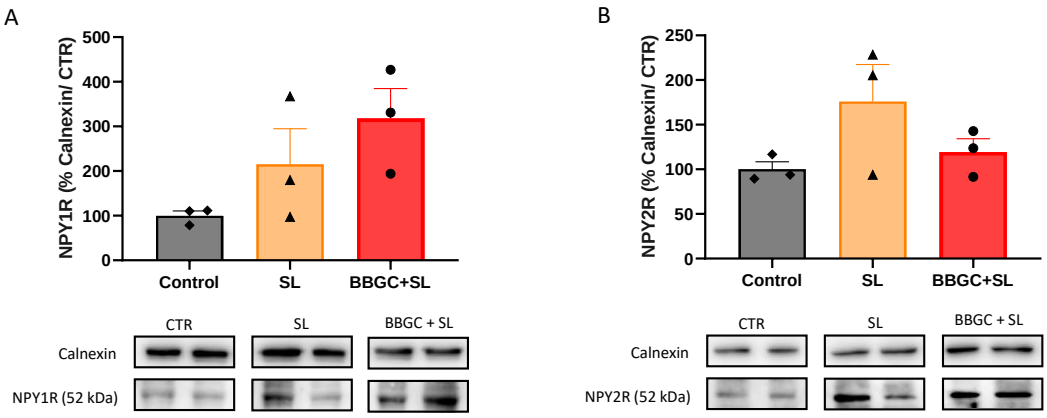

Figure 39 – Maternal glycation seems to not alter NPY signalling in BAT in obese conditions*. NPY1R (A) and NPY2R (B) levels in BAT. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters; BBGC +SL – 45-days-old male obese offspring of dams treated with BBGC. Bars represent mean ± SEM of 3 animals per group and one-way ANOVAs were conducted to compare among the groups.*

Glycated products reduce NPY and dopaminergic signalling in the liver in both lean and obese phenotype

As well as observed in the liver from lean offspring exposed to maternal glycation during lactation, in obese conditions, the decrease in NPY1R levels was also verified ($p<0.001$ vs control; $p<0.01$ vs SL) (figure 40A), as well as the effect on D1R ($p<0.001$ vs control) (figure 40C). The ghrelin receptor did not alter in SL animals from dams treated with BBGC (figure 40B).

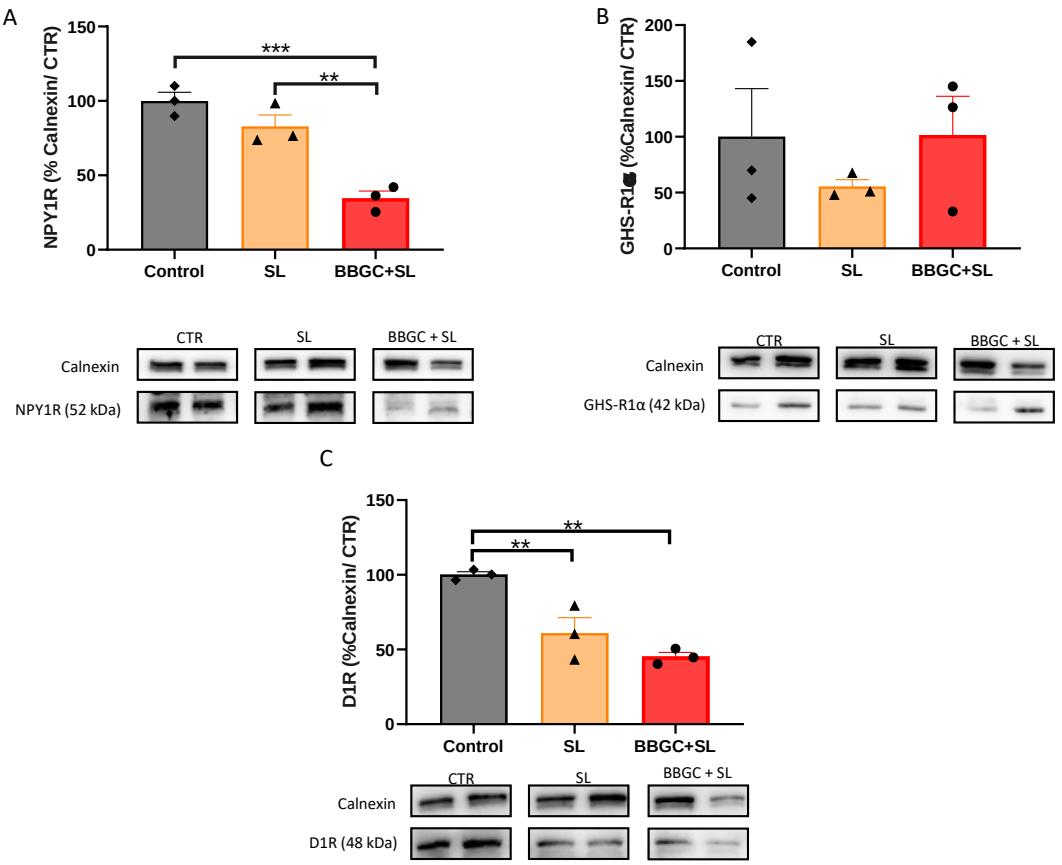

Figure 40 – Exposure to maternal glycated products decreases NPY1R levels in the liver of obese offspring. *Obese offspring of dams treated with glyoxalase 1 inhibitor presented lower levels of NPY1R (A), but GHS-R1α (B) levels were maintained. D1R levels were decreased in both SL conditions (C). Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters; BBGC +SL – 45-days-old male obese offspring of dams treated with BBGC. Bars represent mean ± SEM of 3 animals per group and one-way ANOVAs were conducted to compare among the groups. * p<0.05; ** p<0.01*

Maternal glycation impaired NPY autoinhibitory receptor in the hypothalamus from obese young mice

Contrary to what was observed in lean offspring exposed to maternal glycotoxins, there was no reduction in food consumption in obese animals (Figure 41).

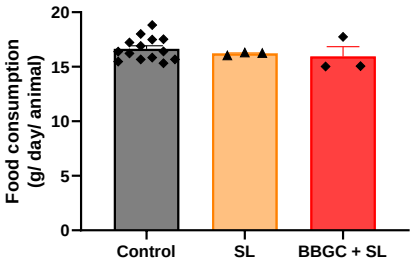

***Figure 41 – Food consumption of obese offspring is unaltered upon maternal glycation exposure**. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters; BBGC +SL – 45-days-old male obese offspring of dams treated with BBGC. Bars represent mean ± SEM of 35 animals in control, 9 animals in SL group and 9 animals in the BBGC+SL group and one-way ANOVA was conducted to compare among the groups.*

However, some hypothalamic alterations could be observed. Regarding NPY1R, as mentioned above, both maternal glycation and early life obesity reduced this receptor. However, when these two conditions are combined, this reduction was not observed (figure 42A). Also, the effect of maternal glycotoxins on D1R was lost in obese young mice (figure 42D). On the other hand, the levels of NPY2R decreased when the obese offspring is exposed to glycotoxins during lactation ($p<0.05$ vs control) (figure 42B). As happens in lean animals, GHS-R1α levels were maintained in obese offspring of dams treated with BBGC (figure 42C).

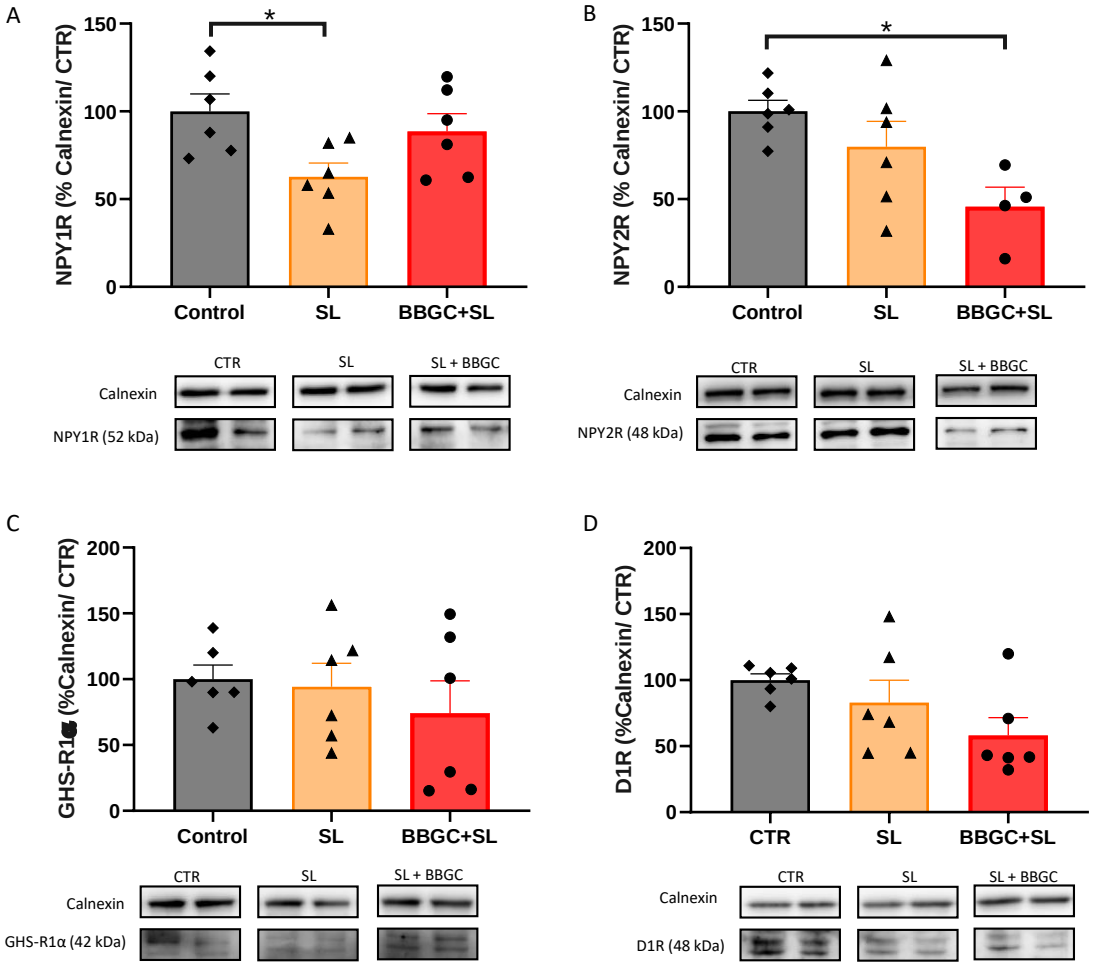

Figure 42 – Obese animals when exposed to glycotoxins during lactation change hypothalamic pathways associated with energy balance. *Maternal glycation prevented alterations in NPY1R levels at the hypothalamic level in early life obesity (A), without affecting GHS-R1α (C) and D1R (D) but decreased NPY2R (B) levels. Representative images of western blot proteins of interest and loading controls (Calnexin) are shown at the bottom. Control – 45-day-old Wistar males from normal litters; SL – 45-day-old Wistar males from small litters; BBGC +SL – 45-days-old male obese offspring of dams treated with BBGC. Bars represent mean ± SEM of 6 animals per group and one-way ANOVAs were conducted to compare among the groups. * p<0.05*

Chapter 5 DISCUSSION

Over the last few years, exposure to obesogenic environments during the perinatal period has been suggested as a risk for the rise of metabolic diseases worldwide. Several studies have been reported showing a higher predisposition to the development of metabolic complications when the organism is exposed to maternal diabetes, obesity, and unhealthy diets. Moreover, higher food consumption during early life also impairs metabolic function. However, the consequences of childhood obesity and unhealthy maternal state on molecular mechanisms regulating energy expenditure and storage remain unclear. To the best of our knowledge, this is the first report scrutinizing the consequences of being exposed to maternal diets rich in sugar and fat and of neonatal hyperphagia on peripheral energy balance regulation. With this work, we also studied the effect of the combination of two obesogenic environments on young mice nutrient-sensing pathways.

In this work, we used an animal model of maternal obesity during the gestational period that was previously validated ([141]). This study showed weight gain in male offspring, which may be associated with molecular alterations in WAT that we observed above. In male offspring of HFHS-fed dams both NPY1R and NPY2R levels are increased. NPY is known to stimulate lipogeneses, upregulating the expression of adipogenic/lipogenic genes such as PPARγ, C/EBP, ap2 and DGAT1, although it is still unclear which receptor triggers these processes since the role of NPY2R is also associated with tissue enlargement. On the other hand, lipolysis is inhibited by the binding of NPY to NPY1R in WAT, decreasing pHSL (ser563) – active form – and CG1-58 levels [72]. Thus, these results suggest an upregulation of lipogenesis and adipogenesis and less lipolysis, which may be related to weight gain. Furthermore, acyl-ghrelin receptor levels are higher in WAT of male offspring exposed to maternal HFHS diet, which may also be a reason for greater adiposity since this hormone, besides promoting adipogenesis, also has an antilipolytic effect [41]. Nevertheless, the levels of D1R, a lipolysis-stimulating receptor, are also elevated. Thus, maternal obesity may trigger compensatory mechanisms to enhance lipid storage, but also counteract the higher exposure to FFA by simultaneously improving energy expenditure in visceral AT. Furthermore, we cannot rule out the modulation of these nutrient-sensing mechanisms by each other, as occurs at the central level in the DA-NPY bidirectional axis, and as already observed in our laboratory, the modulation of NPYRs by the dopaminergic agonist Bromocriptine (unpublished data). Regarding the liver, it has been hypothesized that D1R is associated with lipid mobilization [140]. We observed a reduction of this dopaminergic receptor in the liver of male offspring. This may be a compensatory mechanism to reduce lipid uptake, as a lower ability to oxidate lipids and higher levels of acylcarnitne were previously observed [141]. Indeed, these animals present an increase in Non-alcoholic fatty liver disease (NAFLD) score, indicating lipid accumulation in the liver [141].

Some studies have reported the influence of sex on AT modulation. Indeed, fat distribution depends on gender, being females more prone to accumulate subcutaneous AT (SAT) while males store lipids predominantly in visceral AT (VAT)[143]. For instance, SAT has a lower lipolysis rate but is more efficient on the FFA uptake than VAT [143]. On the other hand, VAT has a lower capacity to store lipids and a more inflammatory environment [143]. Taking this into account, it is expected that the observed molecular mechanisms of VAT differ depending on sex. Here, we observed a decrease in NPY1R levels in female offspring, suggesting a reduction in lipogenic/adipogenic processes, contrary to what occurs in males. But, regarding acyl-ghrelin receptor the alteration observed was similar in both sexes, which raises questions about the mechanisms regulated by acyl-ghrelin in the VAT that still are not fully understood. Although unpublished data from our laboratory suggest that NPYRs are modulated by GHS-R1α in WAT, a possible answer for this discrepancy may also be the gender. Most studies have reported ghrelin as a lipogenic agent, but a few other studies support the opposite [144]. In conclusion, the changes observed in the VAT from male offspring may be distinct comparing to females because SAT has a greater influence on lipid metabolism, while in the male the VAT is the main responsible for energy storage.

Contrary to what occurs in the WAT, the impact of maternal HFHS diet in the liver does not differ between female and male offspring. This suggests that only the fat distribution is dependent on sex. Nevertheless, in the liver of female offspring from obese dams, not only is dopaminergic signaling impaired but NPY1R levels are also decreased. The role of this receptor at the hepatic level is still unknown, so we cannot infer the outcomes of this change.

Besides maternal obesity, neonatal hyperphagia also induces obesity in young mice male accompanied by an increase in the visceral fat mass. In this model of obesity, at the 45th PND, insulin action regarding glucose uptake is not impaired. However, plasma levels of insulin are decreased in hyperphagic animals, indicating that insulin release may be compromised. To corroborate this hypothesis, Robert A. et. al (2002) demonstrated that this animal model at 2 different ages (26 PND and 110 PND) presents an impairment in the release of insulin from β-cells [145].

Early life obesity induced by neonatal hyperphagia also upregulates NP2R signalling in WAT, which potentiates lipogenesis and adipogenesis. On the other hand, contrary to what happens in offspring exposed to maternal HFHS diet, lipolysis is not enhanced by the upregulation of D1R levels. Thus, suggests that, while maternal diet-induced obesity induces other mechanisms regarding lipid metabolism in males, neonatal hyperphagia-induced obesity only promotes WAT enlargement without stimulating energy expenditure, which accentuates weight gain, predisposing to metabolic complications later in life. This may be related, not only to the higher caloric intake from milk but also

to nutritional cues, given that maternal diet was different in both models. It is possible that maternal consumption of fats and sugars stimulates not only energy storage but also energy consumption pathways in the offspring. However, in BAT, a tissue with a high impact in energy expenditure, due to the low number of animals, more studies need to be carried out to conclude whether postnatal overfeeding induces changes in thermogenesis regulation.

Interestingly, both models of early life obesity impair dopaminergic signaling in the liver, suggesting that lipid mobilization is affected by overfeeding (SL models) as well as by the high amount of sugar and fat (HFHS diet-induced maternal obesity).

Several studies demonstrated that small litter is a hyperphagic model [136], [142]. Nevertheless, we do not obverse an increase in food consumption after weaning, despite weight gain. However, at the hypothalamic level, NPY1R levels decline, indicating that food intake stimulation is less triggered or may be an adaptation to the higher amount of food available. Ananda Lages Rodrigues et. al (2011) reported that neonatal hyperphagia reduces NPY hypothalamic content [146]. Moreover, it was reported that in animals from SL, NPY1R has a stronger effect on the suppressing neurons located in the VMH, a satiety center [136], [147]. Summarizing, postnatal overfeeding may prepare the organism for greater food supply by reducing NPY signaling while inducing a superior effect on satiety inhibition by NPY action. However, the long-term impact of such alterations is unknown.

Given that obesogenic diets are often rich in sugars, we also intended to study the effects of exposure to maternal glycotoxins. Until now, studies have used direct MG administration in dams [148], and we aimed to use a less aggressive and more physiological model using BGGC injection. In the study by Francisco et. al (2018), milk triglyceride levels were increased, however, we verified the opposite, suggesting that maternal glycation alters breastmilk composition. However, in the plasma of male and female offspring, this alteration in triglycerides levels was not observed. Moreover, accompanying this, we observed a lower antioxidant capacity in breastmilk from dams treated with BBGC. As described before, AGEs formation debilitates antioxidant defenses [15], and we have here shown that maternal glycation has also an impact on breastmilk and possibly on the detoxification mechanisms of the offspring.

Regarding both male and female offspring from BBGC-treated dams, as occurs in adults exposed to glycotoxins [149], body weight does not increase. Nevertheless, Francisco et. al (2018) showed that offspring from dams administrated with MG present weight gain only after PND 77 [148]. Thus, maternal glycation may predispose to obesity development later in life, which we cannot observe at the 45th PND. In lean animals exposed to glycated products, insulin resistance is not developed, and glucose metabolism is unaffected [149]. Surprisingly, in lean male offspring despite plasma insulin

levels are not altered, kITT indicates an insufficiency in insulin action regarding glucose uptake, suggesting a predisposition to insulin resistance. Moreover, the offspring exposed to MG-induced maternal glycation present hyperinsulinemia, which is a known marker of insulin resistance [148]. Regarding female offspring, plasma insulin levels are decreased, although female offspring of DMSO-treated dams also present this deficiency. Thus, there is no evidence of insulin resistance, which is consistent with several studies that have reported that females are less prone to develop insulin resistance due to hormonal gender differences [143].

Other studies indicate that glycation plays a more powerful role in obesity than in normal conditions. Here we observed that maternal glycation does not alter the molecular mechanisms associated with energy balance in WAT of lean female and male offspring. However, in the liver we also observed that dopaminergic signaling is impaired in both genders, suggesting a lower lipid mobilization in both sexes. These alterations show that not only the amount of food and the nutrient milk composition impact mechanisms in the liver, but also milk composition changes induced by glycation weaken lipid mobilization at the hepatic level. In addition, NPY1R levels are diminished in the liver of male offspring exposed to maternal glycotoxins, but the consequences of this alteration cannot be predicted due to lack of available information. Regarding female livers, DMSO diminishes the levels of NPY1R, GHS-R1α and D1R. Nevertheless, this may be a response to the maternal stress caused by the injection and not a consequence of DMSO. Indeed, diverse studies have shown that females are more prone to develop obesity when exposed to maternal stress than males [150], indicating that females are more susceptible to maternal stress. Moreover, gut microbiota colonization is affected by maternal prenatal stress in a sex-dependent manner, which may influence the endocrine function exerted by the gut and therefore disrupt nutrient-sensing pathways [151].

At central levels, the exposure to maternal glycation had visible effects in the lean phenotype. Indeed, food consumption in male offspring is decreased, which may be a consequence of NPY1R reduced levels in the hypothalamus. Furthermore, NPY neuron-stimulating dopamine receptor (D1R) is increased at the hypothalamic level. This upregulation is extremely related to obesity [88]. Thus, NPY1R decreased levels may be a response to high stimulation of NPY neurons by D1R activation. Therefore, this suggests that despite the lean phenotype exhibited by this male offspring, maternal glycotoxins may affect central mechanisms that will then be responsible for altering the energy balance pathways, possibly having consequences in the periphery, predisposing to metabolic complications development. Regarding females, there is no alteration neither in food intake nor NPY1R and D1R levels. However, acyl-ghrelin receptor levels are reduced while NPY2R levels increase when female are exposed to maternal glycation. This suggests that NPY neurons activity may be reduced, due to less stimulation by

GHS-R1α and more suppression by the autoinhibitory receptor (NPY2R) despite there being no changes in food intake.

In obesity conditions, maternal glycation may have different outcomes. As expected, in breastmilk from BBGC-treated dams of small litters, alterations in triglycerides and antioxidant capacity are similar to those noticed in BBGC-treated dams of normal litters. Surprisingly, we observed that the process of litter reduction also impairs antioxidant defenses in the breastmilk, although this process does not accentuate the poor protection against glycation-induced oxidative stress. Regarding offspring, the weight gain provoked by hyperphagia is lost when the young animals are exposed to maternal glycotoxins. Corroborating this, Neves et. al (2019) showed that in adults, the weight gain induced by HF diet is also lost when MG is administrated [152]. On the other hand, in our obese animals exposed to glycotoxins during lactation, VAT mass is increased. Moreover, plasma triglycerides levels are increased in these obese offspring exposed to glycotoxins during lactation, which is highly associated with obesity and its complications such as cardiovascular diseases. In addition, as observed in obese young mice, plasma insulin levels are decreased, suggesting an impairment in β-cells. However, the disruption of insulin action regarding glucose uptake noticed in the offspring of dams exposed to glycation is not observed in overweight conditions.

As shown, maternal glycation does not alter the mechanisms associated with energy balance in lean animals, however, under obesity conditions maternal glycotoxins impair the NPY2R compensatory mechanism, suggesting a decrease in lipogenesis/ adipogenesis rates promoting a lower energy storage capacity. Thus, the AT environment may be altered into an unhealthy and consequently predisposing to AT dysfunction. Indeed, in adulthood obesity induced by HF diet, exposure to MG impairs adipose tissue expansion causing hypoxia, fibrosis and inflammation [149]. Additionally, processes associated with energy expenditure can also be impaired in obese offspring exposed to maternal glycation. D1R levels are diminished in these animals, suggesting that lipolysis induction is reduced, which may have a negative impact on energy expenditure. So, it appears that exposure to glycotoxins during lactation in obesity prevents the development of storage mechanisms to compensate for higher food consumption and simultaneously impairs energy expenditure. These alterations predispose to the development of T2D or metabolic syndrome due to their contribution to adipocyte hypertrophy, lipotoxicity, hypoxia in AT and insulin resistance.

In the liver, when early life obesity is combined with maternal glycation, lipid mobilization is deceased, as occurs in both conditions separately (postnatal overfeeding and maternal glycation). However, NPY1R levels only decrease when the young mice are exposed to glycotoxins during lactation, suggesting that this pathway may be affected by breastmilk composition but not by overfeeding.

Corroborating this, females exposed to maternal obesity induced by HFHS diet also present a reduction in NPY1R levels. So far, we cannot yet comprehend what are the consequences of NPY1R activation in the liver, which is one of the major limitations of the work since there are many alterations at the hepatic levels.

At central levels, exposure to maternal glycation induces alterations *per se* that are lost with obesity. Indeed, the decrease in food intake only occurs under normal conditions, possibly because in obesity glycotoxins exposure does not decrease NPY1R levels as happens in lean animals and obese mice not exposed to glycotoxins. Moreover, NPY2R levels are also reduced, suggesting weaker inhibition of NPY neurons and therefore higher food intake stimulation and lower energy expenditure rate. Regarding D1R, the increase observed in lean animals does not occur in overweighted ones. As mentioned, this raising in D1R levels is highly associated with obesity, although here this increase was not observed. Overall, maternal glycation aggravates the dysregulation of energy balance in obese animals at the peripheral level, impairing both energy storage capacity and energy expenditure processes. And at a central level, the mechanisms to compensate for increased exposure to food are lost, but more studies are necessary.

Chapter 6 CONCLUSIONS AND FUTURE PERSPECTIVES

Dysregulation of energy balance is one of the major factors that contributes to metabolic diseases development. Insults such as obesogenic environments during the perinatal period program nutrient-sensing mechanisms, disturbing anorexigenic/orexigenic balance and anabolic/catabolic processes. Indeed, the escalation in maternal obesity and western dietary intake may be the route to the actual obesity pandemic.

In this work we characterized, for the first time to our knowledge, the energy balance outcome of early life obesity induced by maternal obesity during pregnancy and by neonatal hyperphagia. Despite the weight gain, young animals exposed to maternal HFHS diet developed compensatory mechanisms regarding energy storage and expenditure, allowing a higher AT plasticity. Postnatal overfeeding seems to affect insulin release and protects AT dysfunction by enhancing energy storage. So, the consequences of postnatal overweight for peripheral mechanisms of energy balance are distinct in both models, suggesting that maternal nutritional cues during lactation have a crucial role in their modulation. At the central level, hyperphagia impairs NPY signaling, suggesting that initially some alteration may be induced to protect against increased consumption. Nevertheless, despite the development of these compensatory peripheral and central mechanisms, modulation of such pathways can become unhealthy later in life, disturbing energy balance and therefore contributing to predisposing to the development of metabolic complications. Further complimentary results are needed to understand the impact of obesity induced during the perinatal period. In females, we need to comprehend the role of postnatal overfeeding on energy balance mechanisms and discloses the potential changes in SAT when females are exposed to an obesogenic environment.

The rise in westernized diets consumption has been abrupt, so to understand the effect of glycation induced by these diets throughout generations, we studied maternal glycation under normal and obese conditions. In a lean phenotype, glycated products seem to have effects initially at the central level, altering energy balance regulation. Nevertheless, when it is combined with an obesogenic environment (hyperphagia), the AT plasticity is compromised, leading to AT dysfunction and lipotoxicity. Accompanied this, at a central level, it seems that there is already a dysregulation established on the control of food intake and energy expenditure.

In conclusion, exposure to obesogenic environments impairs energy balance despite that at first compensatory molecular mechanisms are developed. However, when young animals who have experienced unhealthy motherhood are obese, this disruption is more accentuated, increasing, even more, the risk of developing T2D and other metabolic diseases. Notwithstanding, this work allowed us to understand the powerful role played by pregnancy and lactation in life offspring, especially in how

the balance between energy expenditure and food intake can be disrupted by an unhealthy maternal life.

In the future, it would be interesting to characterize better the systemic metabolic parameters such as insulin resistance, lipotoxicity and also at a more advanced age. Moreover, both studies *in vitro* and *in vivo* are required to understand the crosstalk between gut hormones and neuroendocrine mechanisms in peripheral organs, namely in adipocytes since the crucial role played by these cells in energy storage.

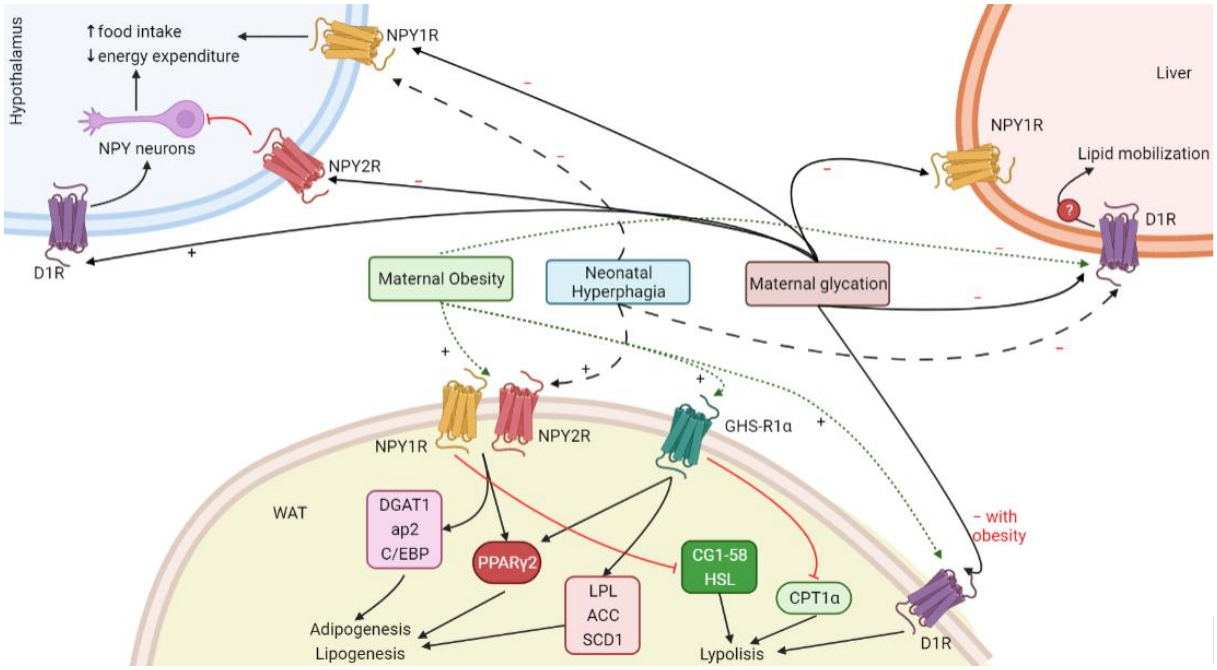

Figure 43 – Schematic representation of maternal obesity, neonatal hyperphagia and maternal glycation effects on WAT, liver and hypothalamus of male. *Created with BioRender.com.*

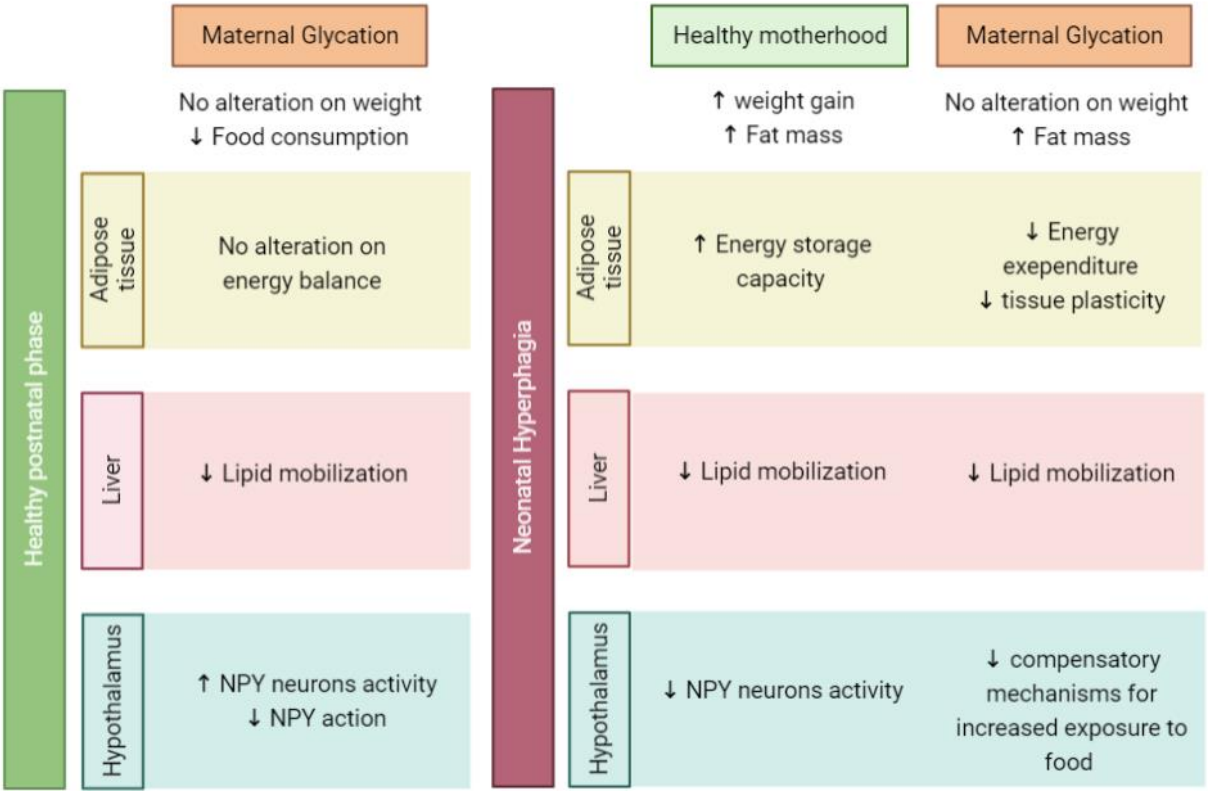

Figure 44 – Consequences of maternal glycation under normal and obese conditions. *Created with BioRender.com.*

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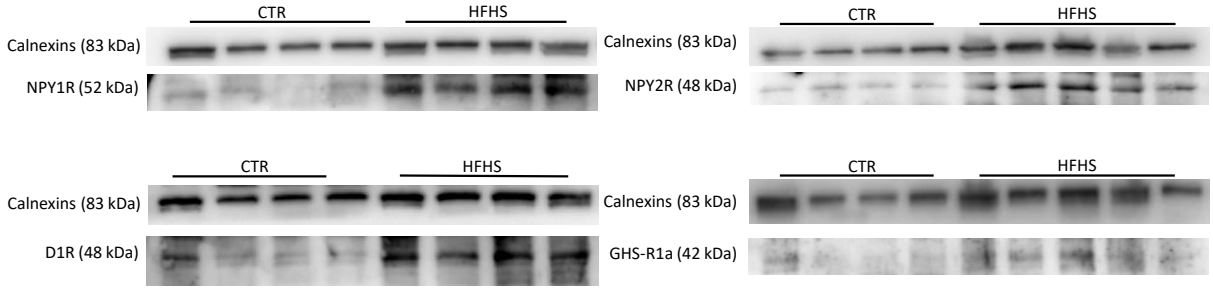
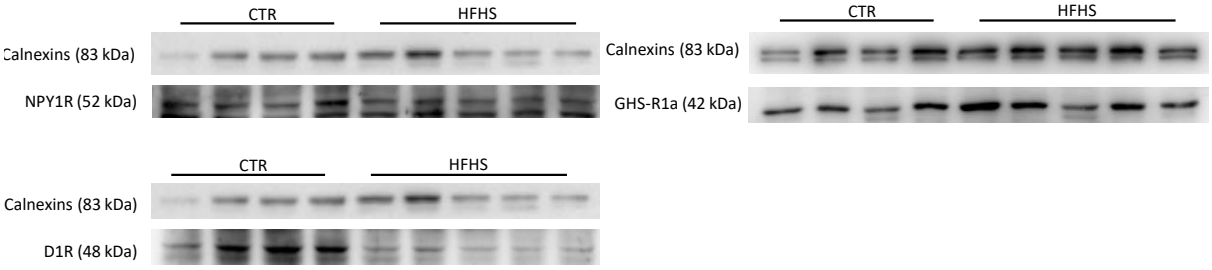
ANNEX I

Maternal Diet-induced Obesity Model

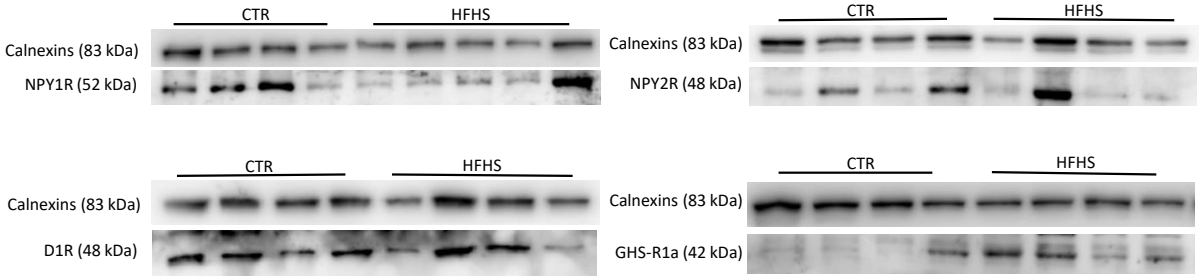
Visceral WAT- Male



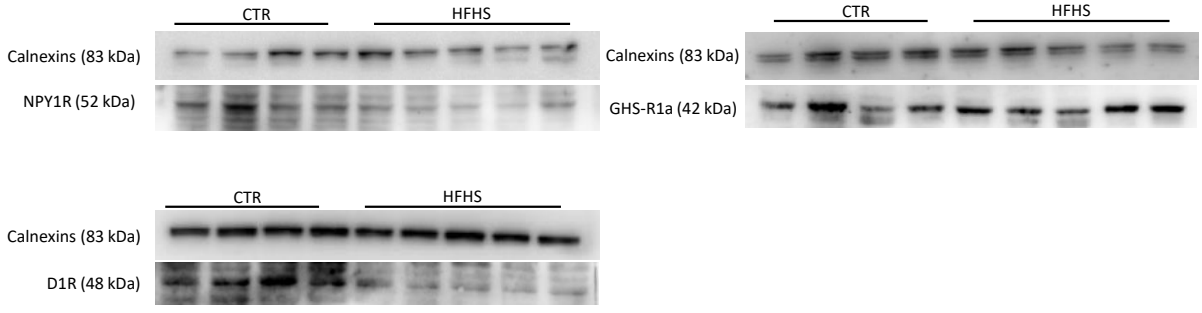
Liver- Male



Visceral WAT- Female

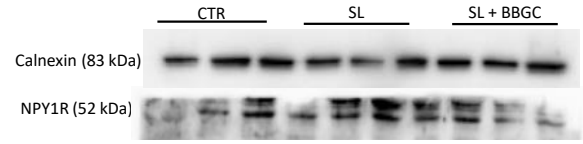


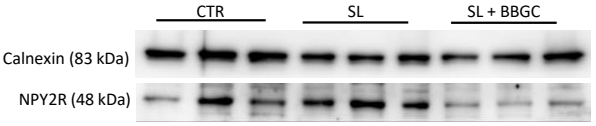
Liver- Female

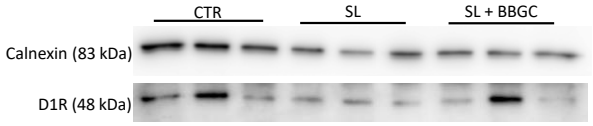


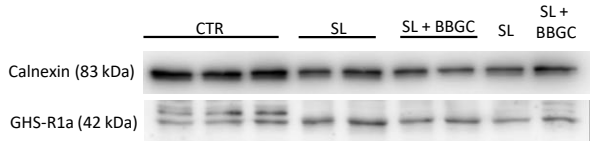
Neonatal Hyperphagia Model And Neonatal Hyperphagia Exposed to Maternal Glycotoxins Model

Visceral WAT

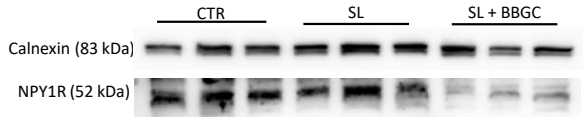


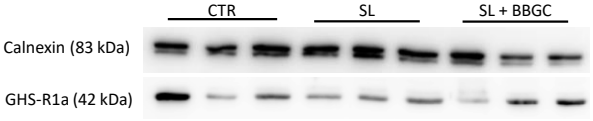


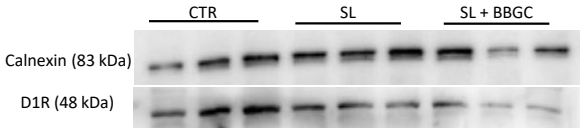




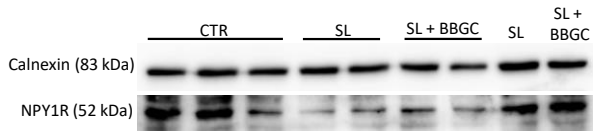
Liver

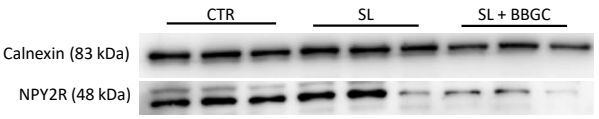


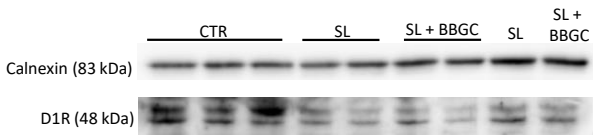


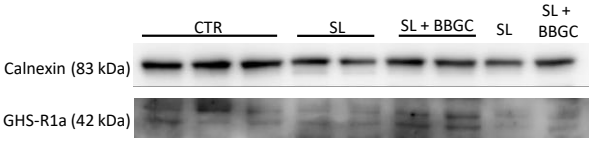


Hypthalamus



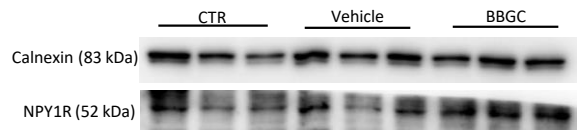


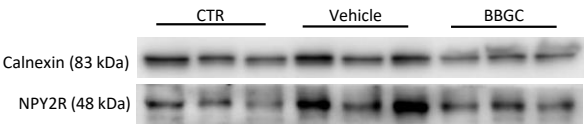


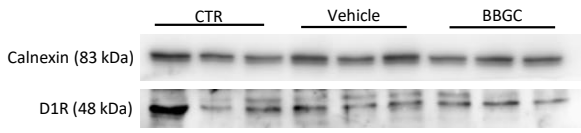


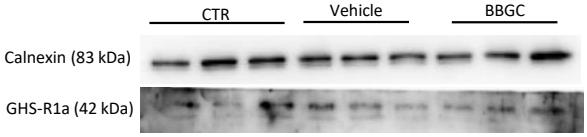
Maternal Glycation Model

Visceral WAT - Male

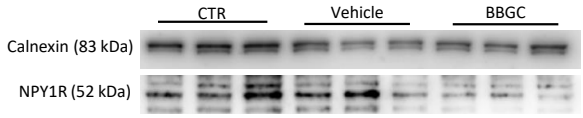


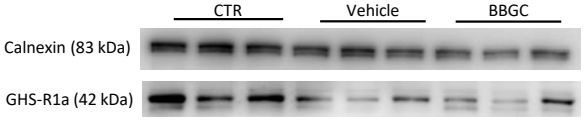


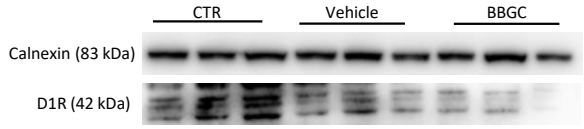




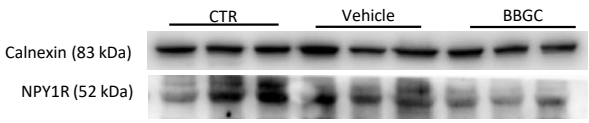
Liver - Male

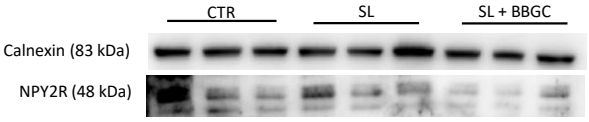


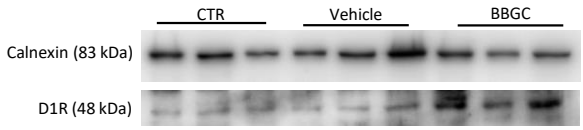


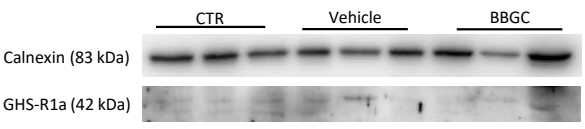


Hypthalamus - Male



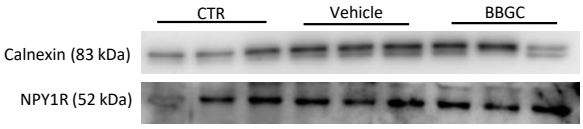


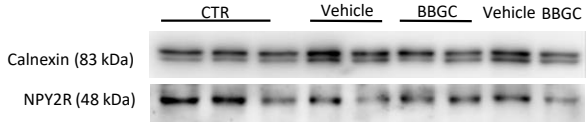


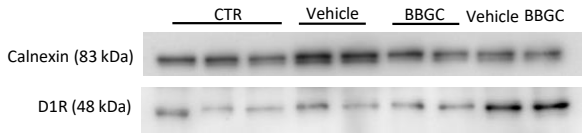


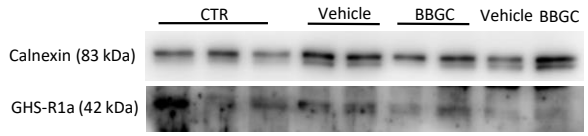
Maternal Glycation model

Visceral WAT - Female

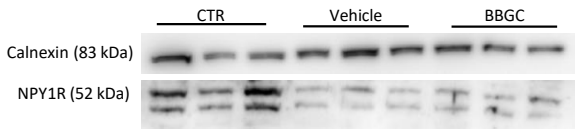


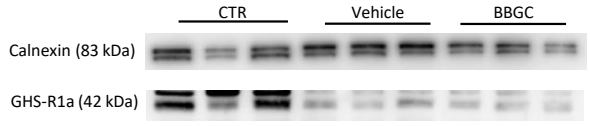


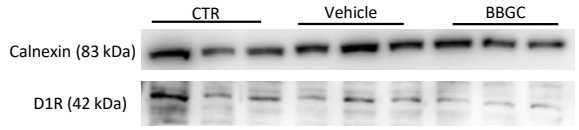




Liver - Female







Hypthalamus - Female