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Andreia Filipa Marreiros Amaro

Lactation Period: a window to neurometabolic alterations?

VOLUME 1

Dissertação no âmbito do Mestrado em Bioquímica orientada pelo Professor Doutor Paulo Nuno Centeio Matafome e pela Professora Doutora Emília da Conceição Pedrosa Duarte e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, Departamento de Ciências da Vida

Julho de 2022

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Resumo

Introdução e Objetivos: A lactação é considerada uma fase importante para o desenvolvimento de doenças metabólicas, como a diabetes, bem como para o risco de alterações neuronais na vida adulta. Embora os mecanismos que potenciam estas alterações não sejam totalmente conhecidos, a dieta e a condição metabólica materna são fatores que contribuem para o seu desenvolvimento. A obesidade infantil é uma problemática cada vez maior nos dias de hoje, que leva a um maior risco de desenvolvimento de síndrome metabólica ao longo da vida. Desta forma, este trabalho tem como principais objetivos – 1) avaliar o efeito da glicação materna durante o período de lactação no neurodesenvolvimento e comportamento da descendência, durante a adolescência tanto em machos como em fêmeas; e, 2) avaliar o impacto da exposição da hiperfagia pós-natal no neurodesenvolvimento e comportamento da descendência, num contexto normal e quando expostos a glicação materna.

Materiais e Métodos: Foram estudados a descendência (machos e fêmeas) de fêmeas Wistar tratadas com S-p-Bromobenzilgutationa ciclopentil diéster – BBGC (5 mg/kg), um inibidor seletivo da Glioxalase 1 (GLO1), durante os primeiros seis dias após o nasimento. Considerou-se ainda um grupo controlo e um grupo veículo tratado com dimetil sulfóxido (DMSO). Com o objetivo de estudar o impacto da hiperfagia pós-natal (SL), ao dia pós-natal (P) 3, o número de descendentes foi reduzido de oito para três, sendo que foi ainda considerado um grupo em que as progenitoras foram administradas com BBGC – SL+BBGC. Entre P5 e P17, foram efetuados vários testes comportamentais para avaliar o neurodesenvolvimento dos descendentes. Após o desmame, foram coletadas amostras de leite materno para medição dos triglicerídeos e quantificação da capacidade antioxidante total. A P43 e P44 foram efetuados testes de comportamento para avaliar a capacidade locomotora, o comportamento ansioso e o processo de recognição de memória na descendência. A P45 foi realizada uma prova de tolerância à insulina, os níveis de triglicerídeos foram medidos seguindo-se a coleta do hipocampo e córtex pré-frontal para estudos moleculares.

Resultados: A glicação materna causa alterações na composição do leite induzindo uma redução dos níveis dos triglicerídeos e da capacidade antioxidante total. Verificou-se ainda que tanto a descendência feminina como masculina não apresentou alterações no peso corporal, nem dos níveis de glicose durante a prova de tolerância à insulina. Contudo, a glicação

materna induziu menores níveis de glioxalase 1 no hipocampo dos descendentes machos acompanhado por uma maior acumulação de MG-H1 e Argpirimidina. Por outro lado, não foram observadas alterações significativas no córtex pré-frontal. A glicação materna acelera o desenvolvimento do sistema vestibular e do sistema olfativo dos machos enquanto induz uma antecipação da abertura dos olhos e da capacidade auditiva em ambos os sexos. A exposição precoce ao BBGC demonstrou reduzir os níveis de ansiedade tanto nos machos como nas fêmeas. Observou-se ainda um aumento dos níveis da expressão dos recetores do $GABA_A$ no hipocampo dos machos. Paralelamente, no hipocampo das fêmeas o aumento dos níveis do recetor do $GABA_A$ foi acompanhado por uma diminuição dos níveis de proteínas envolvidas na sinapse glutamatérgica.

Tendo em consideração o segundo objetivo, a exposição à hiperfagia pós-natal demonstrou induzir excesso de peso na descendência bem como uma redução periférica dos níveis de sensibilidade à insulina. Quando expostos à glicação materna, este efeito foi perdido apesar de se continuar a verificar menor sensibilidade à insulina. Ambos os grupos não demonstraram alterações nos testes de neurodesenvolvimento e, apenas a descendência do grupo SL demonstrou desenvolver um comportamento ansioso. Acompanhado por um aumento dos níveis de proteínas antioxidantes como a glioxalase e a catalase no hipocampo da descendência, que é perdido quando expostos à glicação materna. Verificou-se ainda que ambos os grupos apresentavam maiores níveis de proteínas sinápticas, como a sinapsina e a PSD-95, sem alterações nos níveis de proteínas envolvidas nas sinapses excitatórias (vGLUT1) e inibitórias (vGAT).

Conclusão: A exposição precoce à glicação materna induz alterações na composição do leite, no neurodesenvolvimento da descendência durante a infância e consequentemente a alterações neurometabólicas que aumentam a predisposição para um comportamento menos ansioso na adolescência. A exposição a um ambiente hiperfágico durante o período pós-natal induz alterações metabólicas na descendência, aumentando o risco de desenvolver síndrome metabólica na vida adulta, bem como maior ansiedade na adolescência.

Palavras-Chave: Lactação, neurodesenvolvimento, comportamento, glicação materna, hiperfagia pós-natal, descendência

Abstract

Introduction and Objectives: Lactation is considered a critical phase for the development of metabolic disease and for the programming of neuronal changes in adult life. Although the mechanisms that underlying these changes are not fully understood, maternal diet and metabolic conditions are key factors that contribute to their development. On the other hand, childhood obesity is a growing problem nowadays, leading to a higher predisposition for the development of metabolic syndrome later in adulthood. Thus, this work has two main objectives – 1) study the effect of maternal glycation during lactation period on neurodevelopment and offspring behaviour at adolescence, assessing sex differences; and 2) study the impact of postnatal hyperphagia on offspring neurodevelopment and behaviour, with and without exposure to maternal glycation.

Materials and Methods: We studied male and female offspring of female Wistar rats treated with S-p-Bromobenzylguthione cyclopentyl diester - BBGC (5 mg/kg), a selective inhibitor of Glyoxalase 1 (GLO1), during the first six days of lactation period. A control group and a vehicle group treated with dimethyl sulfoxide (DMSO) were also considered. To study the impact of postnatal hyperphagia (SL), after postnatal day (P) 3, the number of offspring was reduced from eight to three pups. In addition, it was also considered a group in which the mothers were simultaneously treated with BBGC – SL+BBGC. Between P5 and P17, several behavioural tests were performed to assess the neurodevelopment of the offspring. After weaning, breast milk samples were collected to measure triglycerides and quantify the total antioxidant capacity. At P43 and P44, locomotor ability, anxious behaviour and recognition memory processes was assessed in the offspring. At P45 an insulin tolerance test was performed, triglycerides levels were measured and the hippocamps and prefrontal cortex were collected to molecular analysis.

Results: Maternal glycation causes changes in milk composition, inducing a reduction in triglyceride levels and total antioxidant capacity. Our results demonstrates that both female and male offspring do not show changes in body weight or glucose levels during the insulin tolerance test. However, maternal glycation induced lower levels of glyoxalase 1 in the hippocampus of male offspring accompanied with a higher accumulation of MG-H1 and Argpyrimidine. On the other hand, no significant changes were observed in the prefrontal cortex. Maternal glycation accelerated the development of the vestibular and olfactory

systems in male offspring while inducing an anticipation of eye opening and auditory capacity in both sexes. Early exposure to BBGC had an anxiolytic effect both males and females, accompanied with increased levels of $GABA_A$. In parallel, in the female hippocampus, the increase levels of in $GABA_A$ receptor were observed in parallel with a decrease in the levels of proteins at the glutamatergic synapse.

Regarding our second aim, our results demonstrate that postnatal hyperphagia increased weight gain in offspring and induced a reduction in peripheral insulin sensitivity levels. When exposed to maternal glycation, weight gain was lost despite having lower insulin sensitivity. Both groups – SL and SL+BBGC- do not shown changes in neurodevelopmental tests, and only the offspring of the SL group was shown to develop an anxious-like behaviour. It was also observed an increase in the levels of antioxidant proteins such as glyoxalase and catalase in the offspring's hippocampus from SL group, an effect that is lost when exposed to maternal glycation. Additionally, both groups were found to have higher levels of synaptic proteins, such as synapsin and PSD-95, without no changes in the levels of proteins involved in excitatory (vGLUT1) and inhibitory (vGAT and $GABA_A$).

Conclusion: Early exposure to maternal glycation induces changes in milk composition and changes in the neurodevelopment of male and female offspring during childhood and leads to neurometabolic changes and less anxious-like behaviour or more risk-taking in adolescence. Exposure to a hyperphagic environment during the postnatal period induces metabolic changes in the offspring, increasing the risk of developing metabolic syndrome in adulthood, as well as an anxious-like behaviour in adolescence.

Keywords: lactation, neurodevelopment, behaviour, maternal glycation, postnatal hyperphagia, offspring

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Sousa D., **Amaro A.,** Júnior M. F., Pereira S., Rocha M., Barra C., Mello-Gomes R., Oliveira P., Matafome P. O desenvolvimento perinatal em ambientes obesogénicos provoca alterações nas vias de sensibilidade aos nutrientes. **18º Congresso Nacional de Diabetologia, Vilamoura 10-11 de Março.**

Rocha M., **Amaro A.**, Júnior M., Pereira S., Sousa D., Barra C., Mello Gomes R., Oliveira P., Matafome P. A dieta materna hipercalórica durante a gestação e lactação e desenvolvimento da resistência à insulina: papel das glicotoxinas da dieta. **18º Congresso Nacional de Diabetologia, Vilamoura 10-11 de Março.**

Saavedra L. P. J., Silva M. C., Raposo S. R., **Amaro A**., Gonçalves G. D., Piovan S., Matafome P., Mathias P. C. F. Almeida D. L. Os agonistas do PPARα durante o início da vida previnem a acumulação de gordura visceral e resistência à insulina na vida adulta num modelo de obesidade infantil. **18º Congresso Nacional de Diabetologia, Vilamoura 10-11 de Março.**

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Amaro A, Sá-Rocha M, Sousa D., Barra C, Júnior M. F. Monteiro T, Mello-Gomes R, Baptista F, Matafome, P. Maternal glycation during lactation period affect neurodevelopment and behaviour development of offspring ***(in preparation).***

List of Abbreviations

MeS	Metabolic Syndrome
T2DM	Type 2 Diabetes Mellitus
WHO	World Health Organization
DoHaD	Developmental Origins of Health and Disease
CNS	Central Nervous System
IGF	Insulin Growth Factors
IL-6	Interleukin 6
TNF-α	Tumour Necrosis Factor alpha
FOXO	Forkhead Box Transcriptions Factors
PPAR	Peroxisome Proliferator-Activated Receptor
AMPK	AMP-Activated Protein Kinase
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
IGF-1	Insulin-Like Growth Factor 1
BMI	Body Mass Index
AA	Arachidonic Acid
DHA	Docosahexaenoic Acid
STAT3	Signal Transducer and Activator of Transcription 3
PI3K	Phosphoinositide 3-Kinase
AKT	Protein Kinase B
NPY	Neuropeptide Y
POMC	Proopiomelanocortin
MG	Methylglyoxal
AGEs	Advanced Glycation End Products
IgG	Immunoglobulin G
GLO1	Glyoxalase 1
MG-H1	Nd-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine
Arg-P	Argpirymydine
CML	Nε-carboxymethyllysine
CEL	Carboxyethyllysine
RAGE	Receptor for advanced Glycation End Products
ROS	Reactive Oxygen Species

GSH	Glutathione Reductase
ADHD	Attention Deficit Hyperactivity Disorder
ASD	Autism Spectrum Disorder
GDM	Gestational Diabetes Mellitus
BDNF	Brain Derived Neurotrophic Factor
ORX1	Orexin Receptor 1
ORX2	Orexin Receptor 2
PFC	Prefrontal Cortex
DG	Dentate Gyrus
CA3	Cornu Ammonis 3
CA1	Cornu Ammonis 1
IRS	Insulin Receptor Substrate
SOD	Superoxide Dismutase
CAT	Catalase
GR	Glucocorticoid Receptor
HPA	Hypothalamo-Pituary-Adrenocortical
IL-β	Interleukin 1β
GLUT1	Glucose Transporter Protein Isoform 1
DMSO	Dimethyl Sulfoxide
BBGC	S-p-Bromobenzylglutathione cyclopentyl diester
IP	Intraperitoneal
PND	Post Natal Day
SL	Small Litter
EPM	Elevated Plus Maze Test
OPF	Open Field Test
NOR	Novel Object Recognition
RI	Recognition Index
DI	Discrimination Index
AUC	Area under the curve
kITT	Decay of glucose rate during the insulin tolerance test
IRtotal	Total insulin receptor
vGLUT1	Vesicular glutamate transporter 1
vGAT	Vesicular GABA transporter
PSD95	Postsynaptic density protein 95

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Chapter 1 - Introduction

I. Metabolic Syndrome: the new pandemic of 21st century

According to World Health Organization (WHO), Metabolic Syndrome (MeS) is defined when an individual has, at least, three of the following risk factors – abdominal adiposity, hypertriglyceridemia, hypercholesterolemia, hypertension, and impaired glucose, being insulin resistance a key central factor [1]. Moreover, MeS is also considered a major economic burden, which worldwide incidence is increasing and contributing to the higher prevalence of cardiovascular diseases and consequently the risk for type 2 diabetes mellitus (T2DM) [2].

According to WHO, more than 1,9 million adults were overweight in 2016, being 650 million of these obese. In addition, the International Diabetes Federation estimated that 1 in 11 adults suffer from T2DM [3]. The obesity phenotype increases the risk for the onset of T2DM because insulin resistance (IR), a common feature of obesity, contributes to exhaustion of pancreatic cells that result in a hyperglycaemic condition [4]. Currently, T2DM affects 463 million people being estimated that in 2045 more than 700 million people will be diagnosed with this pathology [5].

T2DM is considered a worldwide pandemic, so it has an increasing incidence not only in adults but also in adolescents and children's. It is estimated that over 340 million children and adolescents aged between 5-19 years were considered overweight or obese, showing an increasing prevalence of childhood obesity and a concern to future obesity increase in the world [6].

II. Metabolic programming windows as modulators of offspring development

In the beginning of 80's, the study performed by David Barker's and colleagues defined the "foetal origins of adult disease" hypothesis that demonstrate the long-term risk for adult offspring when exposed to an undernutrition environment in pregnancy [7,8]. Later, the concept of developmental origins of health and disease (DOHaD) was supported by the discoveries on foetal programming by maternal malnutrition, which was demonstrated to cause a low birth weight and imprinting in the new-born a higher risk of developing cardiovascular diseases during life [9]. These findings have demonstrated the importance of preconception, pregnancy, lactation, and infancy-adolescence phases as programming windows to the proper development of the offspring [7,8].

Thus, developmental programming studies have demonstrated that the function of organ systems at adulthood can be defined during early life and that the critical windows of susceptibility to insults differ between organ systems [10]. Therefore, these programming windows are important in the proper differentiation and development of organs involved in maintaining glucose homeostasis, such as the brain. Programming windows are considered important sensitive phases to neuronal connections and may determine central nervous system development (CNS) [10]. Such changes may have long-term consequences for determinant processes in metabolic disorders, such as food intake behaviour, energy expenditure pathways, autonomic enervation tone, neurodevelopment, and behaviour [11]. The insults that occur during these periods could program to different phenotypes at adulthood. However, although there are many studies elucidating the impact of programming windows in new-born's, the mechanisms that underlying these alterations are still unknown.

II.1. Lactation period as a metabolic programming window

The lactation period is considered a critical window of susceptibility to organ maturation and differentiation of the infant. This phase is responsible for supplying important nutrients to ensure the healthy growth of the offspring [10-11]. According to WHO it is recommended the exclusive breast milk feeding during the first 6 months of life to reduce the risk of developing diabetes, obesity and hypertension, among other diseases.

Human breast milk is considered the best source of nutrition for infants since it ensures the proper balance between essential nutrients and bioactive factors, such as hormones like insulin, leptin, insulin growth-factors (IGFs) and their binding proteins [12-14]. In such an early stage, these hormones are not only absorbed at the intestine, but can also cross the blood-brain barrier [15]. However, milk composition is not uniform in its nutritional content and quantity, being dynamic and variable according to several factors [16]. Even in the same individual, breast milk volume and composition can change over the stage of lactation, time of the day and suckling [11].

The stage of lactation includes two major phases: the colostrum and the mature milk. In the first days of lactation the milk or colostrum are composed mainly by immunological functions, containing higher levels of lactoferrin, immunoglobulin A, leukocytes, and specific developmental factors [17]. After the second week of lactation, the mother's milk reaches the mature stage being mainly composed of macronutrients, micronutrients, and growth factors [17]. Thus, maternal milk composition is mainly dependent on the habits and behaviours that the mother adopts during this period.

III. Maternal milk quality and quantity during lactation period as programming factors for offspring metabolic health

Several studies have been performed to understand the effects of offspring under and overnutrition during the lactation period and its impact at adulthood. One common experimental model is the small litter model, in which a reduction of the number of pups per litter is made after birth, leading to a lower competition for milk between pups during the suckling period [18-19]. Therefore, there is a higher consumption of calories and an unbalance between food intake and energy expenditure, which leads to an overweight/obese phenotype [19-20]. *Glavas et al*. showed that overnutrition of the pups during breastfeeding period increases body weight gain and leptinaemia [21]. Moreover, small-litter pups were also shown to develop hyperinsulinemia, hyperphagia and glucose intolerance at adulthood, suggesting that early overnutrition can alter energy homeostasis and increase the susceptibility to obesity and insulin resistance [22]. A study performed by *Junior et al*. demonstrated that overnutrition during this postnatal window can induce oxidative stress and cardiovascular dysfunction, namely cardiac hypertrophy, endothelial dysfunction, reduced intraventricular systolic pressure accompanied with reduced vascularization [23].

On the other hand, offspring undernutrition is defined as an insufficient caloric consumption to supply the body's energy demands. In animal models, it was shown that pups nursed by protein diet-restricted mothers had lower plasma insulin and glucose levels during the lactation period [24]. A recent study in rodents performed by *Miranda et al*., has demonstrated that maternal 50% food restriction during breastfeeding period, although inducing a lean phenotype in adult offspring, lead to hyperphagic behaviour, hypertriglyceridemia, and hypercholesterolemia [25]. Additionally, it was observed that maternal low protein diet can induce an increased hepatic deposition of fatty acids in rodent offspring, with alterations in gene expression and protein levels of key enzymes involved in glycolysis and pathways of fatty acid oxidation in skeletal muscle and adipose tissue [26]. Of note, and according to *Lizarraga-Mollinedo et al*., undernutrition during the suckling period induces glucagon resistance and insulin hypersensitivity in the liver. As consequence, this affects the supply of glucose and ketone bodies to the brain, affecting the synthesis of neurotransmitters and energy production, which can interfere with its proper development [27].

III.1. Influence of maternal metabolic status in milk quality

Although breastmilk is a source of bioactive molecules with benefits for the healthy development of new-born's, there is evidence showing that it is influenced by the maternal metabolic condition during pregnancy and lactation periods [28]. The milk from obese human mothers was found to be richer in long-chain omega-3 polyunsaturated fatty acids with a pro-inflammatory profile, which may influence not only the subsequent cardiometabolic status, but also neurodevelopmental outcomes [29]. Moreover, maternal obesity could also affect breastfeeding due to the excess of adipose mass that impairs the normal function of hormones regulation, and consequently delay the onset of lactogenesis and alter milk production [30]. This metabolic condition is associated with an alteration of immunological factors concentrations in human milk, such as leptin, interleukin-6 (IL-6), insulin, tumour necrosis factor alpha (TNF-α), ghrelin and adiponectin. As consequence, these alterations in human milk could increase the incidence of obesity, insulin resistance, T2DM and other negative metabolic outcomes in offspring [30]. A human study that evaluated the impact of maternal diabetes during breastfeeding demonstrated that women with diabetes are more likely to introduce infant formulas in the first two days of new-born's life when compared with a healthy mother. The alternative of using infant formulas can be explained by a higher difficulty to produce enough milk for the infants being associated with an increased risk to develop metabolic syndrome later in life [31].

In rodent models, a recent study performed by *Sellayah et al.* has shown that maternal obesity can predispose offspring to an obese phenotype with adipocyte hypertrophy [32]. In order to disclose the specific role of breastfeeding, *Gorski et al*. has used a cross-fostering mouse model to compare the differences in pups from obese and lean mothers during lactation [33]. The study showed that the offspring from obese dams, although maintaining an obese phenotype, had improved insulin sensitivity throughout life when fostered with lean dams. On the other hand, pups fed with breast milk from diabetic mothers were shown to have lower body weight and impaired glucose tolerance, impaired insulin secretion and/or insulin intolerance later at adulthood, showing the importance of lactation period to the later metabolic condition [33]. Moreover, male offspring had an upregulation of FOXO and PPAR pathways, while female offspring, besides PPAR pathway activation, also demonstrated an enrichment of AMPK and fatty acids metabolism activation [34].

A study performed by *Nogales et al.,* has shown the effects of a maternal diet rich in fructose (65%), during breastfeeding causes maternal hypertriglyceridemia, insulin resistance (HOMA-IR index) and compromises beta-cell function, which was associated with lower viability index

of the offspring calculated on postnatal day 7, which can possible be explain by the metabolic changes undergone by the mother during the transition from gestation to lactation [35]. In addition, it was also observed that the offspring presented a higher BMI at birth, although, the opposite was observed at the end of lactation period [35]. These findings could be related with the fact that a fructose-rich diet increases fatty acid synthesis and decrease gluconeogenesis, therefore modifying milk content [35-36].

Maternal exposure to metabolic syndrome during lactation also changes appetite profile and endocrine energy balance of the offspring by impairing insulin secretion and increasing serum leptin levels [37]. The levels of selenium are a trace for normal growth and development being involved in insulin-like growth factor-1 (IGF-1) regulation, correct thyroid hormones synthesis, oxidative balance, endocrine regulation of appetite and energy homeostasis [37]. In this context, offspring exposed to maternal metabolic syndrome have lower selenium intake, lower appetite and growth retardation [37]. Similarly, they were shown to present an undeveloped pancreas with a decrease of beta-cell function and low insulin secretion, leading to a phenotype similar to type 1 diabetes mellitus [37]. Moreover, the offspring develop leptin resistance, together with a low amount of brown adipose tissue and underdeveloped muscle and bone mass [37].

III.2. Influence of maternal diet in milk quality

During normal weaning, lipids are the main metabolic fuel during breastfeeding due its importance for the CNS development [38]. Therefore, an appropriated supply of fatty acids, such as n-3 and n-6 long-chain polyunsaturated acids is crucial to ensure a normal development, being involved in the synthesis of important molecules that regulates several signalling pathways [39]. Besides maternal metabolic status, maternal intake of unbalanced diets during pregnancy and lactation periods can also have a negative impact on the metabolic condition of the offspring. Maternal diet during pregnancy and lactation is relevant for fatty acid supply during perinatal period. Some human studies have demonstrated the importance of milk composition for new-born's development. In specific, arachidonic (AA) and docosahexaenoic (DHA) acids are considered important to the appropriate normal growth and development of the brain and visual system [40]. A study performed by *Barrera et al*., as demonstrated that Chilean women, during last stage of pregnancy and across lactation period, had a higher intake of n-6 polyunsaturated fatty acids, and a lower intake of n-3 polyunsaturated fatty acids, namely DHA. This can lead to a reduction of the capacity of the mother to transfer DHA to her offspring, as observed in erythrocytes and breast milk in women

samples [40-41]. Moreover, these fatty acids can modulate energetic metabolism in muscle and adipose tissue, such as insulin action and inflammatory response [42-43].

Some studies have addressed the effects of a maternal high-protein diet in animal models. The offspring were shown to present lower postweaning weight and adiposity accompanied by a lower food intake [44]. Furthermore, the pups from mothers fed with high-protein diets, presented increased blood glucose, insulin, and glucagon levels in fasting conditions after the weaning period [44-45].

Most of the studies in this field have addressed the role of cafeteria diets (diet rich in sugars and fats) in pups' metabolic health. Several experimental studies have shown that diets rich in fat and sugars have negative impacts on offspring metabolic outcomes [11]. In animal models, maternal high-fat diets were shown to cause alterations in milk composition, namely higher insulin, and long-chain polyunsaturated fatty acids levels, being associated with increased offspring growth [46-47]. Although female offspring didn't show any differences, male offspring developed insulin resistance at early adulthood, and presented increased weight gain, insulin intolerance and hyperglycaemia at adulthood. In addition, there is also an increased expression of inflammatory markers on adipose tissue, long-term bone loss and expansion of adiposity at bone marrow [48]. In mice, maternal consumption of cafeteria diet during lactation period was shown to induce obesity and dyslipidemia in the offspring [49-50]. Additionally, in a rat model of maternal diet-induced obesity during lactation, offspring presented lower levels of proteins involved on insulin and leptin hypothalamic signalling, as signalling transducer and activator of transcription 3 (STAT-3), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT). This was associated with increased expression of neuropeptide Y (NPY) and decreased proopiomelanocortin (POMC) levels. Such changes induced an unbalance between hunger/satiety, promoting higher caloric uptake, and increasing the predisposition to obesity at adulthood [51]. The offspring also developed hypertrophy of pancreatic islet cells, further potentiating the development of insulin resistance later in life [51].

Besides the changes of macronutrients consumption in hypercaloric diets, other secondary metabolites may also be involved in their negative effects in the offspring. *Francisco et al*. have shown that the treatment of lactating female rats with methylglyoxal (MG), a glucose metabolism intermediary and precursor of advanced glycation end-products (AGEs), leads to increased offspring body and adipose tissue weight at adulthood, together with glucose intolerance, dyslipidaemia, and impaired β-cell function [52]. Such effects were recently proven to be directly related to glycation, since they were observed in the adulthood of pups

directly treated with MG during the suckling period [52]. Some studies have been shown that the composition of maternal diets, in specific fatty acids, during late pregnancy and breastfeeding, could modulate inflammatory response and contribute to an oxidative stress environment. A recent study performed in piglets has shown that a maternal diet mainly composed by fish oil, which is rich in long-chain n-3 polyunsaturated fatty acids, plays an important role in the regulation of oxidative stress and inflammatory response, enhancing the antioxidant ability but increasing the susceptibility to inflammatory stimulation in offspring [53]. In addition, this type of diet increased the levels of IgG and IL-10 in the colostrum and the level of TNF-α in milk, which could be responsible for improving the anti-inflammatory capability in the offspring [53]. The addition of 3-5% of fish-oil to diet during lactation was also shown to promote the growth of offspring piglets, due the increased secretion of milk fat and immunoglobulins. Furthermore, it was also shown to decrease the levels of IL-1β and increase the expression of IL-10 in the liver and IL-6 and TNF-α in skeletal muscle, alleviating the inflammatory response of the offspring [54-55]. A study performed by *Liermann et al.*, has shown that a diet combined with linoleic acids and saturated fatty acids can increase the inflammatory response with a higher concentration of IL-1β and IL-6 and offspring oxidative response [56].

III.3. Influence of maternal behaviours in milk quality

Besides nutritional cues, exposure to other factors such as stress, cigarette smoking and alcohol during lactation period have also been shown to have a negative impact on offspring metabolic condition. Postpartum stress has negative consequences for lactogenesis and lactation phases, affecting hormone production. Glucocorticoids are not only involved in stress response but also in mammary gland development, lactogenesis and milk production. Furthermore, early life stress induced by maternal separation during lactation period has been shown to be involved in alterations on satiety point, meal duration and meal size, leading to lower food intake in female offspring [57]. Early weaning was also shown to reduce pup's body weight in a sex-dependent manner [58]. The interruption of breastfeeding by exclusive intake of solid foods might contribute to lower body weight observed on the first days of weaning, showing that beyond nutritional challenge, physical interaction between mother and offspring in late lactation period influences body weight gain [58].

Despite many women avoid smoking during pregnancy, most of them restore this habit during lactation period. Several studies have confirmed that nicotine can be transferred to the offspring through breast milk [59]. Moreover, when evaluating infants whose mothers smoked

during pregnancy and lactation period, higher AGEs levels were observed in their skin [60]. Maternal smoking during the suckling period is correlated with foetal growth retardation and impaired development of multiple organ systems, such as the kidney, by inducing injury and fibrosis [61].

A study performed by *Oliveira et al*. has shown that the rat offspring whose mothers were exposed to smoke during lactation presented increased body weight, visceral adipose tissue, and leptin levels [59]. Moreover, these animals showed thyroid dysfunction and a programming for obesity development later in life [59].

Like smoking, the consumption of alcohol during this programming window has serious consequences to the offspring, including the development of metabolic syndrome. A study performed by *Chen et al*, has shown that rodent offspring whose mothers had a heavy alcohol consumption during gestation and lactation, have decreased insulin sensitivity and higher glucose levels, which programs the development of insulin resistance later in life [62]. In addition, the offspring also presented elevated plasma, liver, and muscle triglycerides levels [62]. However, there are few studies that evaluate the effect of alcohol consumption exclusively during lactation. A study performed by *Murillo Fuentes et al*. has shown that maternal consumption of alcohol during lactation period induces a lower body weight gain of the offspring when compared with in utero exposure [63]. Moreover, it was also observed that ethanol consumption significantly decreases milk consumption in both prenatally and postnatally (during breastfeeding) ethanol-exposed litters [63]. These findings were also corroborated by a study performed by *Cheslock et al*., who also observed a lower body weight gain accompanied by a decrease in sucking behaviour [64]. These alterations can be related with the fact that ethanol concentration has been demonstrated to interact in breastfeeding process by inhibiting the secretion of prolactin and oxytocin hormones [63, 65].

IV. The Brain as a major organ of glucose consumption

The central nervous system (CNS) is a dynamic assembly of neurons and non-neuronal cells capable of mediating complex processes, with high energy requirements, using glucose as its main energy substrate [70]. The maintenance and restoration of ion gradients in postsynaptic and action potentials, as well as the uptake and recycling of neurotransmitters, are the main processes that contribute to higher energy expenditure of the brain [71].

During the transmission of the nervous impulse, glucose uptake and aerobic glycolysis is stimulated in astrocytes through the glucose transporter 1 (GLUT1). Lactate is then produced

through aerobic glycolysis and then shuttled to neurons, to be converted into pyruvate and produce energy in mitochondria [70].

In conditions of hyperglycemia or impaired glucose consumption, glucose accumulation can produce methylglyoxal (MG), which is considered the main glycolytic product and can cause cell damage when present in higher concentrations. Furthermore, MG can react with proteins, nucleic acids, lipids, and peptides through non-enzymatic reactions - Maillard reactions. As a result of these reactions, advanced glycation end products (AGEs) are formed, such as Nδ-(5-hydro-5-methyl-4-imidazolon-2-ylornithine (MGH1), Argpyrimidine (Arg), Nεcarboxymethyllysine (CML) and carboxyethyllysine (CEL) [72]. Consequently, these products have an affinity to membrane receptors, more specifically, AGE receptors (RAGE), which can lead to reactive oxygen species (ROS) formation triggering oxidative stress and inflammation [72].

In physiological conditions, cells have detoxifying mechanism such as glyoxalase (GLO) system pathway that is responsible for detoxifying ketonic aldehydes, such as MG and convert them to hemithioacetal, that is glutathione (GSH) - dependent. This intermediate is catalyzed by glyoxalase-1 (GLO1) into S-D-lactoylglutathione. In sequence, glyoxalase 2 converts S-D-lactoylglutathione into D-lactate [72, 73].

As mentioned, both neurons and astrocytes are capable to metabolize glucose. Therefore, these cells are susceptible to the production of MG and consequently formation of AGEs [70]. Since astrocytes are responsible for maintaining brain homeostasis and metabolic processes between neurons and themselves, these cells are well equipped to metabolize MG. However, neurons have lower glyoxalase activity, which is a key factor to impair MG accumulation. Therefore, these cells are more prone to toxicity [70].

During the embryonic period there are important key events that ensure the proper development of newborn brain and are maintained through late adolescence and adulthood. Neurodevelopment begins with the formation of neuronal tube, a process called neurulation, then neurogenesis which consists in the identification of neural progenitors that originate neurons. This process is very complex and dynamic and continues until birth. Next, starts neuronal cell migration or neurogenesis, that is followed by apoptosis to eliminate the excess of synapses and dendritic process. Gliogenesis starts around week 22 that are involved in the production of region-and subtype-specific glia which continues through adulthood [169].

Although in mouse models the pregnancy period is more reduced, the neurodevelopment of the offspring is similar of what it occurs in humans **(Figure 1)**.

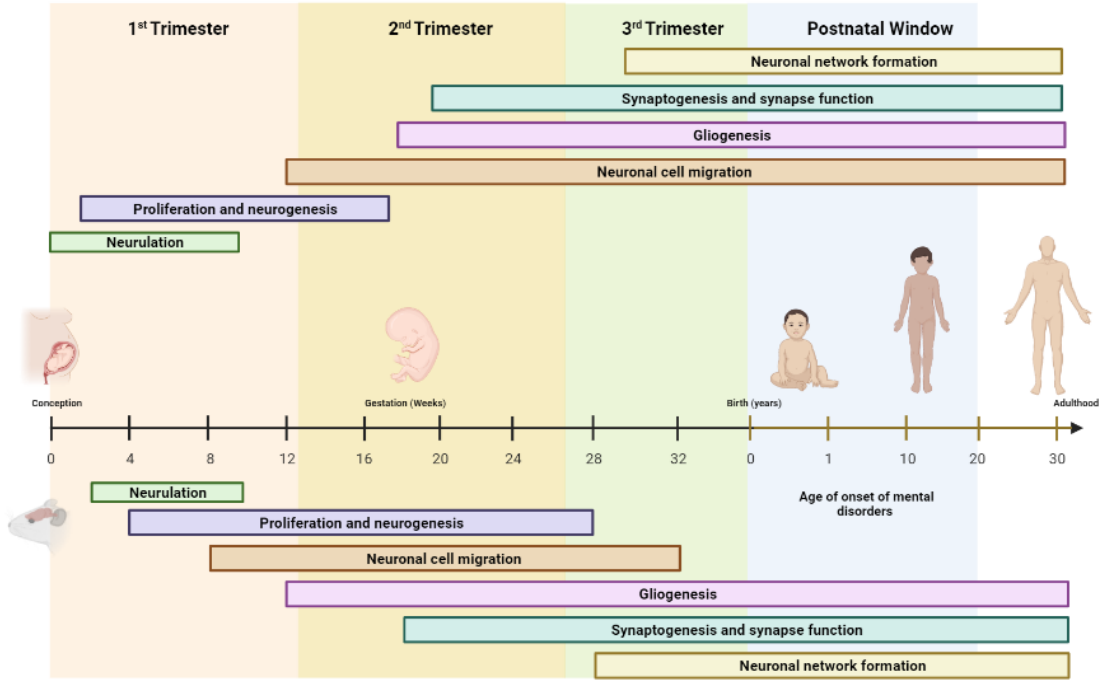

Figure 1 – Neurodevelopment timeline: overview in human and rat model. The process of neurodevelopement starts during the conception period with neurulation process. In rat models, these process only start after the second week of pregnancy. Then, the brain of the foetus suffers several processes of cell proliferation and neurogenesis (in rat models this process occurs during all prengnancy period). At week 12, beggins the migration of neuronal cells that undergoes until adulthood similar to the next processes that starts in the midel of the second trimester namely gliogenesis, synapse function and formation of neuronal network. Image created with Biorender.com

The CNS is one of the most sensitive targets in response to maternal obesity and/or T2DM, being hippocampus and PFC two brain regions very susceptible to these metabolic alterations. The hippocampus is considered a crucial brain region for short and long-term memory formation and spatial navigation. This brain region has several particularities in its composition being formed by a unidirectional tri-synaptic pathway that starts on the cortex and it's projected to the dentate gyrus (DG) then to Corno Amonis 3 (CA3) and subsequently CA1 area [170]. These different areas have their own cellular structure and function. For instance, DG is formed by granule cells whereas CA3 and CA1 regions are formed by pyramidal cells. Additionally, DG is considered the "gateway" to the hippocampus, playing an important role in the process of spatial memory formation [170]. *Lofti et al*. has demonstrated that maternal hyperinsulinemia can cause a hippocampal neuronal death in offspring as displayed by decreased neuronal density in hippocampal sub-regions [93]. Moreover, *Kim et al*. showed that maternal overnutrition could increase the proliferation of astrocytes and a decreased

ability of neurons migration which suggests an alteration on proliferation capacity in these cellular population of the CNS [74]. Moreover, it was also demonstrated that chronic maternal hyperglycemia could increase RAGE expression and hippocampal excitability in the offspring [75]. In a context of diabetes during pregnancy the expression of neural markers such as msi1, a marker of neural stem cell, could be affected in male but not in female offspring hippocampus. Additionally, the expression of doublecortin that is involved in the migration of neuronal precursors shown to be increased in both male and female offspring [86].

Prefrontal cortex (PFC) is considered the most important region that are involved in the development of cognitive functions [171]. This region can be divided into two main subregions that play an important role in several functions, in specific, medial PFC and orbitofrontal cortex that contribute to cognitive function and emotional control. Although neurons of PFC are generated before birth, the differentiation and maturation of its neurons and synaptic connections occurs until adulthood [171]. Therefore, during this period, there is a continuous production and elimination of neurotransmitters, receptors, and transporters [171].

V. Influence of lactation period in offspring neurodevelopment and behaviour

V.1. Maternal metabolic condition and risk of offspring neurodevelopmental diseases

The maternal metabolic condition, besides increasing the risk of metabolic diseases in the offspring **(Figure 2),** has also been suggested to contribute to a higher risk of psychiatric disorders, such as attention deficit hyperactivity disorder (ADHD), autism spectrum disorders (ASD), anxiety, depression, and schizophrenia [76-78, 89].

In humans, maternal obesity has shown to account for a two-fold increased risk of having a child with ADHD [76]. Children of obese or overweight mothers have an increased risk of having more symptoms related with this disease, such as inattention and difficulty to deal with emotions [76]. Maternal diabetes has been further associated with an induction of cognitive impairments and an increased susceptibility to neuropsychiatric disorders [77]. A study involving 212 children with ADHD has shown that gestational diabetes was associated with an over two-fold increased risk of developing this neuropsychiatric disease, and a recent meta-analysis showed that pregestational diabetes could also increase the risk of ADHD in the offspring by 44% [76, 78]. Moreover, maternal diabetes has also shown to be positively associated with ASD risk in offspring [79-81]. A systematic review and meta-analysis published in 2018 involving 16 studies, reported that children's ASD was positively associated, not only with gestational diabetes mellitus (GDM), but also with diabetes before pregnancy [79-80].

In rodent models, maternal high-fat diet-induced obesity has been shown to increase anxiety behaviour and reduce sociability in female offspring, which is a common feature of neurodevelopmental disorders such as ASD and ADHD [82]. Maternal obesity has been associated with increased offspring risk of disrupted emotions like fear and sadness, being also involved in increased internalizing behaviours associated with depression and anxiety [83]. These changes in development were accompanied by neuroinflammation, namely in the prefrontal cortex, due to increased expression of proinflammatory cytokines in the first days of life and latter at adulthood, together with increased hippocampal microglial activation and lipid peroxidation [83-84]. Maternal obesity has also been shown to impair neuronal plasticity in the offspring by decreasing neurogenesis, and synaptic function [75].

In animal models of diabetes during pregnancy and lactation, new-borns from diabetic dams have higher expression levels of AGEs and pro inflammatory markers in the forebrain, hippocampal RAGE levels, neuronal activity, and oxidative stress, contributing to behavioural and memory alterations at early adulthood [85]. A study performed by *Sousa et al*. showed altered expression of neuronal markers, such as neural stem cell markers in males and neuronal precursors in both male and female offspring of diabetic mothers. Such alterations were associated with an early delay in offspring development, affecting the vestibular system, balance, strength, coordination, and locomotion, as well as impaired short-term memory in female offspring at infancy [86]. Maternal diabetes also impacts on glyoxalase-methylglyoxal (GLO-MG) pathway, increasing the maternal circulating levels of MG. This was associated to premature neurogenesis and depletion in embryonic mouse cortical neural precursor cells, which can predispose to cognitive dysfunction, impairment of visual reception, motor skills, and adaptative communication and socialization [87-88].

Importantly, the studies currently available are exclusively focused on the perinatal period, including pregnancy and lactation, and little is known about the specific role of each of these programming periods in the ethology of neurodevelopmental and psychiatric disorders.

V.2.Maternal diets and risk of offspring neurodevelopmental diseases

Maternal high-fat diets considered unbalanced or obesogenic have been shown to induce neurophysiological changes, such as impaired brain glucose regulation, changes in the levels of excitatory and inhibitory amino acids and neurotransmitters, neuroinflammation, oxidative stress and alterations in the structural integrity of the blood-brain barrier that can directly or indirectly impair hippocampal-dependent learning and memory operations **(Figure 2).** As already detailed, fatty acids unbalance may trigger a proinflammatory and oxidative

environment. In addition, this type of diet increased the levels of IgG and IL-10 in the colostrum, which could be responsible to improve the anti-inflammatory capacity of the offspring. The addition of 3–5% of fish-oil to sow feed during lactation, was shown to promote the growth of offspring piglets, due the increased secretion of milk fat and immunoglobulins [91-92].

Interestingly, in animal models, maternal cafeteria diets during breastfeeding were demonstrated to have different outcomes in the offspring. *Wright et al.* demonstrated that there is an improvement on memory performance in male offspring while, on the opposite, female offspring have an impairment on memory tasks [96]. In addition, other studies in piglets and mouse models have shown that maternal exposure to western diets during perinatal period impairs spatial learning and memory, raising the question about the mechanisms and factors associated with neurodevelopment deficits [97-99].

The specific contribution of the lactation period has been addressed in animal models. Offspring of high-fat diet-induced obese rodent models present decreased sociability, and increased anxiety-like behaviour and hyperactivity, which are ADHD-like symptoms [100]. Also, maternal high-fat diet during suckling period has shown to increase the expression of pro-inflammatory cytokines, IL-1, IL-6, and TNF-α and glucocorticoids receptors in the offspring hippocampus, and consequently, leading to a higher anxious behaviour [101]. In the study performed by *Teixeira et al.* in rodents, offspring hippocampal oxidative stress caused by maternal cafeteria diet, from lactation period until adulthood, was also associated with anxious-like behaviour, memory impairment and decreased social interaction, despite having a better performance in locomotion evaluation [102]. A recent study performed in minipigs during gestation and lactation periods has demonstrated that inflammation caused by maternal western diets decrease neuronal activity in hippocampus and cortex, consequently affecting learning and cognitive and memory processes [103].

Maternal cafeteria diets consumption during pre and postnatal periods, not only increase oxidative stress and inflammation, but also affects the normal function of several neurotransmitters, such as serotonin and dopamine, leading to a predisposition to neuropsychiatric diseases, disturbances in locomotion and anxious behaviour [104-105]. As well, exposure to maternal high-fat diet for 9 weeks (including gestation and lactation periods) induced an increased expression of brain-derived neurotrophic factor (BDNF) in offspring dorsal hippocampus and an increase in $GABA_A$ α2 subunit and serotonin receptors 5-HT1A in the ventral hippocampus, which was also associated with increased anxious-like behaviour [106]. In addition, in rodent models, it was shown that the preference for maternal high-fat

diets consumption during pregnancy and breastfeeding, instead of a low-fat diet, increases circulating plasma leptin levels in the offspring [107]. Interestingly, the cross-fostering of the pups during lactation period, from a standard to a high-fat maternal diet, was shown to have similar negative outcomes by increasing the circulation of leptin levels, suggesting the importance of this period in the modulation of appetite/reward systems and the metabolic status of the offspring [107].

Regarding the effects of sugars, *Erbas et al*. showed that, in rodents, a long-term exposure to maternal fructose diet during pregnancy and lactation periods impairs sociability in male offspring. Moreover, it was observed an impairment of locomotor activity and memory performance in both male and female offspring, which may be correlated with a development of autistic social deficits [107]. The long-term exposure to fructose diet increases circulating TNF-α levels, in both male and female offspring rats. In addition, there was a significant reduction of neuronal markers related with neuronal growth and synaptic formation in the hippocampus [107]. In accordance, maternal consumption of a diet rich in Maillard Reaction Products during lactation period, which introduces considerable quantities of AGEs into a diet, accelerated male offspring neurodevelopment, improved working memory but inducing an anxious-like behaviour [109].

Although maternal high-fat diets have consequences to the neurodevelopment of the offspring, maternal malnutrition can also have negative impacts. Some experimental studies in rodents have shown that maternal protein restriction during the perinatal period can negatively affect neurogenesis, cell migration, myelination, and plasticity, which can have consequences in exploratory behaviour, anxiety, and learning and memory abilities [110]. A study performed by *Batista et al.* in rodent models has shown that maternal protein malnutrition during pregnancy and lactation periods induces a decrease in male and female offspring vocalizations, impairs social discrimination and decreases social play behaviours, which are associated with ASD phenotype [111]. In addition, maternal protein-restricted diet during these periods has demonstrated similar negative outcomes in the offspring to a diet rich in fat, with an impairment in learning and motivation [112]. Moreover, this poor nutrition of the offspring during breastfeeding period can cause a dysregulation of gene expression in the medial PFC, which can be associated with behavioural alterations associated with neurodevelopmental disorders [113].

V.3.Maternal behaviours and risk of offspring neurodevelopmental diseases

The exposure to stress or stress mediators, such as glucocorticoids, during critical periods of development has an impact in the neuroendocrine and behavioural systems of the brain, being a relevant risk factor to psychiatric disorders later in life, such as anxiety and depression [114-116] **(Figure 2).** Infants of depressed mothers were shown to have behavioural difficulties, like socialization, and cognitive delays, with a higher risk of developing psychiatric disorders during the adolescence period [117-118]. On the other hand, studies also showed that relaxation interventions during the lactation period could have positive effects on maternal psychological state, breast milk intake, milk composition, and infant behaviour and growth [119]. Early behaviour or temperament of breastfed infants has been associated with higher maternal breast milk cortisol levels, suggesting that mothers may shape infant behaviour by the transmission of bioactive factors in milk [120-122]. Mothers who were less stressed or doing relaxation intervention during lactation period, had longer and better-quality time to physically bond with their infants [122]. Consequently, this stimulated or facilitated infant sleep [119].

In rodents, maternal exposure to social stress during lactation period depresses maternal care, increases maternal anhedonia and anxiety, and induces a mild growth impairment in the offspring [123-124]. Such effects were associated with a modulation in gene expression of the neuropeptide's orexin and its receptors (Orx1r and Orx2r), which are involved in maternal care and depressive behaviour, modulating the development of mood and anxiety disorders in the offspring [125]. Deprivation of maternal care during perinatal period can have marked effects on emotional development of the offspring [126]. Animal studies have demonstrated that maternal deprivation or neglect during lactation induce a higher risk for future psychiatric illness, accompanied by increased anxiety-like behaviour and aggression, delayed conditioned-fear extinction and decrease empathy [126]. These behavioural alterations have been linked with alterations in BDNF signalling in CNS which can be responsible for neuronal dysfunction. Moreover, early weaning could also upregulate circulating corticosterone levels in the PFC region which induces higher anxiety at adulthood [126]. In accordance, it has also been shown an anxious-like behaviour in male and female offspring after prenatal maternal glucocorticoid administration, although the role of lactation was not specifically addressed [127-128].

As mentioned above, maternal cigarette smoking during late gestation and lactation periods may have negative consequences for the offspring. Maternal nicotine exposure was shown to impair hippocampal neurogenesis, potentially affecting anxiety-like behaviour and impulsive decision-making later in life, increasing the incidence of ADHD or addictive disorders [129-

130]. As well, the maternal consumption of alcohol during lactation has a negative impact in the brain function, being associated to decreased cognition and difficulties in completing tasks [131]. Exposure to alcohol through milk induces hyperactivity and deficits in learning and memory in young mice and exposure to alcohol in milk formula during early postnatal period also increases offspring microglial activation in hippocampus, potentially impairing offspring immune function and cognition later in life [132].

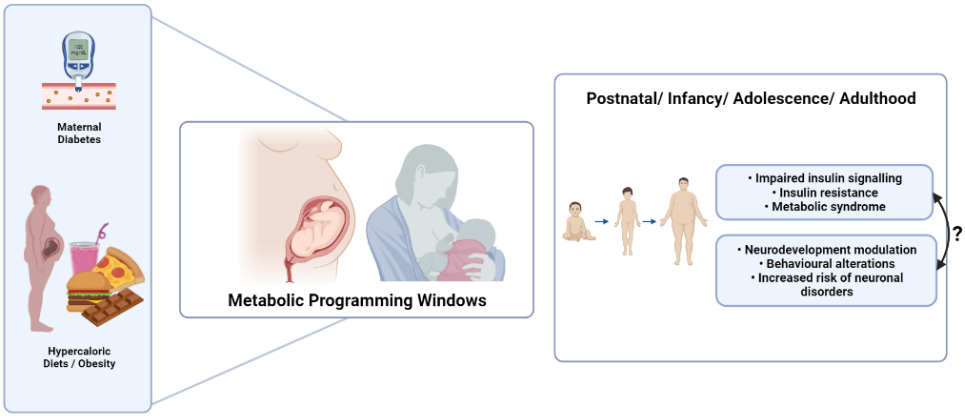

Figure 2- Maternal metabolic condition, diet and behaviours adopted during perinatal period may predispose to the offspring for metabolic syndrome such as insulin resistance and metabolic syndrome at adulthood. Moreover, these insults can also have an impact in offspring neurodevelopment and increase their susceptibility to behavioural alterations and neuronal disorders. Image created with Biorender.com

VI. Possible mechanisms upon lactation period underlying the risk of neurodevelopmental diseases at adulthood

VI.1. Insulin Resistance

The metabolic condition and diet of the mother during lactation are crucial for the correct development of the offspring, being insulin an important growth factor for brain development. In pregnancy, alterations of mother's insulin sensitivity due to obesity and/or diabetes could influence the neurodevelopmental state of the offspring. Reduction of neuronal plasticity, decreasing neurogenesis, and synaptic function in the hyperinsulinemic offspring of diet-induced dams was shown to be associated to an impairment of hippocampal insulin resistance [134]. The exposure to maternal high-fat diets during lactation was shown to activate mitogen-activated protein kinases (AMPK) in hippocampus and lead to central insulin resistance [135-

136]. Lower insulin signalling in the hippocampus could be linked to chronic inflammation, oxidative stress, and impaired neuronal growth and transmission, which may impact learning and memory processes [135-136]. AKT is a downstream molecule of IRS and plays a crucial role in insulin signalling. Maternal high-fat diet or obesity decreases AKT phosphorylation in the dorsal hippocampus of adult rat male offspring, which is associated with lower cognitive function [137-138] **(Figure 3)**.

VI.2. Oxidative Stress and inflammation

Maternal obesity and diabetes are associated with chronic inflammatory processes, resulting in higher secretion of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, inducing inflammation in the offspring hippocampus, and being associated with neurodevelopmental and neuropsychiatric disorders [139-140].

Maternal high-fat diets during lactation period can also stimulate the activation of inflammatory pathways, such as JNK, and increased TNF-α mRNA expression in adolescent mice hippocampus [141]. In addition, the exposure to these diets during the postnatal period can reprogram the hippocampal neurogenesis later at adolescence period, increasing the levels of inflammatory markers, such as TNF-α, IL-1β and IL-6 in hippocampus, hypothalamus, and prefrontal cortex. This inflammatory environment can induce oxidative stress and increase ROS in the offspring, while reducing the levels of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, being also associated with an impairment of spatial memory [140-142].

The upregulation of pro-inflammatory cytokines in the first months of life can influence the development of neural pathways regulating behaviour, such as the hypothalamic-pituitary-adrenal (HPA) axis, serotonergic system and BDNF levels [126]. The upregulation of HPA axis, impairs glucocorticoid receptor (GR) leading to higher susceptibility to stress, depression, and anxious behaviours [105]. Early weaning of rodents induces persistent anxiety and heightened HPA activity. The higher circulating levels of corticosterone can act on GR-expressing neurons, such as PFC neurons, and consequently this can modulate anxious-like behaviour during development [143]. Moreover, early weaning could also increase the accumulation of ROS species in the hypothalamus specially in female offspring, while male offspring presents higher levels of nitric oxide [58]. Consequently, this stress environment can also interfere with the activity of antioxidant enzymes in offspring's hippocampus and hypothalamus. More specifically, it was observed a decrease of superoxide dismutase (SOD) activity in hippocampus of female offspring, while in males the effect was more pronounced at hypothalamus,

promoting a dysregulation in the ratio of SOD/CAT activity [58]. In addition, GLO1 activity has been shown to be upregulated at female offspring's hippocampus, while in male offspring this effect was observed in the hypothalamus [58].

The consumption of maternal trans-fat diets during pregnancy and lactation periods, besides increasing the production of proinflammatory cytokines, activates glucocorticoid receptors in the hippocampus of adult offspring, which could also lead to an anxious-like behaviour [101]. Moreover, maternal high-fat diet exclusively during lactation can lead to instability and loss of spines in cerebral cortex at juvenile offspring, even when there is a shift of the diet after weaning. This can cause a synaptic impairment and brain dysfunction at adulthood, which is associated with increase oxidative stress and lipid peroxidation during this period [144] **(Figure 3)**.

VI.3. Alteration of brain-derived neurotrophic factor

The last week of gestation and the first two weeks of postnatal development encompass a critical period for hippocampal neurogenesis. BDNF is considered an important regulator of brain synaptic transmission, plasticity, and growth playing a critical role in hippocampal long-term potentiation, which is a long-term enhancement of synaptic efficacy underlying learning and memory [145]. Since spatial learning and memory are dependent on alterations in synaptic plasticity and dendritic spine maturation, nutritional manipulation during development is likely to affect key neurotrophins and synaptic proteins which could be important in determining behaviour. In adult rodent models, it has been demonstrated a correlation between the decreased expression of BDNF in hippocampus and cortex and negative outcomes in learning and memory processes [147-148]. Cellular actions of BDNF are mediated by binding to its receptor, and by activating cytoplasmic signalling pathways of kinases such as AMPK. AMPK have a regulatory role in neuroplasticity, proliferation, and cell differentiation [146]. The exposure to cafeteria diets during pregnancy or lactation periods potentiates an increase of AMPK expression in hippocampal region. This AMPK upregulation may suggest that the BDNF intracellular signalling could be upregulated to increase long-term potentiation via positive feedback. Consequently, this could affect memory performance at adolescence and adulthood [136, 141]. Interestingly, although the exposure to an obese environment during the perinatal period may promote the expression of genes related with synaptic plasticity like BDNF, it is accompanied by a decreased ratio of synaptophysin-to-synaptotagmin, a marker of impaired synaptic plasticity and cognitive function, suggesting that BDNF upregulation beyond normal levels may also be deleterious for synaptic plasticity [147].

The consumption of maternal high-fat diet may decrease the expression of BDNF in early phases of brain development. The exposure to this obesogenic environment during lactation period leads to a persistent decrease of BDNF expression in male offspring hippocampus resulting in an impairment of spatial memory [138]. In young animals from diet-induced obese dams, the lipid peroxidation rate was found elevated in the hippocampus, suggesting that the reduced levels of BDNF could be due to oxidative stress [148]. The consumption of high-fat diet during the postnatal period, has been shown to induce similar negative outcomes, suggesting that this period is crucial in the modulation and expression of BDNF in offspring [149] **(Figure 3).**

VI.4. Modulation of GABAergic signalling

The balance between excitatory and inhibitory synaptic transmissions is essential for the continual remodelling of neural networks, which is required for synaptic plasticity that is important for cognitive processes, such as learning and memory [168].

y-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter, which regulates excitatory glutamatergic neurons [150]. $GABA_A$ receptor is an ionotropic receptor that mediates fast GABA responses by opening Cl^- channel that, when activated, increases intracellular uptake of Cl^-, leading to membrane hyperpolarization and consequently, reduction of neuronal excitability [150].

It has been shown that the maturation of GABAergic neurons is more faster than the glutamatergic excitatory neurons, and GABA is the major excitatory neurotransmitter before the maturation of glutamatergic synapse [151]. So, the depolarization is mediated by GABA receptors, which are responsible to activate calcium-sensitive signalling processes important to neuronal differentiation and maturation, during brain development. The expression of GABA receptors is prominent at the time and regions where neurogenesis persisted, such as hippocampus, during the first postnatal weeks [152].

$GABA_A$ receptors have been linked to learning, memory, and mental development, being GABA serum and plasma levels considered a potential peripheral marker for depression and other stress-related disorders at adulthood [153, 154]. Maternal separation during lactation period has shown to induce a decreased expression of $GABA_A$ receptors in prefrontal cortex and evoked anxiety and depressive-like behaviour in adulthood [155, 156].

Maternal high-fat diet exposure in perinatal period was shown to induce a downregulation of GABAergic neurons in prefrontal cortex of the offspring and consequently behavioural alterations, such as anhedonia, depressive- and anxiety-like behaviour, and memory

impairment [157]. The consumption of maternal high-fat diets during pregnancy and lactation periods can be associated with a lower hypothalamic expression of genes involved in GABA system, which can interfere with brain modification and plasticity [158]. Interestingly, other animal studies of maternal high-fat diets during lactation have also demonstrated that increased expression of $GABA_A$ receptor in the dorsal hippocampus could be involved in the risk of anxiety-like behaviour at adulthood, although the mechanisms of its increased expression were not addressed [106].

It has also been suggested that maternal high-fat diet enhances offspring hedonic feeding [159]. Since GABA neurons can mediate the reward system, changes in offspring GABAergic system may alter offspring reward system and influence feeding behaviour later in life [160]. Nevertheless, further studies on the remodelling of the reward system during early life are needed.

GABA synthesis is regulated by glutamic acid decarboxylase, which in adult brain exist in two isoforms (GAD65 and GAD67). A decreased expression of GAD67 has been associated with individuals with schizophrenia and ASD [161, 162]. Therefore, a decreased GABA synthesis can negatively affect several physiological functions such as locomotor activity and cognitive performance, which are characteristic of schizophrenia and autism diseases [163, 164]. A long-term exposure of maternal fructose intake diet during lactation period has shown to cause a significant decrease in the levels of GAD67 in the brain of male offspring, which was accompanied by neurodevelopmental and behavioural alterations in the offspring characteristic of autism disease [108].

Specific glucose metabolites have also been shown to modulate GABAergic activity, namely the MG and its detoxifying enzyme GLO1 [72]. However, MG was suggested to function as a $GABA_A$ agonist in physiological concentrations, modulating the anxiolytic behaviour. It was demonstrated that the low-dose treatment with MG can activate $GABA_A$ receptors in primary neurons promoting a decrease in anxious-like behaviour [167]. In adult mouse models, GLO1 expression was associated with an increase of anxious-like behaviour [166]. GLO1 overexpression increases the metabolization of MG and consequently leads to an increased anxious behaviour due a decreased $GABA_A$ receptor activation [167]. Nevertheless, such studies were performed in adult mice and using physiological methylglyoxal concentrations. Further studies on maternal glycation during perinatal period could be important to address the importance of these mechanisms during the suckling period and the risk of anxiety at adulthood **(Figure 3)**.

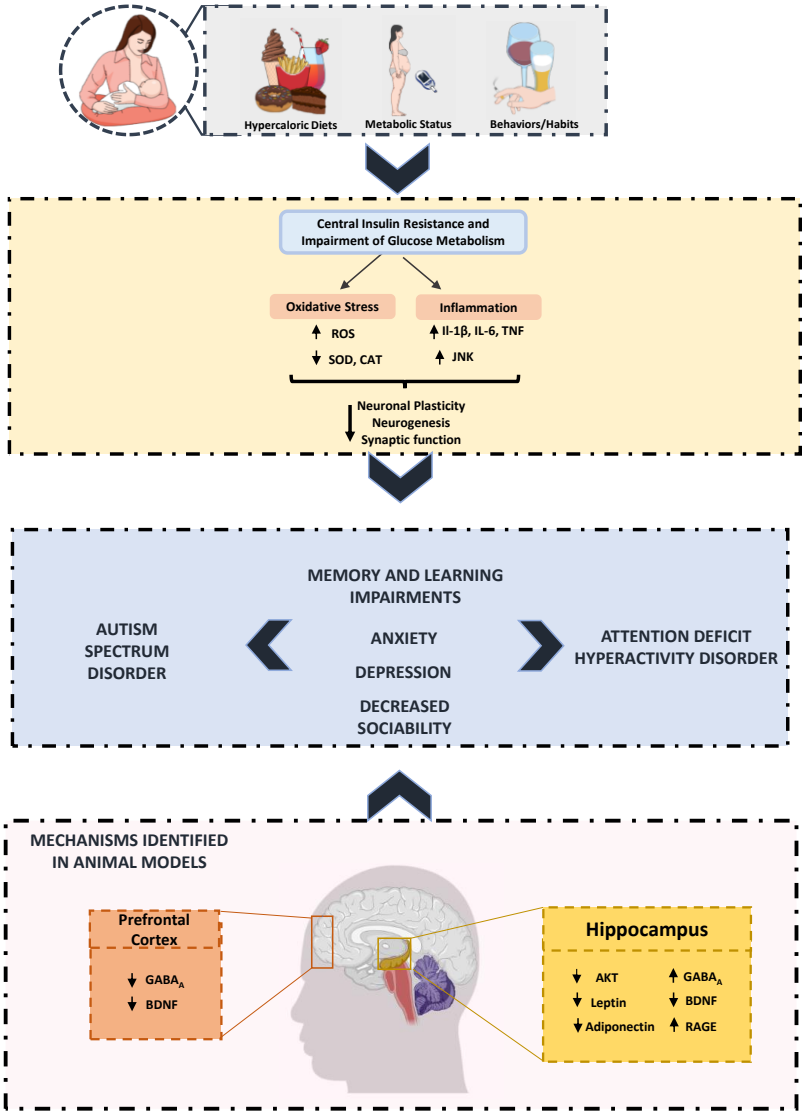

Figure 3 - The possible mechanisms underlying neurodevelopmental and behaviour alterations are still poorly understood. Nevertheless, the consumption of hypercaloric diets, the metabolic status and the behaviours adopted by the mother during breastfeeding period, may predispose the offspring for the development of central insulin resistance and impairment of glucose metabolism. As consequence, this can potentiate alterations in BNDF signalling, and GABAergic in different central brain regions, affecting the proper neurodevelopment of the offspring . Adapted from *Amaro et al.* [172].

Chapter 2 - Scientific Framework

The first periods of life are crucial in shaping metabolism and brain function, but childhood metabolic syndrome and consequently neurodevelopmental disorders are dramatically increasing.

Offspring neurodevelopment and behaviour at adulthood may also be modulated by alterations that occur during perinatal period, increasing the susceptibility to development of neuronal disorders such as depression, anxiety, and memory and learning impairments at adulthood [172]. Lactation period, in particular, has been considered an important programming window in the modulation of offspring CNS. However, the mechanisms that underlying these alterations are still unknown [11].

It has been demonstrated that maternal milk composition has a critical role on the proper development and maturation of CNS, and its nutrient content level influence the neurodevelopment and consequently the behaviour at adulthood [136, 149, 157]. Obesity and metabolic alterations have been associated with the later risk of developed behaviour diseases at adulthood. Indeed, maternal diabetes demonstrates to negatively impact offspring neurodevelopment and behaviour, in a sex-dependent manner [86]. However, how these alterations are related is still undetermined.

Some studies have been demonstrated that the imbalance of excitatory and inhibitory synapses could be involved in offspring neurodevelopment and behaviour alterations. In fact, some studies had demonstrated that glycation at adulthood could modulate the GABAergic signalling and consequently affect the anxious-like behaviour [167]. However, if these alterations are observed in the first period of life are still unclear. Thus, it would be interesting to disclose the effect of maternal glycation on offspring GABA response and its later behaviour development.

Interestingly, it has also been suggested that maternal metabolic state, diets and behaviours adopted during this period could induce sex-specific responses in the offspring. Thus, it is also important to disclose the existence of potential sex-differences responses.

Nevertheless, the impact of glycation in adult models has been shown to be higher in obese than in lean models. Thus, it is also important to disclose if the postnatal impact of glycation is different in conditions of hyperphagia-induced obesity, a model that is not affected by major nutritional changes in the milk.

Chapter 3 - Main Objectives

This work is focused in two important goals: firstly, we aim to evaluate the impact of maternal glycation during lactation period on offspring neurometabolism, neurodevelopment and behaviour and the mechanisms that could underpin these alterations. The second aim is to disclose the impact of maternal glycation during breastfeeding period, in the context of postnatal hyperphagia, on offspring neurometabolism, neurodevelopment and behaviour.

Specific objectives

I. To characterize the effects of maternal glycation during lactation on offspring neurometabolism and metabolic status.
II. To evaluate the effect of maternal glycation during lactation on offspring neurodevelopment and behaviour at adulthood.
III. To disclose the impact of maternal glycotoxins in central mechanisms of neurometabolism.
IV. To evaluate the impact of postnatal overweight in neurometabolism and metabolic status in male offspring.
V. To evaluate the impact of maternal glycation in brain offspring development and behaviour after postnatal overweight.

Chapter 4 - Materials and Methods

Experimental Design – In Vivo Study

All procedures involving animals was performed according to good practices of animal handling, with the approval of the Institutional Animal Care and Use Committee (ORBEA 13/2018) and the procedures were performed by licensed users of Federation of Laboratory Animal Science Associations—FELASA, conformed to the guidelines from the Directive 2010/63/EU of the European Parliament for the Protection of Animals Used for Science Purpose.

Female lactating dams and offspring

Pregnant Wistar rats were housed under standard animal conditions (ventilation; 22-24ºC temperature; 55% humidity; 12h/12h light/dark cycle) and ad libitum access to food and water. To study the effects of maternal glycation, 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) [90], in the first six days after birth, whereas control dams were injected with the vehicle dimethyl sulfoxide (DMSO) **(Figure 4).**

Additionally, to study the effect of childhood obesity it was used a model of hyperphagia. On the third day after birth, a small-litter (SL) protocol was induced where the number of pups was reduced from 8 to 3 pups per dam to induce postnatal hyperphagia **(Figure 5).**

Also, another experimental group of animals was considered, in order to study the effect of maternal glycation in the context of postnatal hyperphagia – SL+BBGC **(Figure 4 and 5).** After birth, lactating dams were administered with BBGC during the first six postnatal days and, in addition, at PND3, the number of pups were reduced from 8 to 3 per dam.

On postnatal day (PND) 21, both male and female offspring were separated from the mother until postnatal day 45 and fed with standard diet. At weaning, milk samples were collected to biochemistry analysis. The offspring were housed in standard conditions as mentioned above.

During this period, body weight was monitored weekly at PND0, PND4, PND7, PND14, PND21, PND35 and PND45. At PND45, after five hours of fasting, an insulin tolerance test was performed, and the levels of triglycerides were measured. After blood collection, animals were anesthetized with an IP injection of ketamine/chlorpromazine, euthanized by cervical displacement and the hippocampus and PFC were collected to molecular analysis **(Table 1).**

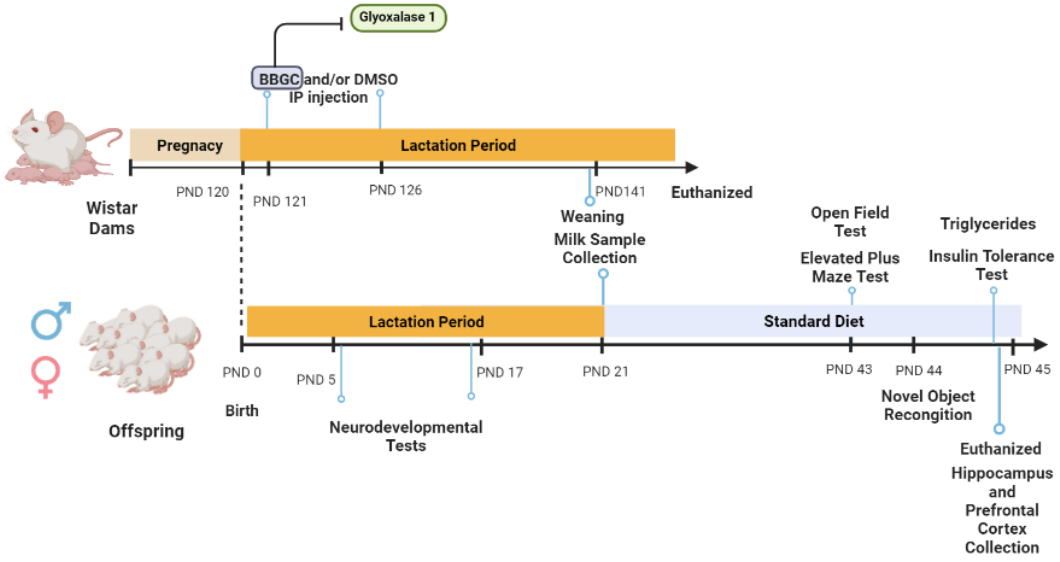

Figure 4 - In vivo study maternal glycation experimental design. Offspring whose mothers were treated with BBGC were studied until PND45. During the first days after birth offspring were subjected to neurodevelopmental tests (PND5-PND17). After weaning, offspring were fed with standard diet. At PND43 and PND44 offspring were tested at elevated plus maze, open field tests and novel object recognition test. At PND45, an insulin tolerance test was performed, triglycerides were measured, and hippocampus and prefrontal cortex were collected for molecular analysis. Image created with Biorender.com

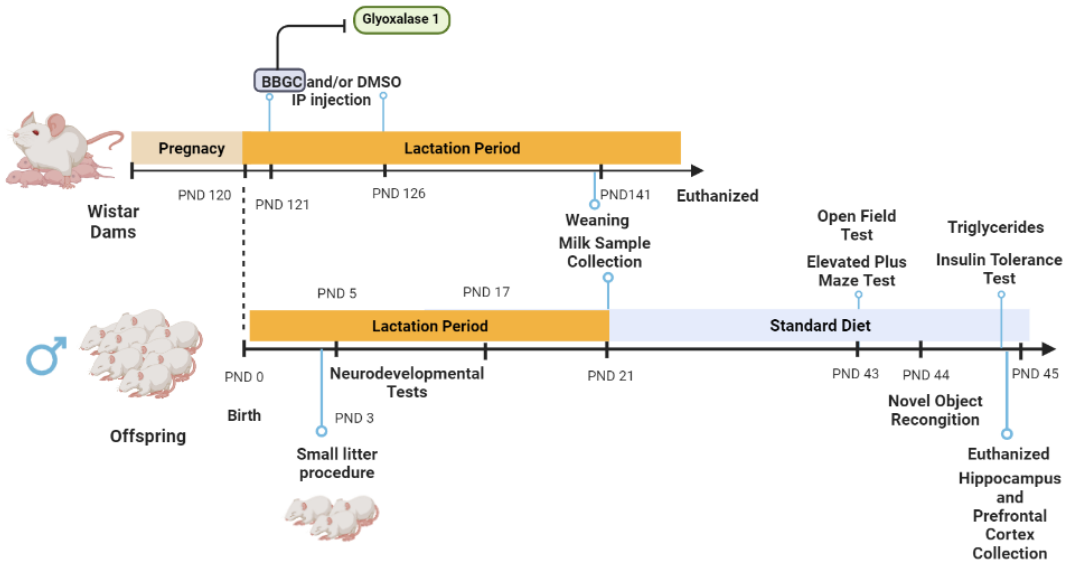

Figure 5 - In vivo study neonatal hyperphagia experimental design. At PND3, offspring were reduced from 8 to 3 pups per dam and studied until PND45. During the first days after birth offspring were subjected to neurodevelopmental tests (PND5-PND17). After weaning, offspring were fed with standard diet. At PND43 and PND44 offspring were tested at elevated plus maze, open field tests and novel

object recognition test. At PND45, an insulin tolerance test was performed, triglycerides were measured, and hippocampus and prefrontal cortex were collected for molecular analysis. Image created with Biorender.com

Developmental behavioural testing

The offspring of the five experimental groups were submitted to developmental tests from P5 to P17, as previously described [86]. Behaviour was monitored each day during the light phase of the light cycle and under dim white light.

Cliff Aversion (PND5-PND9)

Pup ability to retract from the edge of a cliff was assessed as indicative of pup vestibular system development. Animals were placed on an elevated flat surface only with their snout and forepaw digits hanging over the edge. The time for the pup to turn away from the cliff and move its paws and snout away from the edge was counted up to 30 s **(Figure 6)**.

Figure 6 – Evaluation of vestibular system development. Offspring were tested on cliff aversion test between PND5 and PND9.

Locomotion (PND5-PND10)

Pup locomotion ability was assessed by placing the animals on a flat surface in the center of a 13 cm diameter circle. The time for the animal to fully exit the arena with all four limbs was recorded up to 30 s.

Nest Seeking behaviour (PND5-PND8)

The ability to identify and locate the maternal scent depends on the display of adequate olfactory, motor and discriminatory ability to discern nest bedding from home bedding. Pup ability to discriminate their nest bedding by olfaction was determined by using a rectangular arena (26 × 14 cm) divided into 3 compartments: the center compartment where the pup was placed; the home bedding goal on one side, where there was home nest bedding; and a fresh bedding goal on the opposite side, where there was fresh clean bedding in a similar amount to

home bedding. Each animal was placed in the central compartment at a 90° angle from the goal compartment for a first trial and after a 30 s intertrial interval, the pup was again tested for a second trial. Each trial was performed with the animal facing opposite sides of the equipment to balance possible side turning preferences. The latency to goal was scored by the time the animal took to transpose the apparatus home bedding goal mark with both snout and forelimbs. The cut-off time of each trial was 120 s. Latency to goal was evaluated as an average between the two trials, resulting in one score per pup, per day **(Figure 7)**.

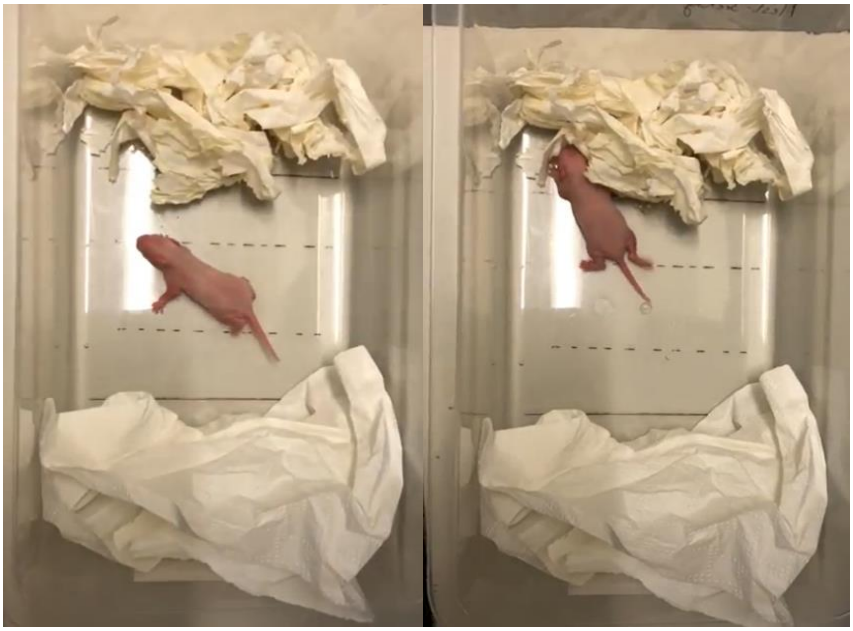

Figure 7 – Evaluation of olfactory, motor and discriminatory ability. Offspring were tested on nest seeking behaviour test between PND5 and PND8.

Wire Suspension Test (PND10-PND14)

Pup forelimb strength was assessed through the wire suspension test. The pups were placed against a horizontal wire rod and were allowed to grasp it with both forepaws. Time was scored after the release of the animal until it felt to a padded drop zone. The cut-off time for this test was 10 s **(Figure 8).**

Figure 8 - Evaluation of forelimb strength was assessed through the wire suspension test. Offspring were tested on wire suspension test between PND10 and PND14.

Auditory Startle (PND11-PND14)

Auditory startle test was used as a measure of auditory system development, which indicates maturation of somatosensory, vestibular and/or proprioceptive function. Animals' capacity to produce full body startle response to a loud finger snap at a 10 cm distance was evaluated and the percentage of animals that responded per litter, per day was calculated.

Eye Opening (PND12-PND17)

Eye opening was monitored by observation of pups and the percentage of pups with eyes opened, per litter, per day, was calculated.

Late infancy behavioural assessment

All animals were gradually adapted to manipulation over several days prior to experimentation. One hour before behavioural experiments all animals were habituated to the experimentation room under dimmed red light, controlled temperature and ventilation. Experiments were performed during the light phase of the light cycle.

Open Field test - OPF - (PND43)

At PND43, the OPF was performed to assess the locomotor ability. Animals were placed facing the wall in the center of the arena and were left to explore the arena (45 × 45 × 40 cm) for 10 min. Test analysis was performed through the Any Maze software to evaluate the animal locomotor pattern of exploration, average speed and distance travelled.

Elevated – plus maze - EPM – (PND43)

At PND43 animal anxious-like behaviour was assessed with the EPM test, as previously described [86]. This test evaluates rodents' conflict between preferring protected areas (closed arms), and their innate motivation to explore new environments (open arms). For this test, animals were left at the maze for exploration during 5 min. Analysis of animal performance in the test was achieved by "Observador" software (University of Athens, Medical School, Department of Pharmacology) and by the analysis of the time and number of entries in the open arms.

Novel object recognition test – NOR – (PND44)

At PND44, NOR test was performed to evaluate animal ability to distinguish between a familiar and a novel object, being an indicator of short-term recognition memory. The test was composed by a familiarization and a test trial. The familiarization trial had a duration of 10 minutes while the test trial had a duration of 3 minutes with a 4 h intertrial interval. The animal performance was measured through the Recognition Index (RI): [RI = TN/ (TN + TF)], and also by the Discrimination Index (DI): DI = (TN − TF)/(TN + TF) where TN is the time exploring the novel object, and TF the time exploring the familiar one.

Estrous cycle

To evaluate the estrous cycle of female Wistar offspring, at PND 45 days were subjected to a vaginal smear, made with an inoculating loop soaked in phosphate buffer saline 1x (PBS 1x) (137 mM NaCl, 2.1 mM KCl, 1.8 mM KH_2PO_4 and 10 mM Na_2HPO_4, at pH 7.4). After collecting the cells, they were placed in microscope slides, which were then fixed and stored in 96% Ethanol until observation in the light microscope LEICA DM 4000B (Leica, Wetzlar, Germany) with the 10x objective lens (Plan 63x/0.25 PH1).

Milk Sample Collection and determination of Total Antioxidant Capacity and Triglycerides

On postnatal day 21, female dams were anesthetized and injected with oxytocin (Facilpart) with a concentration of 2,5 UI/mL after 6h of fasting. Milk samples were collected, triglycerides were measured, and total antioxidant capacity was assessed with an Assay Kit (ab65329, Abcam) according to the manufacturer's instructions.

Determination of offspring plasma Insulin Levels

At PND45, 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 stored at -80˚C and used to perform the Rat Insulin ELISA Kit (Mercodia, Uppsala, Sweden).

Preparation of samples extracts and Western Blot

For the preparation of the samples' extracts of hippocampus and prefrontal cortex, a homogenization buffer was prepared: 0,25 M Tris-hydrochloride (Tris-HCl) and 100 mM sodium chloride (NaCl), with the adjustment of pH to 7.4, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol-bis(2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA), 20 mM sodium fluoride (NaF), 10 mM β- glycerophosphate, 2.5 mM sodium pyrophosphate and sodium metavanadate ($NaVO_3$), 1% Triton X-100, 10 mM phenylmethylsulfonyl fluoride (PMSF) and 5% protease inhibitor. The tissues were disrupted using TissueLyser systems (Quiagen, Germany), followed by the centrifugation at 14 000 rotations per minute (rpm), for 20 minutes at 4ºC. The pellet composed of cellular membranes and nucleic acids were discarded and the supernatant collected.

To quantify the protein concentration in the extracts, BCA Protein Assay Kit was carried out. Then, the samples were diluted in 2x Laemmeli buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue), with the adjustment of pH to 6.8.

Samples were sonicated and boiled at 90ºC for 2 minutes. Electrophoresis was performed with 8%, 10% or 12% polyacrylamide gels, according to protein molecular weight, composed with resolving (0.75 M Tris-HCl, 0.2% SDS, pH 8.8) and stacking buffer (0.25 M Tris-HCl, 0.2% SDS, pH 6.8), both of which are supplemented with acrylamide, MiliQ water, ammonium persulfate (APS) and N-tetramethylethylenediamine (TEMED).

Samples with a concentration of 15 µg of total proteins were loaded onto SDS-PAGE and electroblotted into PVDF membrane (Advansta, San Jose, CA, USA). Membranes were blocked with TBS-T 0,01% and BSA 5%, 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, Chicago, USA). The quantification of the bands obtained was performed through Image Quant 5.0 software (Molecular Dynamics). The results were expressed as a percentage of vehicle and control.

Statistical Analysis

The results are presented as mean + standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism 9 (GraphPad Software, Inc, San Diego, USA). The normality of the data was assessed with Shapiro-Wilk normality test. Accordingly, data were analysed with non-parametric Kruskal-Wallis test or with parametric one-way ANOVA followed by Tukey´s post-hoc test.

Table 1 – Primary and secondary antibodies used in Western Blotting

Antibody	Molecular Weight	Host	Dilution	Company
Anti-GLO1	21 kDa	Rabbit	1:1000	Abcam
Anti – IR total	90 kDa	Rabbit	1:1000	Santa Cruz
Anti – MG-H1	-	Mouse	1:500	Hycult Biotech
Anti – Argpyrimidine	-	Mouse	1:500	Nordic
Anti- PSD95	95 kDa	Rabbit	1:5000	Cell Signaling
Anti-NeuN	50 kDa	Rabbit	1:5000	Abcam
Anti-Synapsin	74 kDa	Mouse	1:5000	Synaptic Systems
Anti – vGAT	62 kDa	Mouse	1:1000	Abcam
Anti – vGLUT1	57 kDa	Rabbit	1:1000	Abcam
Anti - GABAa	52 kDa	Rabbit	1:1000	Abcam
Anti-Glutaminase	66 kDa	Rabbit	1:1000	Cell Signaling
Anti - Catalase	60 kDa	Rabbit	1:1000	Santa Cruz
Anti - Calnexin	83 kDa	Goat	1:1000	Sicgen
Anti - GADPH	37 kDa	Goat	1:1000	Sicgen

Chapter 5 - Results

I. Effect of maternal glycation on offspring brain and sex-specificities

Maternal glycation causes alterations on milk composition content

Several studies demonstrated that alteration on milk composition content could influence the development of the new-born [28].

In fact, in female dams that were treated with BBGC (BBGC group) milk analysis showed to have decreased triglycerides levels compared with the control ($p<0.05$), accompanied with a lower total antioxidant capacity ($p<0.05$), demonstrating that maternal glycation influences milk composition, during breastfeeding period. Although it was not observed a significant statistical difference in triglycerides levels between dams treated with vehicle and BBGC ($p=0.8$) it was observed a tendency to have lower total antioxidant capacity when compared with vehicle group ($p=0.1$) **(Figure 9).**

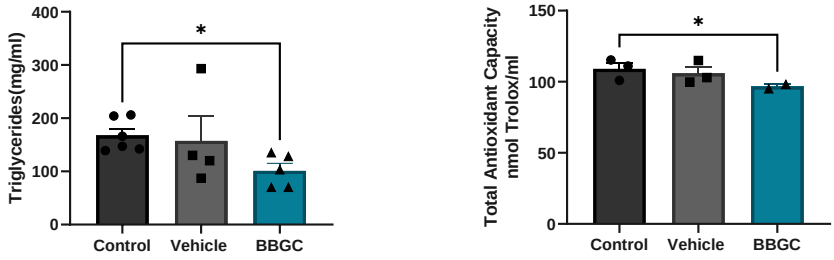

Figure 9 – Maternal glycation caused alterations on milk composition content. Milk from dams treated with BBGC showed lower levels of triglycerides and total antioxidant capacity. The results are shown as mean ± SEM of 3 a 5 animals per group and Kruskal-Wallis comparisons were conducted to compare among the groups. 1 symbol $p<0,05$.

Maternal glycation causes metabolic alterations in male but not in female offspring

The effect of maternal glycation in offspring body weight composition was monitored weekly during the 45 days. During this period, it was observed that body weight composition in male offspring was not affected during lactation period, although at PND21 the BBGC group showed a tendency to have a lower body weight comparing to the control group ($p=0.06$). Moreover, after lactation period, the BBGC offspring maintained a similar body weight composition compared with both control and vehicle groups **(Figure 10).**

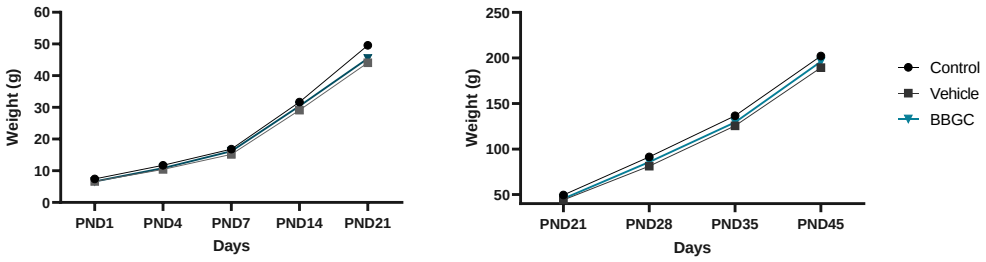

Figure 10 – Male offspring body weight composition was not affected by maternal glycation during and after breastmilk period. The results are shown as mean ± SEM of 22 a 35 animals per group and Kruskal-Wallis or one-way ANOVA comparisons were conducted to compare among the groups

However, during the insulin tolerance test (ITT), the decay of glucose rate among the 45 minutes of IIT performance (K_{ITT}) was lower in the male offspring of the dams that were injected with BBGC, showing a lower decay ($p<0.001$) when compared to vehicle group. However, the levels of insulin plasma remained unaltered compared with the control and vehicle groups. Nevertheless, there were not observed any alteration on triglycerides levels **(Figure 11).**

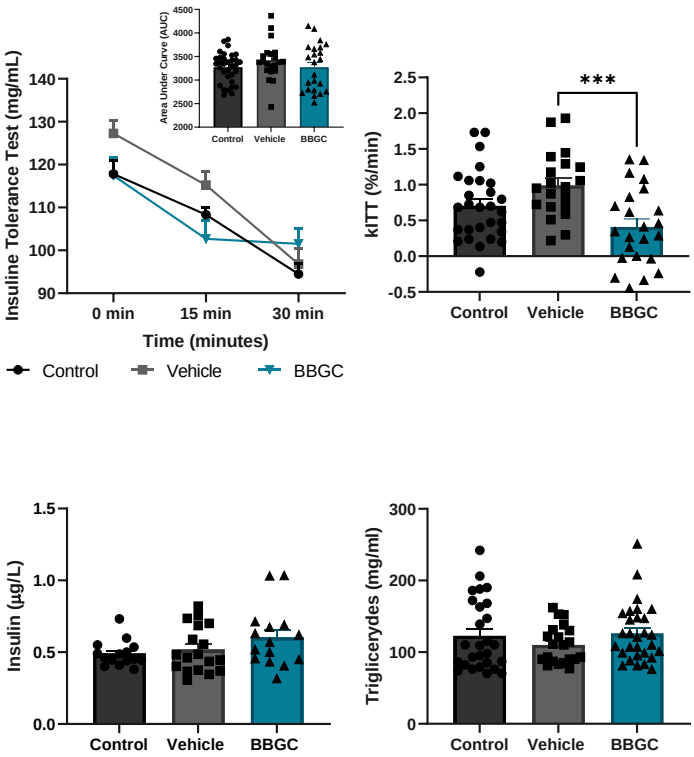

Figure 11 – Maternal glycation affected insulin sensitivity in male offspring. On PND45, an insulin tolerance test was performed in the offspring. Although there were no alterations on AUC, there were a significantly decrease of glucose rate among of the time - kITT. Moreover, no alterations were observed on plasma insulin levels, as well as triglycerides levels. The results are shown as mean ± SEM of 22 a 35 animals per group and Kruskal-Wallis or one-way ANOVA comparisons were conducted to compare among the groups. 3 symbols p<0,001.

Since the hippocampus and PFC are two brain regions responsive to insulin, it were evaluated the levels of total insulin receptor in both tissues. At hippocampus region, the levels of expression of insulin receptor demonstrated a trend to be increased when compared to control (p=0.06) and vehicle (p=0.1) groups while in PFC no alterations were found **(Figure 12).**

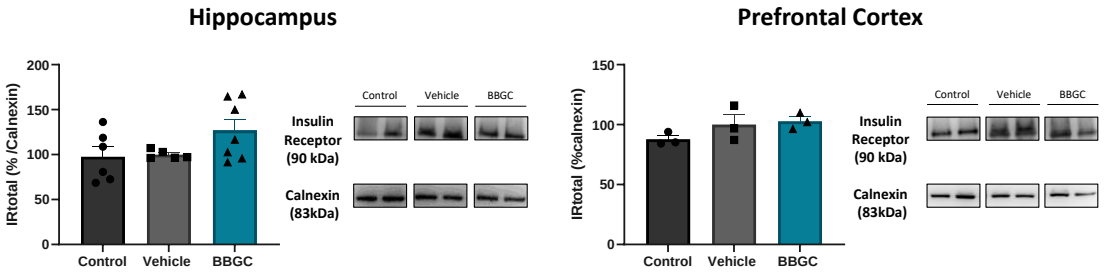

Figure 12 – Maternal glycation does not caused alterations on insulin signaling in male offspring hippocampus and PFC. The results are shown as mean ± SEM of 3 a 7 animals per group and one-way ANOVA comparisons were conducted to compare among the groups.

Similar to male offspring, body weight was monitored on female pups weekly. The group which dams were treated with BBGC showed no alterations on body weight composition during and after breastfeeding period comparing with the control and vehicle groups **(Figure 13).**

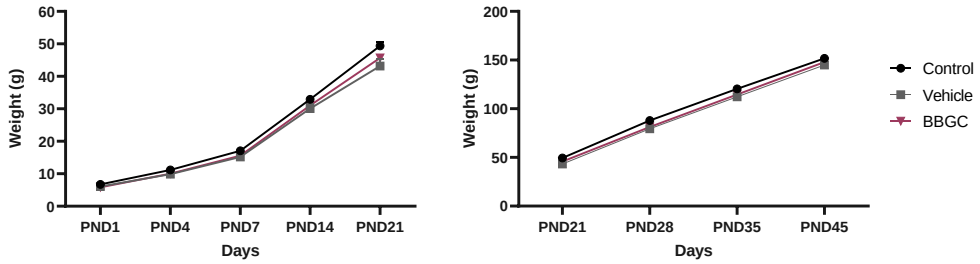

Figure 13 – Female offspring body weight composition were not affected by maternal glycation during and after breastmilk period. The results are shown as mean ± SEM of 7 to 15 animals per group and Kruskal-Wallis or one-way ANOVA comparisons were conducted to compare among the groups.

The evaluation of metabolic parameters such as the response to insulin during the ITT, similarly to male offspring, did not show alterations when compared to control and vehicle groups, which was corroborated with AUC. Moreover, it was not observed alterations on the decay of glucose rate among of the time - kITT. However, plasma insulin levels were lower in both vehicle and BBGC groups compared with the control group ($p<0,01$ and $p<0,01$, respectively). While the levels of triglycerides remained unaltered when compared to both control and vehicle groups **(Figure 14).**

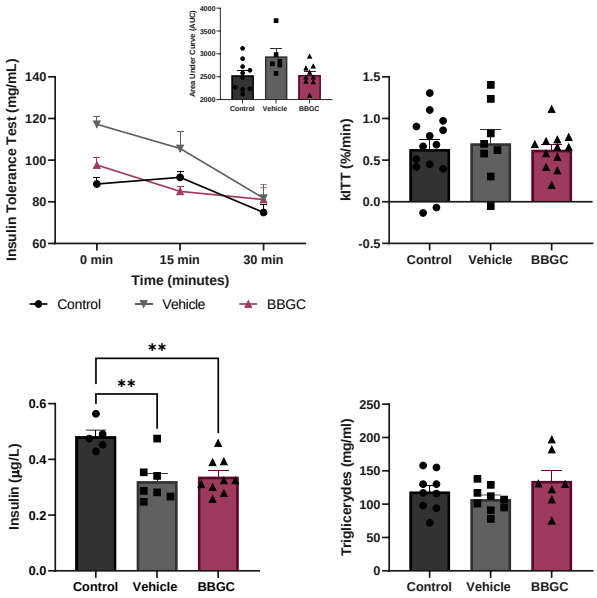

Figure 14 - Maternal glycation does not caused alterations on female offspring. The results are shown as mean ± SEM of 7 a 15 animals per group and Kruskal-Wallis or one-way ANOVA comparisons were conducted to compare among the groups. 2 symbols p<0.01.

To analyse potential alterations in insulin signalling at CNS, the levels of total Insulin receptor – IR total - were evaluated in female hippocampus and PFC, demonstrating no alterations, in both tissues, when compared with control and vehicle groups **(Figure 15).**

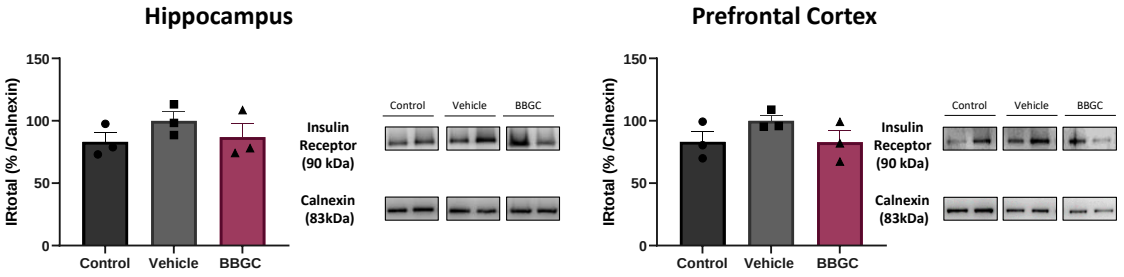

Figure 15 – Maternal glycation does not caused alterations on insulin signaling in female offspring hippocampus and PFC. The results are shown as mean ± SEM of 3 animals per group and one-way ANOVA comparisons were conducted to compare among the groups.

Maternal glycation impacts on offspring neurodevelopment

The perinatal period is a critical offspring developmental window, but the effects of maternal glycation on offspring neurodevelopment remain unknown. During the first PND both female and male offspring were subjected to several developmental tests to assess any alteration.

Offspring were tested for their latencies to home-bedding on the nest seeking test. Male offspring did not show alterations when compared with the vehicle group, although the vehicle showed an impairment in this behaviour only on PND6 compared with the control ($p<0.01$). In the tests evaluating the development of the vestibular system, namely cliff aversion test, maternal glycation induced a better performance compared with the vehicle at PND5 ($p<0.01$), PND7 ($p<0.05$) and PND8 ($p<0.01$) and with the control PND7 ($p<0.05$) suggesting increased maturation of this system. However, an impairment on locomotor ability was detected when compared with the vehicle group, at PND11 ($p<0.01$) and PND12 ($p<0.01$).

In the wire suspension test, male offspring of BBGC groups showed to have a better performance at PND10 ($p<0.05$) and PND11 ($p<0.01$) comparing with the control and at PND11 ($p<0.05$) comparing with vehicle group, respectively, indicating a higher strength in their upper forelimbs **(Figure 16).**

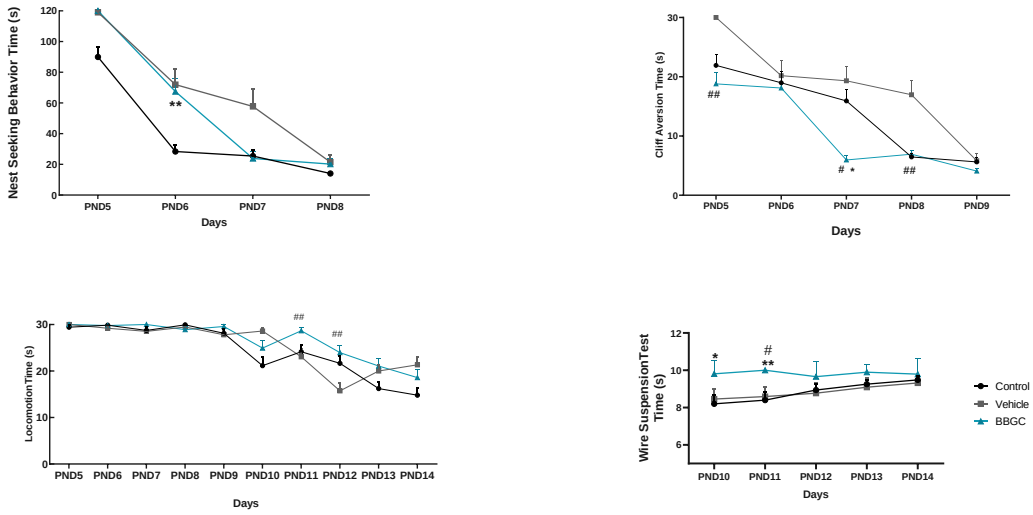

Figure 16 – Maternal glycation affects offspring neurodevelopment in male offspring. Pups were tested to several behavioural testes from PND5 until PND17. The results are shown as mean ± SEM of 22 a 35 animals per group and Kruskal-Wallis comparisons were conducted to compare among the groups, #BBGC vs vehicle and *BBGC vs control. 1 symbol $p<0,05$; 2 symbols $p<0,01$.

Male offspring had a significant delay of auditory startle response at PND12 ($p<0.05$), however at PND13 ($p<0.05$) all the pups had their hearing ability developed. Moreover, it was also observed that offspring from BBGC group had an anticipation of eye-opening day compared with the vehicle group ($p<0.05$), suggesting that maternal glycation accelerated the acquisitions of these developmental milestones **(Figure 17).**

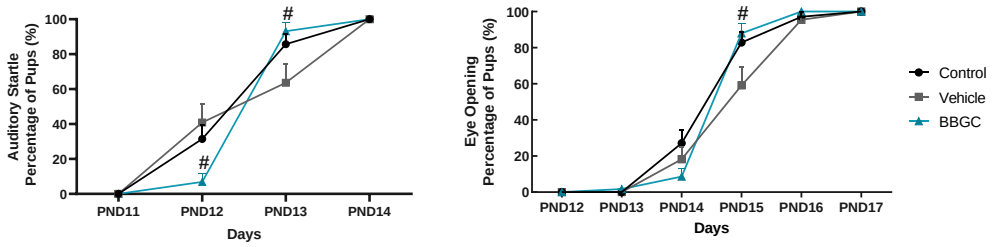

Figure 17 - Maternal glycation induced an antecipation of auditory startle capacity and of eye opening day. The results are shown as mean ± SEM of 22 a 35 animals per group and Kruskal-Wallis comparisons were conducted to compare among the groups. #BBGC vs vehicle and *BBGC vs control groups. 1 symbol $p<0.05$.

Concerning female developmental tests, it was observed that maternal glycation leads to an impairment on nest seeking test when compared with the control at PND8 ($p<0.01$), although no differences were observed between control and vehicle groups. The evaluation of vestibular system development through cliff aversion test also shown no alterations between BBGC and control groups, as well as no alterations on locomotor ability. However, in wire suspension test, similarly to male offspring, female pups presented a better performance at PND10 ($p<0.05$) and PND11 ($p<0.05$) comparing with the vehicle group and at PND11 ($p<0.01$) comparing with the control group, suggesting a higher strength in their upper forelimbs **(Figure 18).**

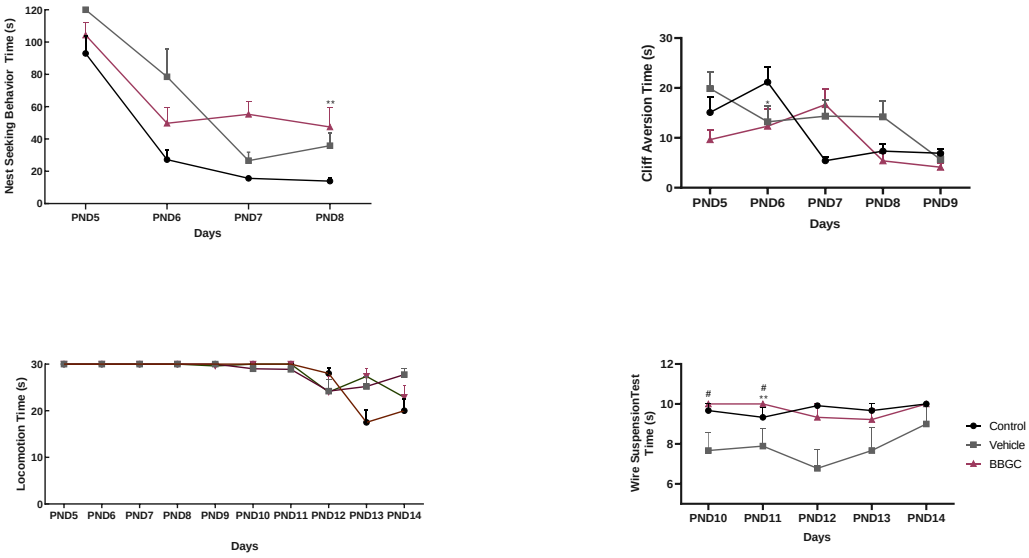

Figure 18 – Maternal glycation induced higher strength in female offspring upper limbs. Female pups were subject to developmental tests from PND5 until PND17. The results are shown as mean ± SEM of 7 a 15 animals per group and Kruskal-Wallis comparisons were conducted to compare among the groups. #BBGC vs vehicle and *BBGC vs control groups. 1 symbol p<0,05; 2 symbols p<0,01.

Interestingly, female pups from BBGC group shown a delay of the auditory startle response comparing with the control group (p<0.01) at PND12, a transitory effect since at PND13 all the offspring of BBGC group presented an auditory startle response. Although a delay on eye opening was observed at PND14 when compared with the control group (p<0.05), at PND15 a higher percentage of female with eyes fully opened was detected when compared with the vehicle group (p<0.01). **(Figure 19).**

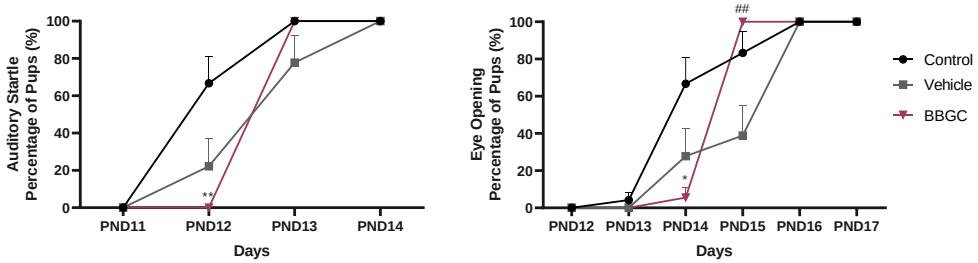

Figure 19 - Maternal glycation induced an antecippation of eye openning day. The results are shown as mean ± SEM of 7 a 15 animals per group and Kruskal-Wallis comparisons were conducted to compare among the groups. # BBGC vs vehicle and * BBGC vs control groups. 1 symbol p<0,05; 2 symbols p<0,01.

Maternal glycation impacts on adolescent male and female offspring anxiety-related behaviour

At PND43 and 44 behavioural tests were performed in the offspring. Since EPM and NOR are tests dependent on proper locomotor activity, locomotion was assessed at PND43 in the OPF by placing each animal in an open arena and by letting the animal freely explore it for 10 minutes. Male offspring from BBGC group showed to travel a greater distance compared with the control ($p<0.0001$) and vehicle ($p<0.01$) groups. Furthermore, this was accompanied with a higher average speed compared to both control ($p<0.0001$) and vehicle ($p<0.05$) groups demonstrating hyperlocomotion and a disinhibited phenotype.

Anxious-like behaviour was evaluated by EPM test, where animals were left for 5 minutes in an elevated plus shape platform to explore the open and/or closed arms. Male offspring from BBGC group spent more time exploring open arms compared with the control ($p<0.05$) and vehicle ($p<0.01$) groups, further suggesting a disinhibition-like behaviour and an anxiolytic-like effect of BBGC exposure, though no alterations in the number of entries in the open arms.

NOR test was performed to evaluate recognition memory in the offspring. The recognition index (RI) is an index of memory retention, while the Discrimination Index (DI) allows to analyse the discrimination between the novel and familiar objects. A score higher than zero indicates more time spent with the novel object and a score lower than zero indicates more time spent with the familiar object. The results shown that male offspring from BBGC group presented a RI between 70 and 90%, similar to the control and vehicle groups. Furthermore, it was also observed that male offspring from BBGC group and both control groups had a positive DI, demonstrating that maternal glycation does not affect short-term memory **(Figure 20).**

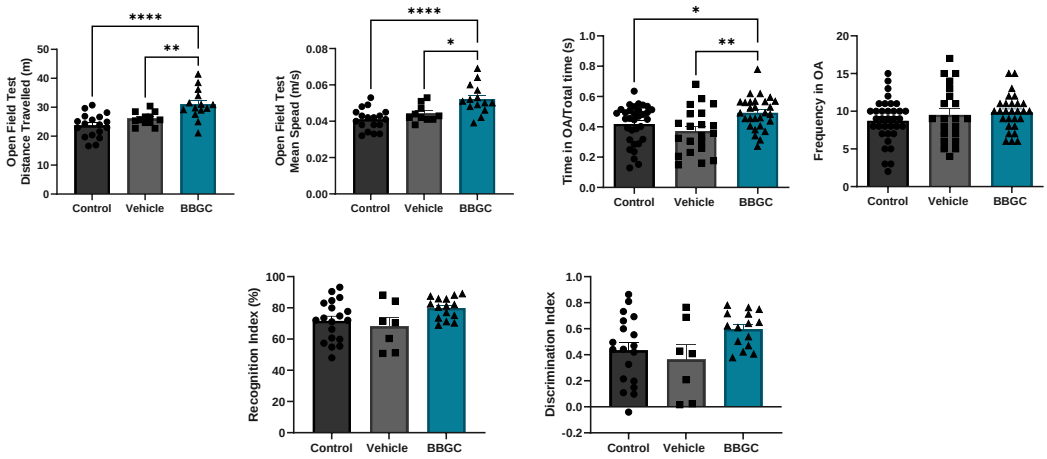

Figure 20 – Maternal glycation induced in male offspring a desinhibition-like behaviour. Offspring were tested in the OPF and EPM test at PN43 and in the NOR test at PND44. The distance travelled (m) by the offspring in the open field and the mean speed (m/s) were recorded. The time spent by the offspring in the open arms of the EPM test and the number of entries in the open arms was noted. Offspring were tested in the NOR test for the assessment of Recognition Index and of Discrimination Index. The results are shown as mean ± SEM of 22 a 35 animals per group and Kruskal-Wallis comparisons were conducted to compare among the groups. 1 symbol p<0,05; 2 symbols p<0,01; 3 symbols p<0,001; 4 symbols p<0.0001.

Female pups from BBGC group did not shown alterations on distance travelled, as well as mean speed compared with the control and vehicle groups demonstrating no alterations in locomotor activity.

Interestingly, female pups from BBGC group, similarly to males, spent more time exploring the open arms at elevated plus maze arena comparing with the vehicle group($p<0.05$), without alterations on the number of entries in the open arms demonstrating that maternal glycation induces and anxiolytic effect in the offspring.

Additionally, female pups from BBGC group had a RI between 70 and 90% and a positive score on DI identical to control and vehicle groups, showing that maternal glycation does not affect short-term memory in the offspring **(Figure 21).**

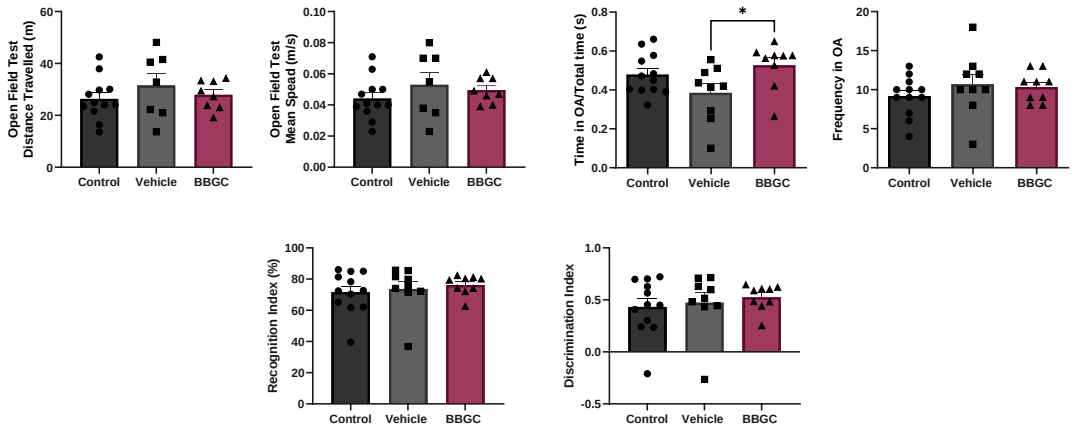

Figure 21 – Maternal glycation induced in female offspring an anxiolytic effect. Offspring were tested in the OPF and EPM test at PN43 and in the NOR test at PND44. The distance travelled (m) by the offspring in the open field and the mean speed (m/s) were recorded. The time spent by the offspring in the open arms of the EPM test and the number of entries in the open arms was recorded. Offspring were tested in the NOR test for the assessment of Recognition Index and of Discrimination Index. The results are shown as mean ± SEM of 7 a 12 animals per group and Kruskal-Wallis comparisons were conducted to compare among the groups. 1 symbol $p<0,05$.

Regarding female behavioural findings it was important to evaluate if these alterations could be due to female estrous cycle phase. Changes in reproductive hormone levels can influence behaviour, namely estrus phase are associated with significant changes in behaviour compared to other stages in the estrous cycle. Females from BBGC group were distributed through the different stages of the cycle suggesting that the cycle phase did not influence the behaviours outcomes.

Table 2 – Analyses of female estrus cycle

Estrus cycle phase	Female Offspring		
	Control	Vehicle	BBGC
Proestrus	2/12	0/7	0/9
Estrus	3/12	2/7	2/9
Metestrus	7/12	5/7	6/9
Diestrus	0/12	0/7	1/9

Maternal glycation leads to an accumulation of AGES in the offspring in a sex- and brain region-specific manner

Maternal glycation causes alterations offspring development and behaviour, but the putative underlying mechanisms are still unknown.

Since female dams were treated with a selective inhibitor of GLO1 during breastfeeding period, it was evaluated if this protein induced alterations in AGEs levels in offspring hippocampus and PFC. GLO1 is responsible for detoxifying MG to consequently avoid the formation of AGEs, such as MG-H1 and Arg-P [72].

Offspring male hippocampus from BBGC group had significantly lower levels of GLO1 compared with the control ($p<0.01$) and vehicle ($p<0.05$) groups, although catalase, which is an antioxidant enzyme, did not shown alterations. Subsequently, pups from BBGC group presented significantly higher levels of MG-H1 compared with the control ($p<0.01$) and vehicle ($p<0.01$) groups, as well as Argpyrimidine (Arg-P) ($p<0.05$), demonstrating that maternal glycation leads to a higher accumulation of AGES in male offspring hippocampus **(Figure 22).**

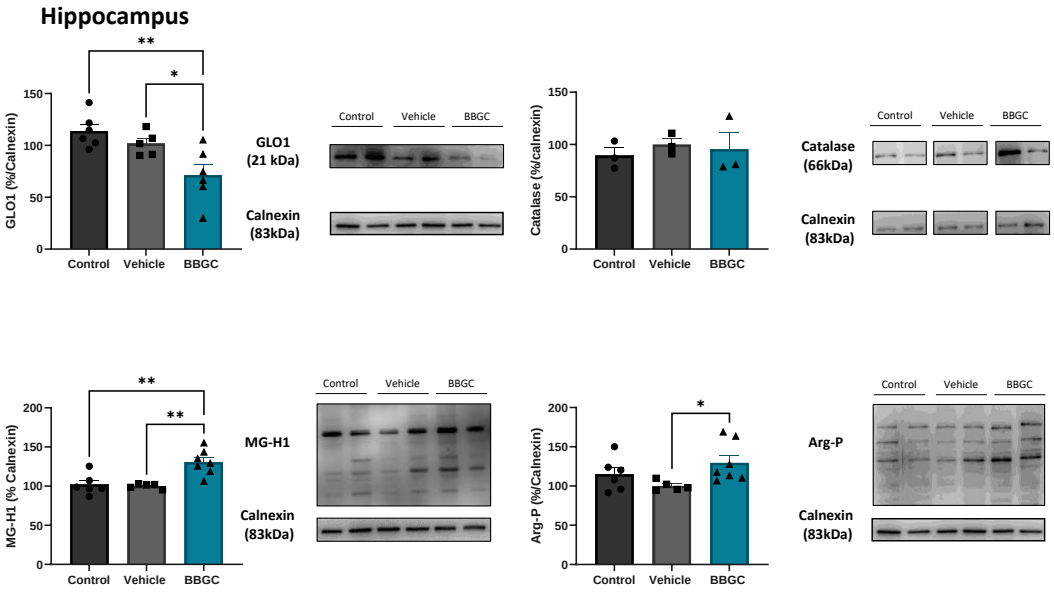

Figure 22 – Maternal glycation leads to a higher accumulation of AGES in male offspring hippocampus. GLO1 levels were shown to be significantly decreased, whereas higher levels of MG-H1 and Arg-P were found. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol $p<0,05$; 2 symbols $p<0,01$.

However, when evaluating the levels of these proteins at the offspring PFC from BBGC group, no alterations were observed at antioxidant proteins, GLO1 and catalase, and consequently in the formation of AGEs, such as MGH1 and Arg-P **(Figure 23).**

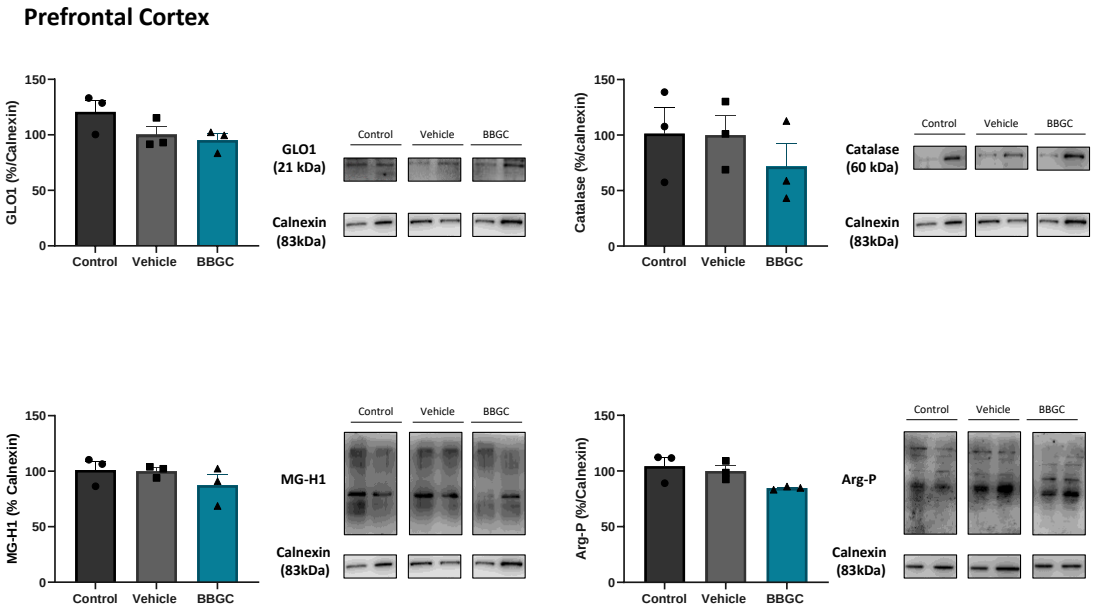

Figure 23 - Maternal glycation does not induced alterations on GLO1 pathway in male offspring PFC. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups.

Female offspring from BBGC group presented significantly lower levels of GLO1 compared with the control ($p<0.05$) and vehicle ($p<0.05$) groups. However, like in male offspring no alterations were found regarding catalase levels. Interestingly, female offspring in opposition to what was detected in males, showed no alterations in the levels of AGEs, such as MG-H1 and Arg-P, suggesting that the activation of compensatory mechanisms could be responsible to avoid their formation **(Figure 24).**

Hippocampus

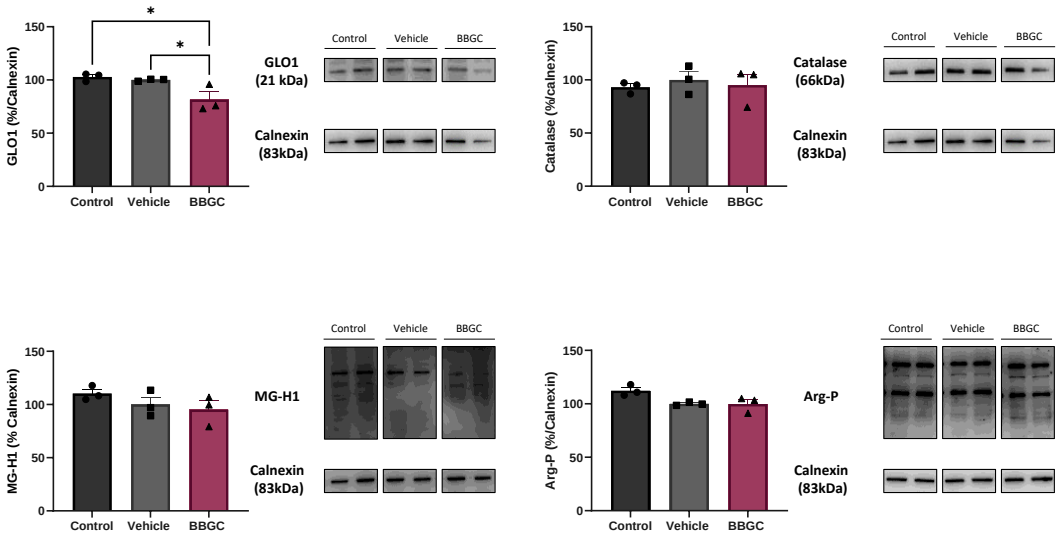

Figure 24 - Maternal glycation decreased GLO1 levels in female offspring hippocampus, although without alterations on AGEs formation. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol p<0,05.

Interestingly, when evaluating the levels of GLO1 in female offspring PFC from BBGC group, although there was a trend to have lower levels when compared to the vehicle group (p=0.0938), no alterations was observed regarding catalase levels. However, higher levels of MG-H1 were observed when compared to the control (p<0.01) and vehicle (p<0.01) groups, as well as Arg-P (p<0.05), but only versus the control group. Thus, contrarily to male offspring, female PFC apparently is more susceptible to maternal glycation **(Figure 25).**

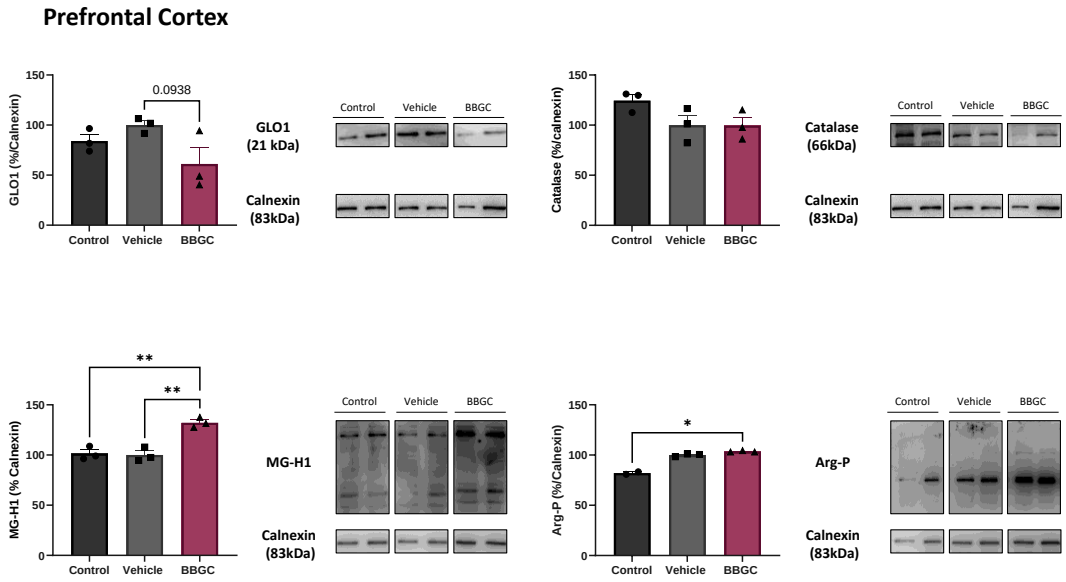

Figure 25 - Maternal glycation increased the formation of AGES on female offspring PFC. Although there was a trend on GLO1 levels, there was increased levels of MG-H1 and Arg-P in female pups from BBGC group. The results are shown as mean ± SEM and one-way ANOVA test comparisons were conducted to compare among the groups. 1 symbol p<0,05; 2 symbols p<0,01.

Maternal glycation impacts on $GABA_A$ receptor and VGAT levels in the offspring hippocampus, in a sex-dependent manner

Aiming to associate behavioural with molecular alterations, we were interested into evaluate if maternal glycation could lead to synaptic loss, and for that we evaluated two proteins located at pre and post synaptic sites – synapsin and post synaptic density protein 95 (PSD95). respectively. Synapsin is a protein involved in the regulation of neurotransmitters release at synapses. While PSD95 is a scaffold protein that is located at excitatory postsynaptic neurons.

Male offspring from BBGC group showed a trend to have higher levels of synapsin when compared to the vehicle group (p=0.0760), without alterations in the PSD95 levels.

Additionally, we evaluated the expression of proteins present at excitatory and inhibitory synapses. Male offspring hippocampus from BBGC group did not showed alterations in the levels of glutaminase, an enzyme responsible to converted glutamine to glutamate, as well as in the levels of vesicular glutamate transporter (vGLUT1) responsible to transport glutamate in presynaptic neurons, suggesting that maternal glycation does not cause alterations at excitatory level.

Several studies have been demonstrating that glycation potentiates the activation of $GABA_A$ receptors [167]. Interestingly, in male offspring hippocampus from BBGC it was found

significantly higher levels of $GABA_A$ receptor compared with the control ($p<0.001$) and vehicle ($p<0.05$) groups. In addition to that, the vesicular GABA transporter (vGAT), located at presynaptic neuron, also presented a significantly higher expression compared with control ($p<0.01$) and vehicle ($p<0.01$) groups. These results suggest that maternal glycation may cause an imbalance in excitatory/inhibitory synapses in hippocampus of male offspring **(Figure 26).**

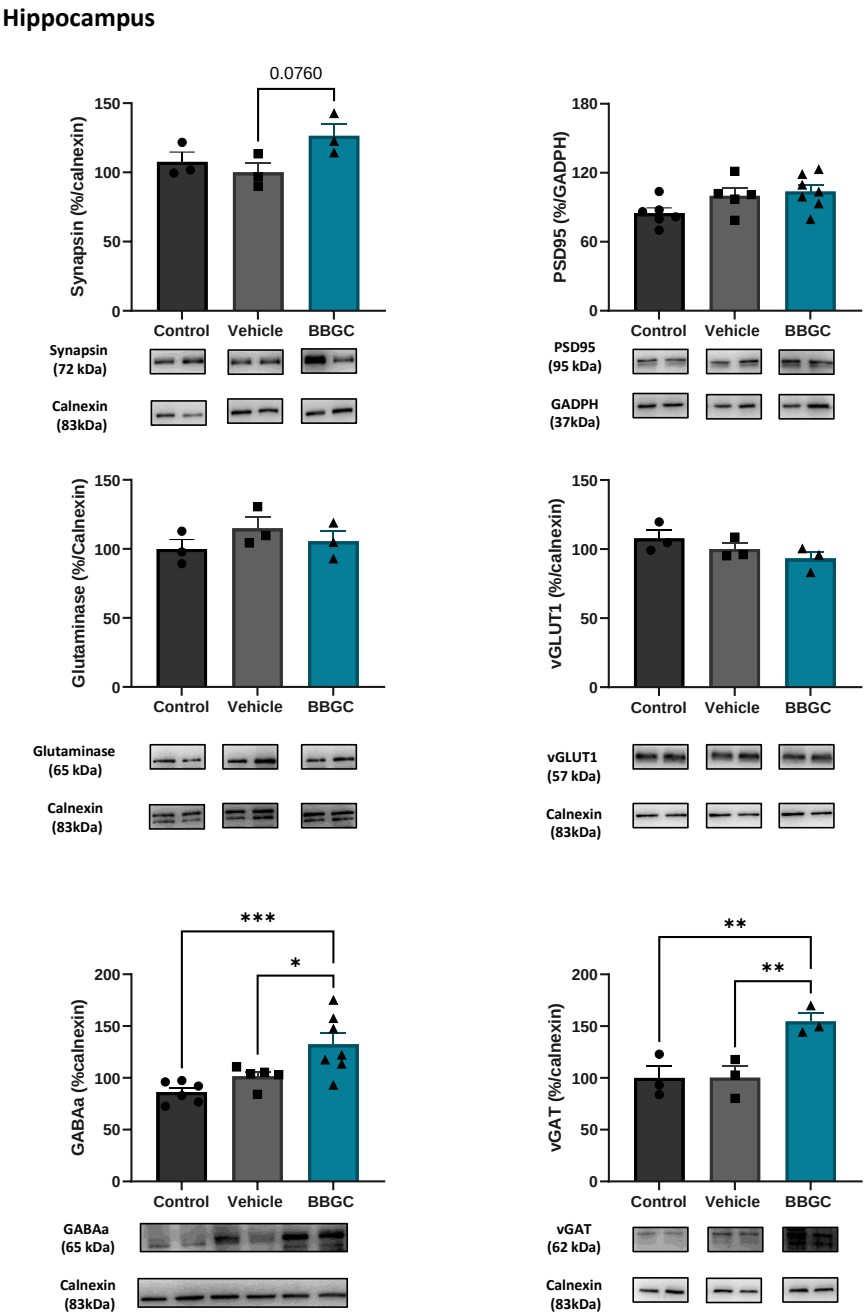

Figure 26 – Maternal glycation increased GABA receptor and vGAT levels in male offspring hippocampus. There were no alterations on proteins related with excitatory synapses. However, there was significantly higher levels of GABA receptor and GABA vesicular transporter (vGAT) when compared

to vehicle and control groups. The results are shown as mean ± SEM and one-way ANOVA test was conducted to compare among the groups. 1 symbol p<0,05; 2 symbols p<0,01; 3 symbols p<0,001.

Interestingly, these alterations were not observed in the PFC from male offspring whose mothers were treated with BBGC, since there were not observed changes in synapsin and PSD95 levels. Moreover, glutaminase and vGLUT1 protein levels also did not showed alterations. Furthermore, the levels of GABA and its vesicular transporter, vGAT also remained unaltered. These results suggest that maternal glycation affects the brain in a region-specific manner, in which the hippocampus is more vulnerable to maternal glycation comparing to PFC **(Figure 27).**

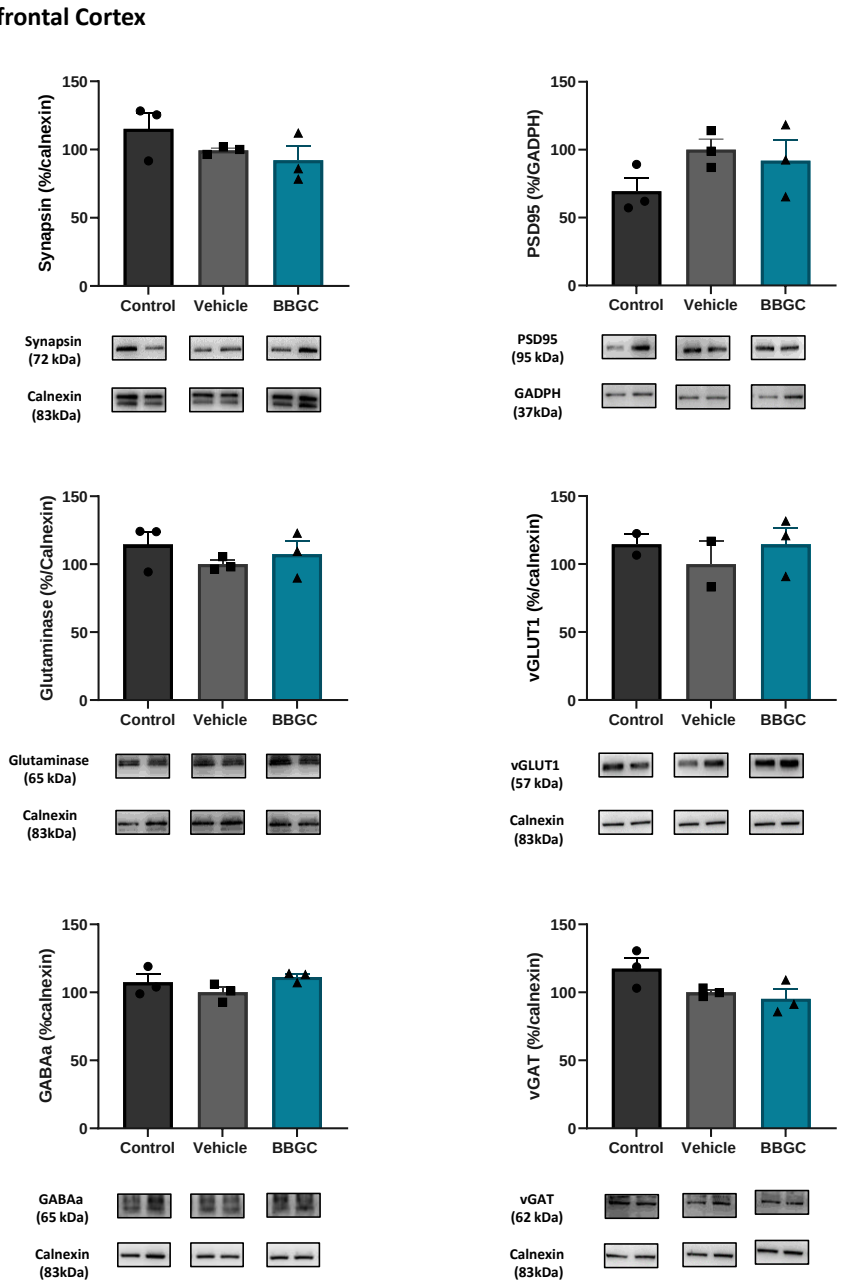

Figure 27 - Maternal glycation does not induced alterations on key proteins presented in glutamatergic and GABAergic synapses in male offspring PFC. The results are shown as mean ± SEM and one-way ANOVA test comparisons were conducted to compare among the groups.

Regarding female offspring from BBGC group, the levels of synapsin at hippocampus were significantly lower compared with the vehicle group ($p<0.01$). At postsynaptic level, PSD95 levels were also shown to be significantly decreased compared with control ($p<0.05$) and vehicle ($p<0.01$) groups.

Concerning the levels of proteins involved in glutamatergic synapse, glutaminase levels at hippocampus were significantly lower compared with the control ($p<0.05$) and vehicle ($p<0.05$) groups, although without alterations on vGLUT1 transporter content.

Nevertheless, the levels of $GABA_A$ receptor were significantly increased when compared with control ($p<0.01$) and vehicle groups ($p<0.01$). Interestingly, when evaluated the content of vGAT which, as mentioned, is responsible to transport GABA into vesicles at presynaptic neurons, there was not observed alterations when compared with both control and vehicle groups **(Figure 28).**

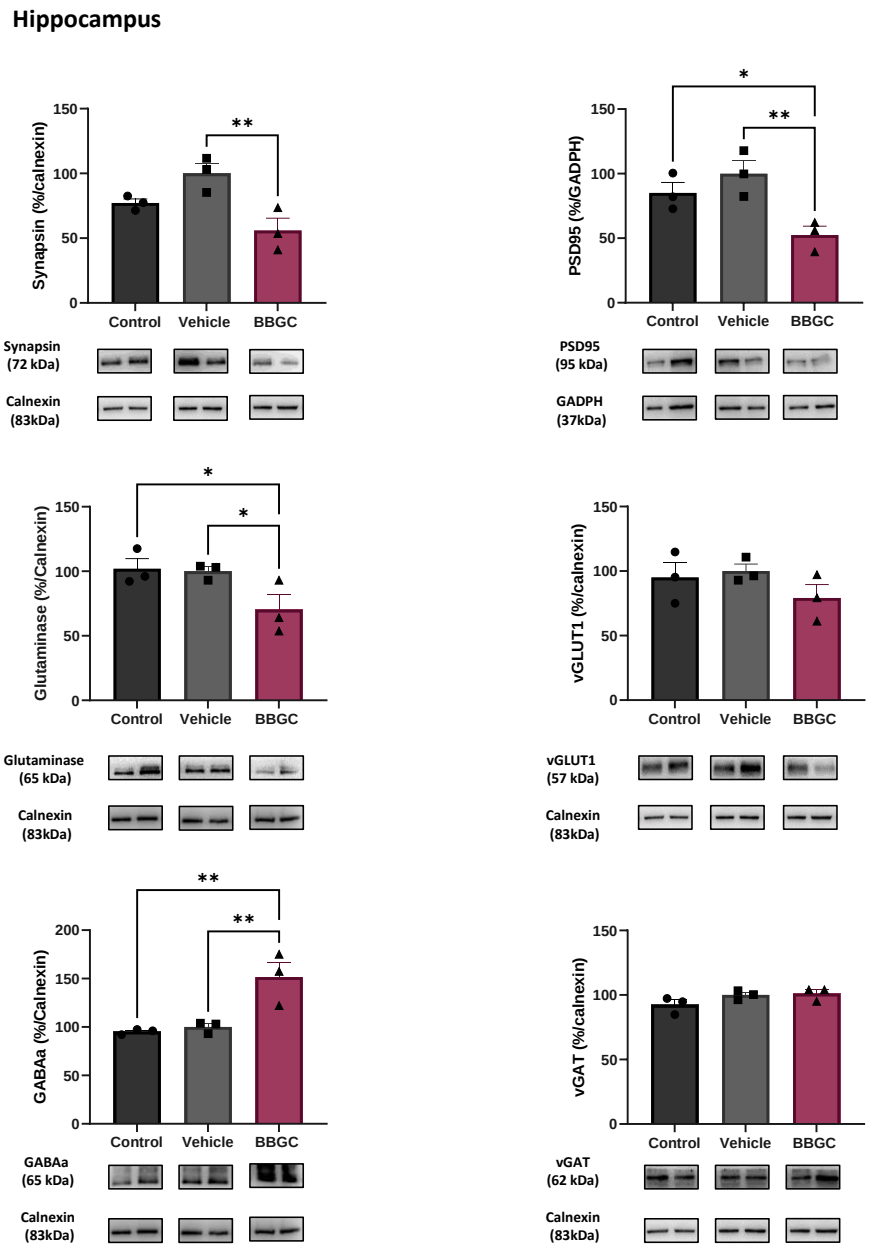

Figure 28 - Maternal glycation decreased synapsin, PSD95 and glutaminase levels and increases $GABA_A$ receptor levels in female offspring hippocampus. It was observed a significant decreased of synapsin and PSD95 proteins when compared to control and vehicle groups, as well as glutaminase protein levels while the levels of $GABA_A$ receptor were higher. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol p<0,05; 2 symbols p<0,01.

PFC from female offspring were observed to have similar alterations with hippocampus. Although synapsin levels was showed differences between control and vehicle groups

($p<0.01$), this effect was more pronounced in female offspring from BBGC group when compared with the vehicle group ($p<0.001$). In addition to that it was also observed lower levels of PSD95 ($p<0.05$) compared with the control group. Furthermore, glutaminase and vGLUT1 levels were shown to have lower levels but only when compared with the control ($p<0.05$) group. Additionally, no alterations were observed between control and vehicle groups.

However, in the opposite to what it was observed in female hippocampus, the levels of $GABA_A$ receptor remained unaltered. Furthermore, the levels of vGAT were significantly higher when compared with the vehicle group ($p<0.05$), although this effect was also observed when compared the control with the vehicle group ($p<0.05$) **(Figure 29).**

Prefrontal Cortex

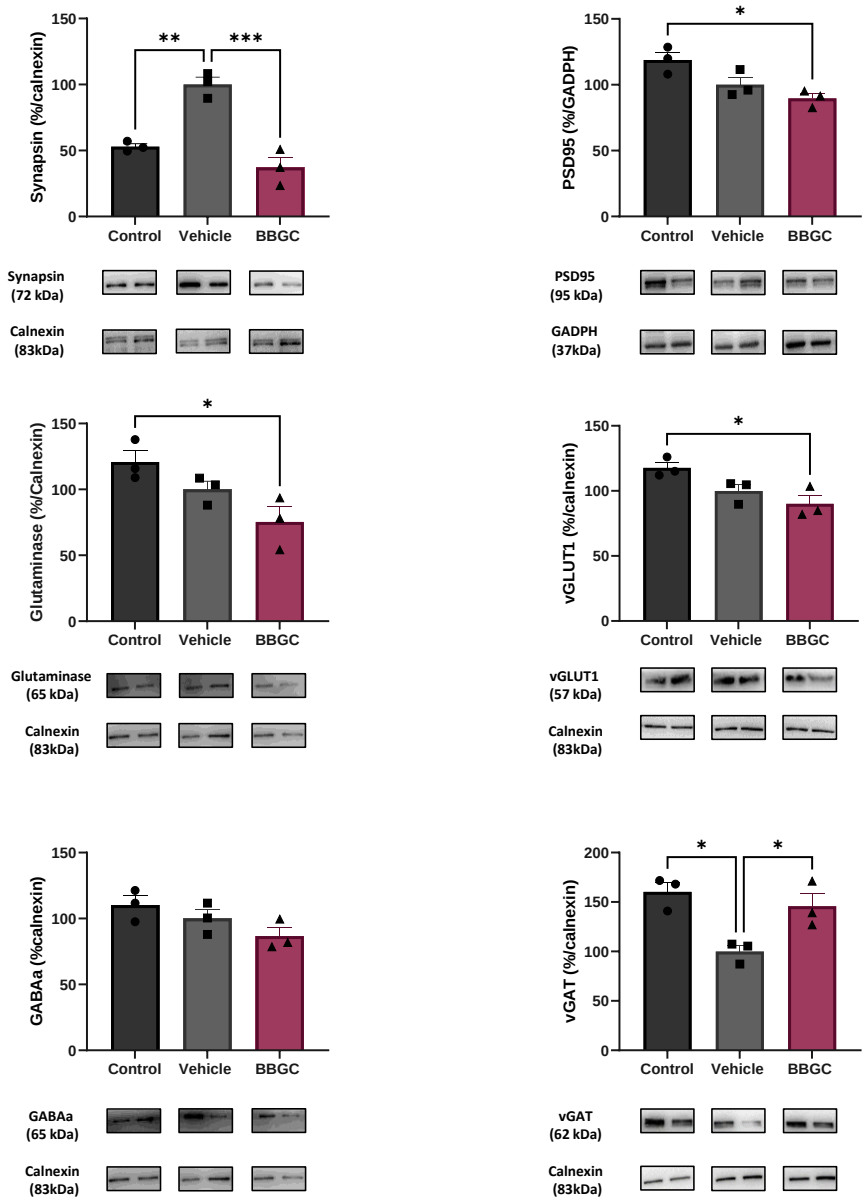

Figure 29 – Maternal glycation decreased synapsin and increases vGAT levels in female offspring PFC. Female pups from BBGC group were shown to have lower synapsin levels compared with the vehicle group. In the opposite, maternal glycation was shown to significantly decreased the levels of PSD95, glutaminase and vGLUT1 compared to the control group. Furthermore, maternal glycation was shown to increase vGAT levels compared with the vehicle group. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol p<0,05; 2 symbols p<0,01; 3 symbols p<0,001.

Maternal glycation does not induce alterations in the levels of NeuN neuronal markers in the offspring brain

Considering it was observed synaptic alterations in male and female offspring at different brain regions, the next aim was to evaluate if maternal glycation induced neuronal loss. Thus, NeuN, neuronal marker was used as indicative of neuronal cell loss.

Male offspring from BBGC group did not shown alterations on NeuN levels in both hippocampus and PFC regions **(Figure 30).**

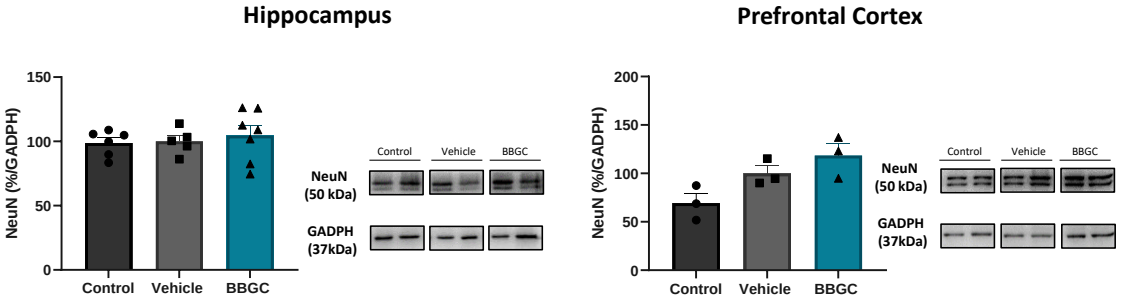

Figure 30 – Maternal glycation did not cause neuronal loss in male offspring hippocampus and PFC. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups.

Furthermore, female from BBGC group, also did not shown alterations on NeuN levels in both hippocampus and PFC regions, suggesting that maternal glycation does not cause neuronal loss **(Figure 31).**

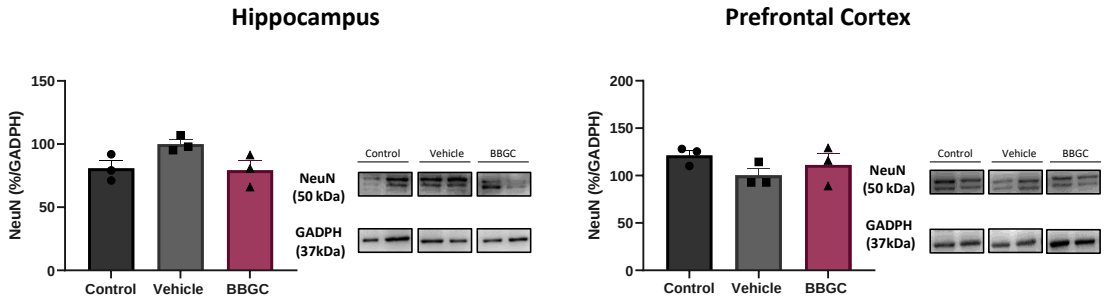

Figure 31 – Maternal glycation did not cause neuronal loss in female offspring hippocampus and PFC. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups.

II. Impact of maternal glycation in the brain of overweight offspring induced by postnatal hyperphagia

The next (and second) aim of this work was to evaluate if maternal glycation could have an impact in neurodevelopment and behaviour of male offspring that was exposed to an hyperphagic environment during postnatal period. As described, this was achieved through a small litter protocol, where the number of pups were reduced from 8 to 3, at PND3.

Maternal glycation causes alterations on milk composition in conditions of postnatal hyperphagia

Postnatal hyperphagia could influence the composition of milk and consequently affect the development of the new-born.

The levels of triglycerides and total antioxidant capacity was evaluated in milk samples analysis. Interestingly, it was observed that in conditions of postnatal hyperphagia, maternal glycation (SL+BBGC) induce a significantly reduction of triglycerides content when compared with the control ($p<0.05$). Moreover, maternal glycation for itself was shown to induce trend in reduction of triglycerides levels when compared with small litter group (SL) ($p=0.0817$).

Additionally, total antioxidant capacity was observed to have significantly lower levels in a context of hyperphagia (SL) and maternal glycation together with postnatal hyperphagia (SL+BBGC) when compared with the control group ($p<0.05$) suggesting that postnatal hyperphagia induce a reduction of total antioxidant capacity in milk by itself **(Figure 32).**

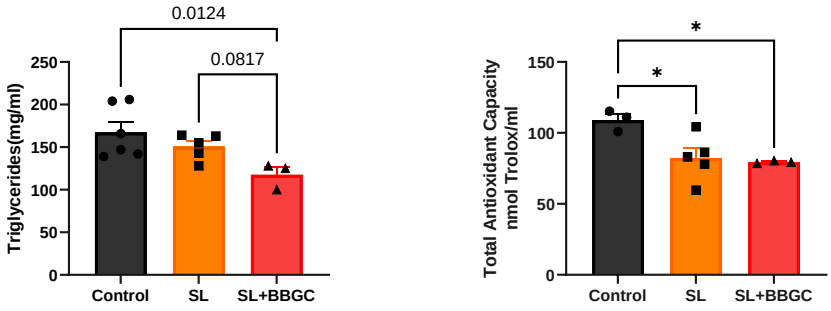

Figure 32 – Postnatal hyperphagia together with maternal glycation caused alterations on milk composition. Milk from dams treated with BBGC presented lower levels of triglycerides compared with the control and SL group. Moreover, there was a significant reduction of total antioxidant capacity levels in both groups of postnatal hyperphagia – SL and SL+BBGC – compared with the control group. The results are shown as mean ± SEM of 3 a 5 animals per group and one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol $p<0,05$.

Maternal glycation impacts on offspring body weight and metabolic state in conditions of postnatal hyperphagia

Several studies have been demonstrated that postnatal hyperphagia could increase the risk of obesity in life [19-20]. Indeed, it was observed that SL presented a significantly higher body weight gain compared with the control, from PND4 ($p<0.05$) during lactation period until weaning period (PND21) ($p<0.001$). This effect was maintained after weaning period where SL offspring showed to have a higher body weight in comparison to the control group at PND45 ($p<0.01$).

Interestingly, the offspring whose dams were treated with BBGC – SL+BBGC- showed to have significantly lower body weight during the first weeks of breastfeeding, PND4 ($p<0.001$), PND7 ($p<0.001$), PND14 ($p<0.01$) and on weaning day ($p<0.05$) in comparison with the SL group. After weaning, it was also observed that SL+BBGC offspring presented a significant lower body weight, at PND35 ($p<0.05$) when compared with SL group **(Figure 33).**

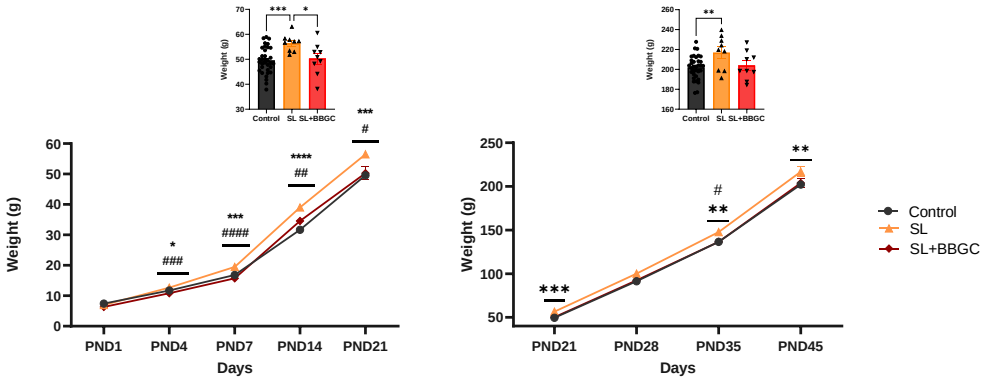

Figure 33 – Postnatal hyperphagia induced offspring overweight that were lost when exposed to maternal glycation. The results are shown as mean ± SEM of 9 to 20 animals per group and one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol $p<0,05$; 2 symbols $p<0,01$; 3 symbols $p<0,001$; 4 symbols $p<0.0001$.

Nevertheless, no alterations on insulin sensitivity were observed, during the insulin tolerance test, as observed by the AUC and the plasma glucose disappearance rate (kITT). However, offspring from SL group had significantly lower plasma insulin levels when compared to the control group ($p<0.01$), which were maintained in the SL+BBGC group ($p<0.05$). Furthermore, despite SL did not cause alterations on triglycerides levels, exposure to BBGC caused an increase of their plasma levels ($p<0.01$) **(Figure 34).**

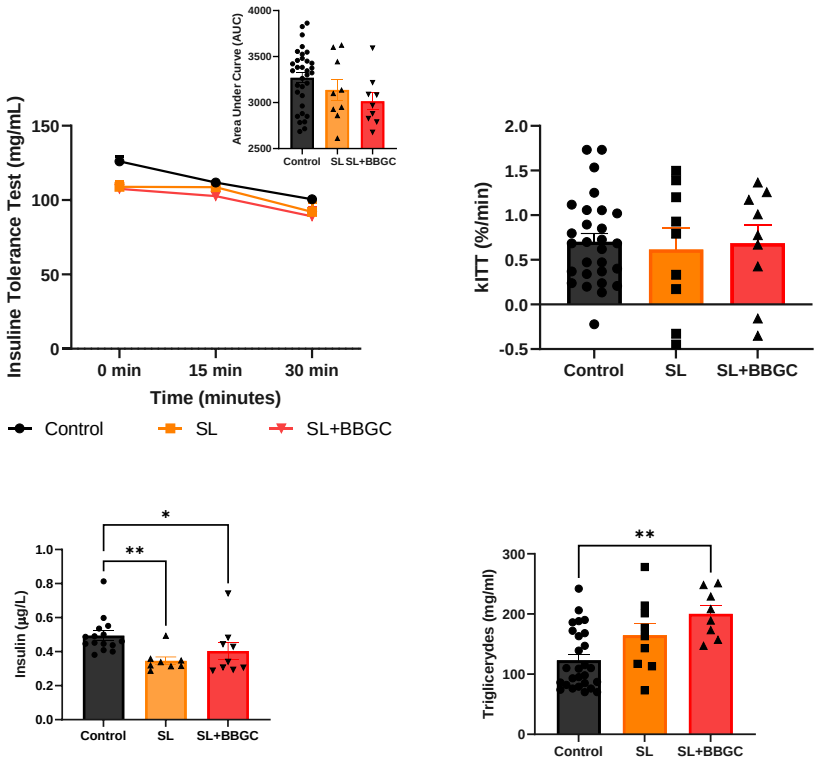

Figure 34 – Postnatal hyperphagia and glycation affected insulin response in male offspring. The results are shown as mean ± SEM of 9 a 20 animals per group and Kruskal-Wallis or one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol p<0,05; 2 symbols p<0,01.

Insulin signalling in CNS is known to be involved in the regulation of energy balance and peripheral glucose homeostasis. In the hippocampus of SL offspring, the levels of total insulin receptor were significantly lower when compared with the control group (p<0.05), an effect that was maintained after exposure to glycation (p=0.0686). In PFC the effects of SL were not observed, but SL+BBGC group showed a trend to have lower levels of total insulin receptor when compared to SL group (p=0.06) **(Figure 35).**

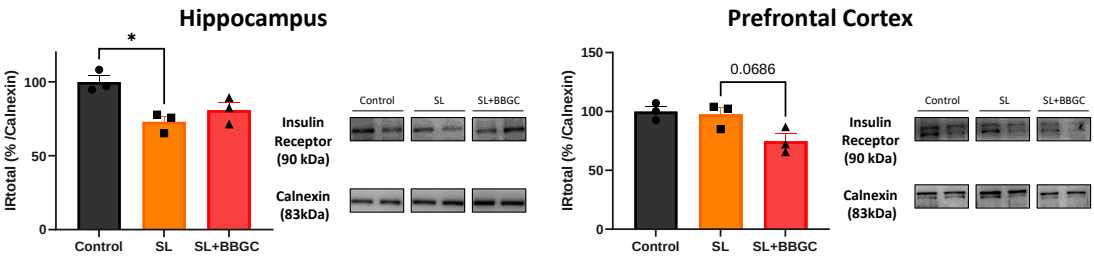

Figure 35 - Postnatal hyperphagia modulated insulin activity in hippocampus male offspring. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol p<0,05.

Maternal glycation does not impact on neurodevelopment of overweight offspring

Since the aim of this work was to evaluate if maternal glycation impacts the neurodevelopment of the offspring exposed to postnatal hyperphagia, all the groups were subjected to several developmental tests as already described.

Offspring were tested for their latencies to home-bedding on the nest seeking test. SL+BBGC pups demonstrate an anticipation on this behaviour compared to the control group, but only on the first day of testing ($p<0.01$). Cliff aversion test was performed to evaluate the development of vestibular system, but no alterations were observed. Moreover, maternal glycation associated with postnatal hyperphagia – SL+BBGC group – caused a significant impairment in locomotion performance when compared with the control group, at PND 12 ($p<0.01$) and PND13 ($p<0.01$). On the other hand, no significant alterations in the wire suspension test were observed, although offspring from SL group shown a trend to present a worse performance **(Figure 36).**

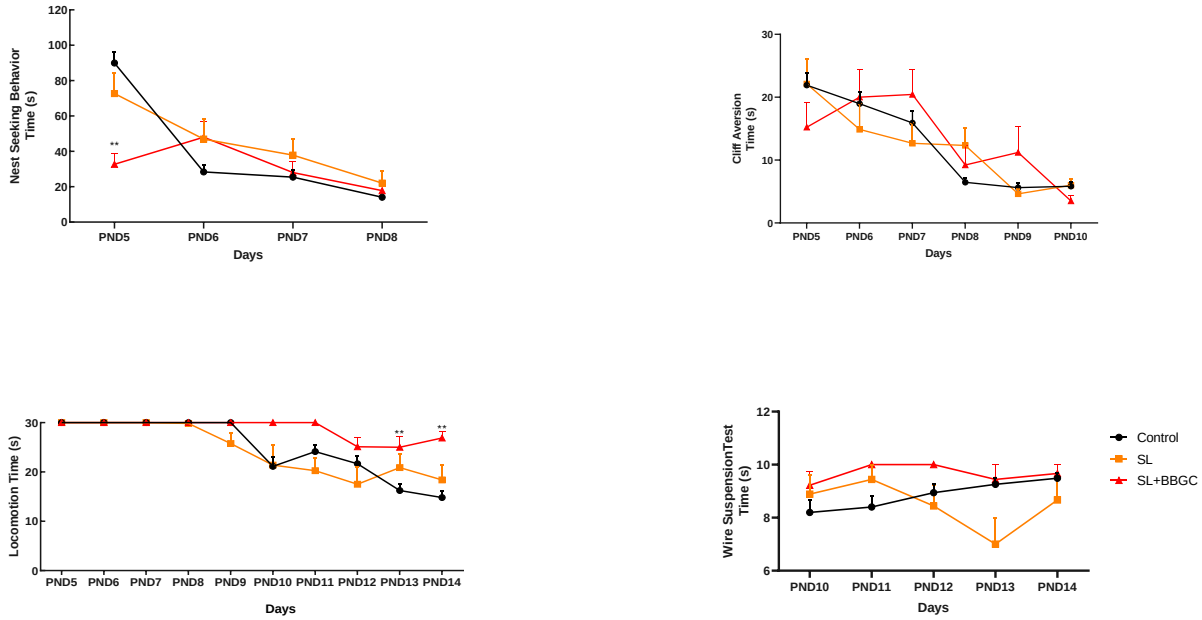

Figure 36 – Maternal glycation, in conditions of hyperphagia affected locomotor activity of male offspring. The results are shown as mean ± SEM of 9 a 20 animals per group and Kruskal-Wallis test comparisons were conducted to compare among the groups. 1 symbol $p<0,05$; 2 symbols $p<0,01$.

Offspring from SL and SL+BBGC groups, did not show alterations in the development of auditory ability, and in eye opening day, comparing to the control and SL groups **(Figure 37).**

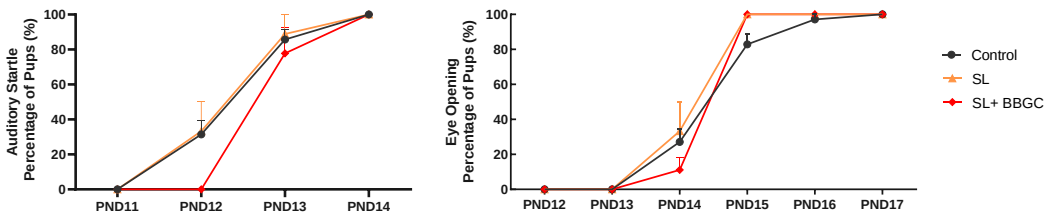

Figure 37 - Maternal glycation does not induced alterations of auditory startle response and eye openning day in hyperphagic rats. The results are shown as mean ± SEM of 9 a 20 animals per group and Kruskal-Wallis comparisons were conducted to compare among the groups.

Postnatal hyperphagia induced an anxious-like behaviour in the offspring

On PND43 and PND44 offspring were tested in EPM and NOR tests to evaluate anxious-like behaviours and recognition memory, respectively. Since animal performance in these tests are dependent on the absence of locomotor problems, pups were tested in OPF in which each animal circulated freely in an open arena for 10 minutes. Regarding the distance travelled and the mean speed, no statistically differences were observed between the experimental groups, indicating no alterations in locomotor activity.

Concerning EPM test, animals were left for 5 minutes in an elevated plus platform to freely explore the open and/or closed arms. Offspring from SL group spent less time in the open arms comparing with the control group ($p<0.05$), without no alterations in the number of entries in the open arms. This behaviour was not changed by exposure to maternal glycation.

On the other hand, when evaluated the NOR test, offspring from SL and SL+BBGC groups do not shown alteration on recognition index, an indicator of memory retention, as well as on discrimination index, demonstrating that postnatal hyperphagia associated or not with maternal glycation does not affect short-memory process at adolescence period **(Figure 38).**

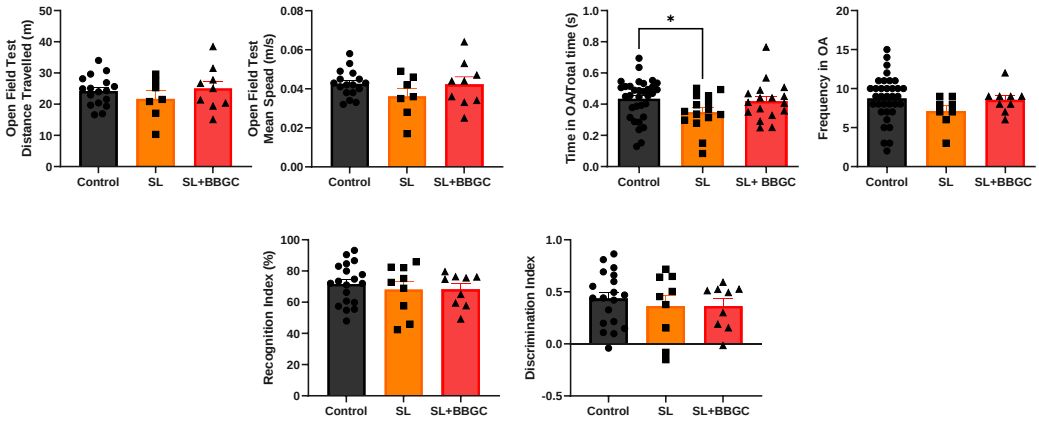

Figure 38 – Postnatal hyperphagia induced an anxious-like behaviour on male offspring. Offspring were tested in the OPF and EPM test at PN43 and in the NOR test at PND44. The distance travelled (m) by the offspring in the open field and the mean speed (m/s) were recorded. The time spent by the offspring in the open arms of the EPM test and the number of entries in the open arms was recorded. Offspring were tested in the NOR test for the assessment of Recognition Index and of Discrimination Index. The results are shown as mean ± SEM of 7 a 13 animals per group and Kruskal-Wallis comparisons were conducted to compare among the groups. 1 symbol p<0,05.

<u>Postnatal hyperphagia triggers mechanisms of protections in the hippocampus that are not observed in PFC</u>

Although were not observed significant alterations on offspring development, postnatal hyperphagia induced an anxious-like behaviour, despite the neuronal alterations that influences this behaviour are still unknown. Thus, we aimed studying if postnatal hyperphagia causes alterations in antioxidant defences, and consequently leads to an accumulation of AGES in hippocampus and PFC regions.

In the hippocampus of the offspring from SL group, it was observed a significantly increased in the levels of GLO1 and catalase proteins when compared with the control group ($p<0.05$; $p<0.01$, respectively). These changes were accompanied with no alterations on MG-H1 and Arg-P levels when compared with the control group.

Interestingly, the increase of GLO-1 and catalase levels were lost when overweight offspring was exposed to maternal glycation, being observed a significant decreased of GLO1 and catalase levels compared with SL group ($p<0.05$; $p<0.05$, respectively), although no alterations were observed in MG-H1 and Arg-P levels **(Figure 39).**

Hippocampus

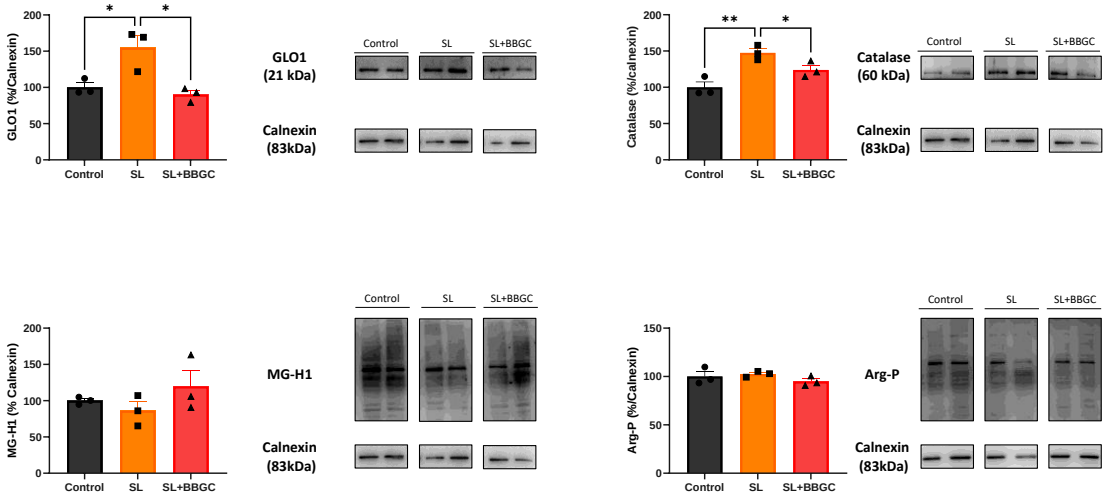

Figure 39 – Offspring exposed to postnatal hyperphagia activated mechanisms of protection on hippocampus that are lost when exposed to maternal glycation. Offspring from SL group was shown to have higher levels of GLO1 and catalase proteins when compared with the control group. Interestingly this effect was lost when offspring whose dams were treated with BBGC. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol $p<0,05$; 2 symbols $p<0,01$.

Regarding the results observed in PFC, no alterations were observed on the levels of GLO1, catalase and MG-H1 in pups from SL and SL+BBGC groups **(Figure 40).**

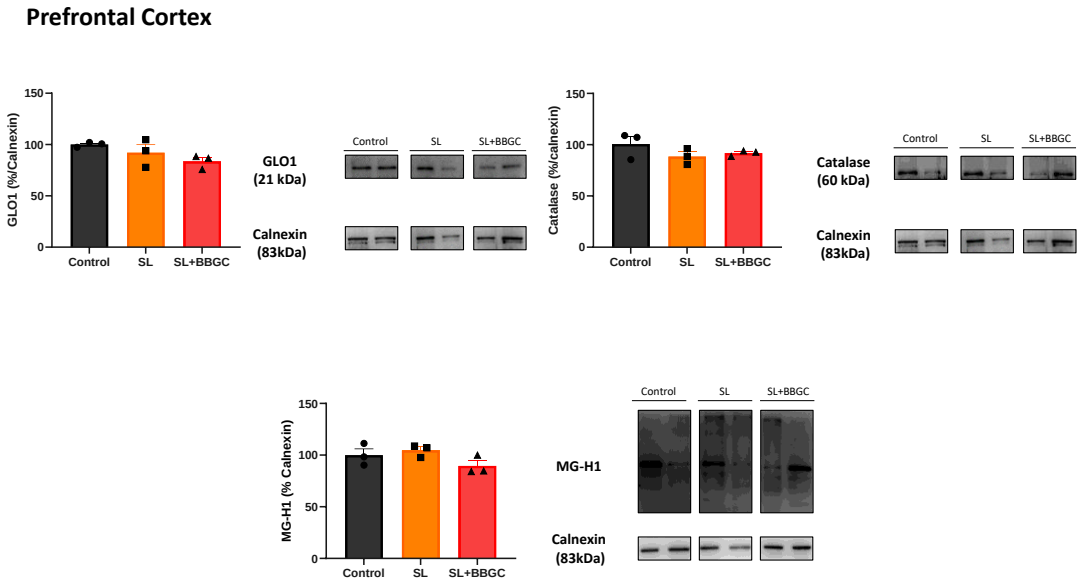

Figure 40 - Postnatal hyperphagia does not caused alterations on AGEs accumulation on offspring PFC. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups.

<u>Postnatal hyperphagia increases synaptic marker content in offspring hippocampus but not in the PFC</u>

The next aim was to evaluate whether postnatal hyperphagia induces changes at synaptic levels, as well as the balance between glutamatergic and GABAergic synapses and if these alterations occur in specific brain regions.

In hippocampus, offspring from SL group shown to have higher levels of synapsin when compared to the control group ($p<0.05$). Moreover, at postsynaptic level, PSD95 shown to present higher levels compared to the control group ($p<0.05$). Regarding, excitatory synapse, postnatal hyperphagia (SL) shown to induced a trend to have higher glutaminase levels when compared with the control group ($p=0.0975$) without no alterations on its vesicular transporter, vGLUT1 content. Furthermore, no alterations on GABA receptor and vGAT transporter were observed.

Concerning offspring from BBGC group – SL+BBGC, synapsin levels were increased when compared with SL group ($p<0.05$), and a similar effect was observed at postsynaptic cell through the increase levels of PSD95 ($p<0.01$), however, no alterations on glutaminase and vGLTU1 or neither on $GABA_A$ receptor and vGAT were observed **(Figure 41).**

Hippocampus

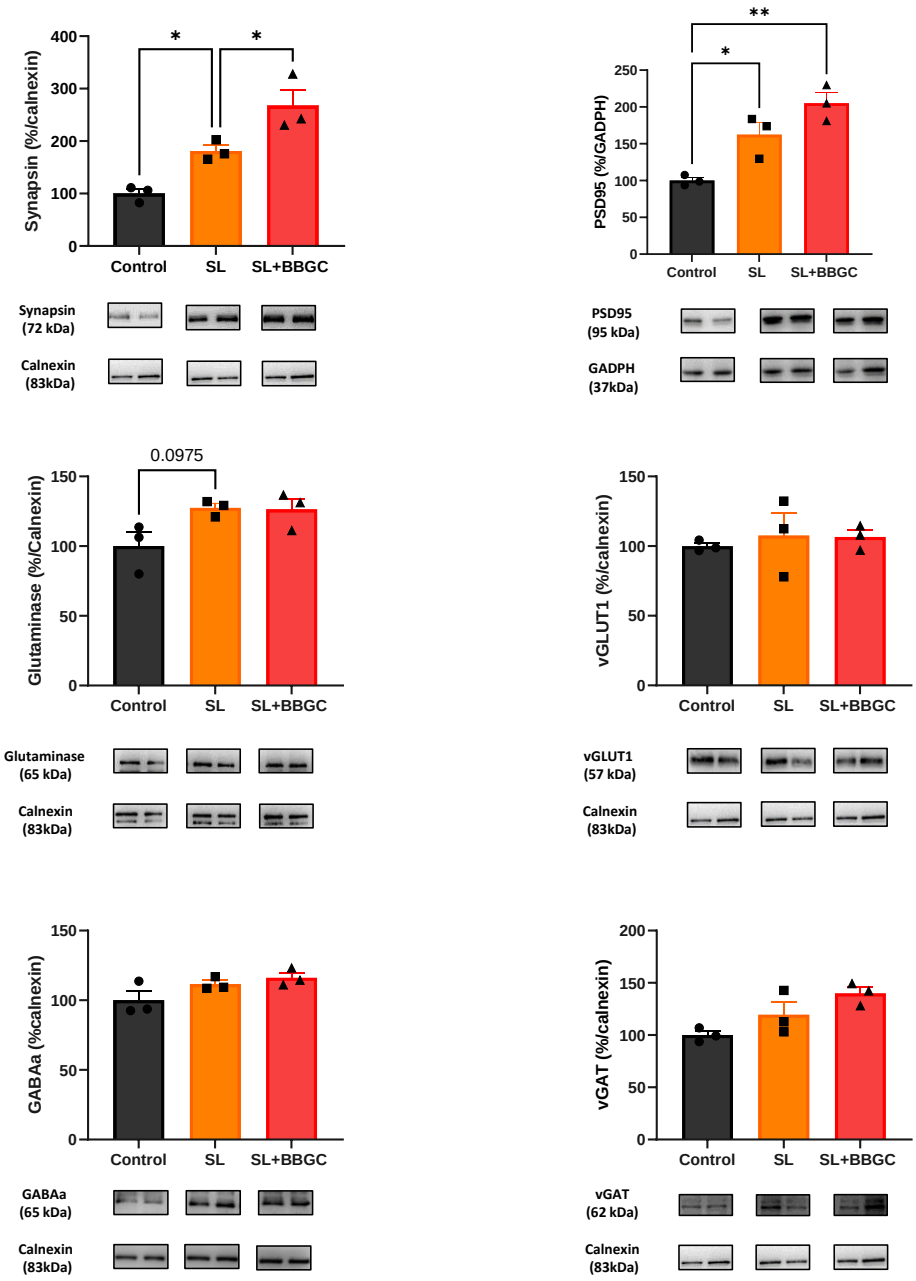

Figure 41 - Postnatal hyperphagia increased the levels of synapsin and PSD95 that were more pronounced in conditions of maternal glycation in offspring hippocampus. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol p<0,05; 2 symbols p<0,01.

Concerning PFC region, offspring from SL group was shown a trend to have lower levels of synapsin whencompared to control group (p=0.0761), accompanied without no alterations in

PSD95 levels. Furthermore, there were not observed alterations at the level of glutamatergic synapse, evaluated through glutaminase and vGLUT1 proteins. Regarding GABAergic synapse, offspring from SL group presents a trend to have higher levels of $GABA_A$ receptor when compared with the control group ($p=0.0517$), although without alteration on its vesicular transporter, vGAT content.

Interestingly, when exposed to maternal glycation, offspring from SL group – SL+BBGC – maintained a trend to have lower levels of synapsin ($p=0.0682$) compared with the control without no differences to SL group. Moreover, offspring from SL+BBGC group presented significantly lower levels of glutamate transporter (vGLUT1) when compared with the SL group ($p<0.05$), as well as a trend to increased GABA receptor levels when compared with the SL group ($p=0.1575$) **(Figure 42).**

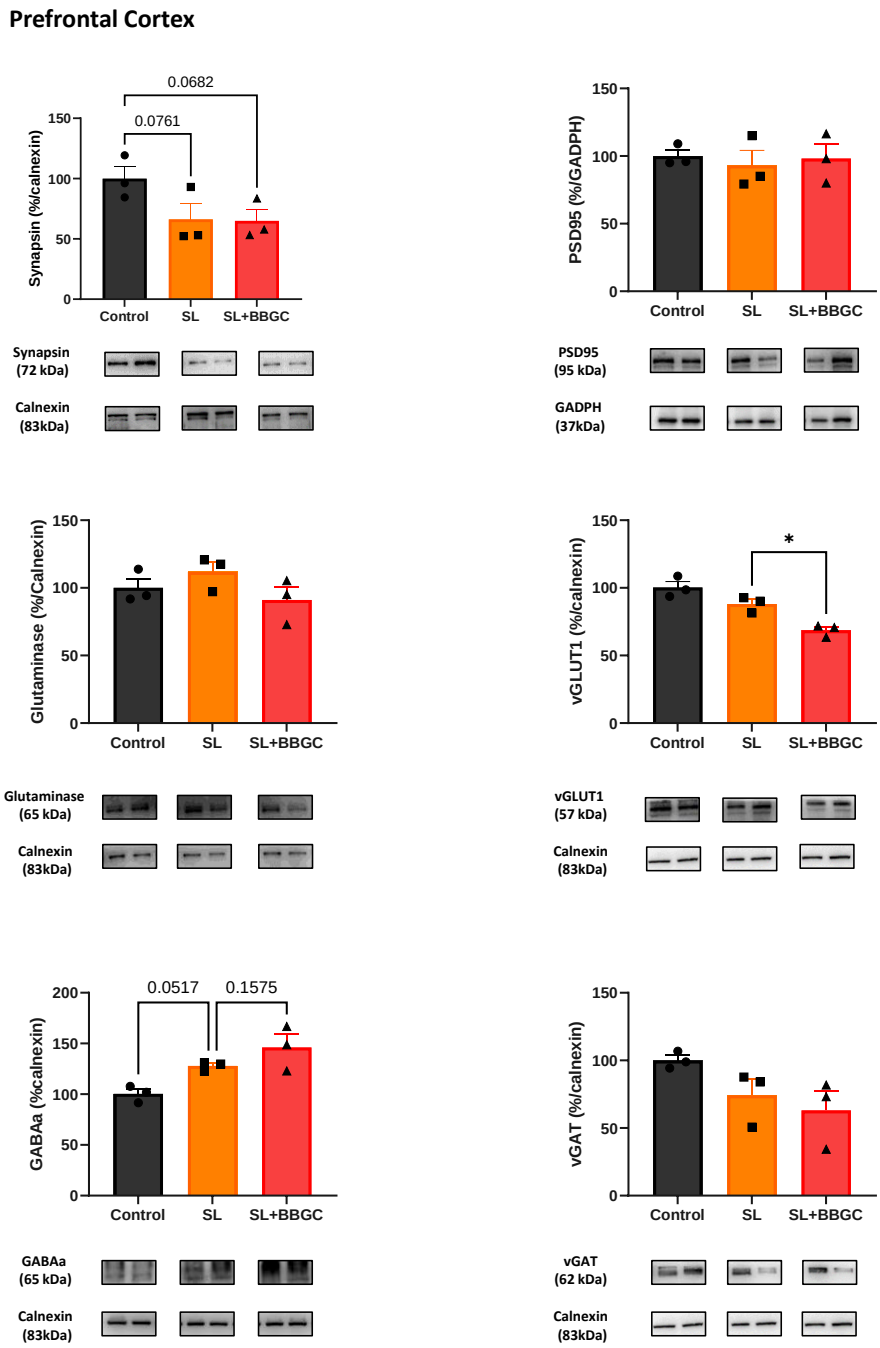

Figure 42 – Maternal glycation under condition of hyperphagia decreased vGLUT1 on offspring PFC. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups. 1 symbol p<0,05.

<u>Postnatal hyperphagia does not cause neuronal loss in offspring hippocampus and PFC</u>

In order to disclose if postnatal hyperphagia could affect offspring neuronal loss in hippocampus and PFC regions, the levels of NeuN were evaluated.

In the offspring hippocampus from SL group no alterations were observed in NeuN in hippocampus and PFC. Furthermore, maternal glycation in conditions of postnatal hyperphagia have shown to not induce alterations in this marker **(Figure 43).**

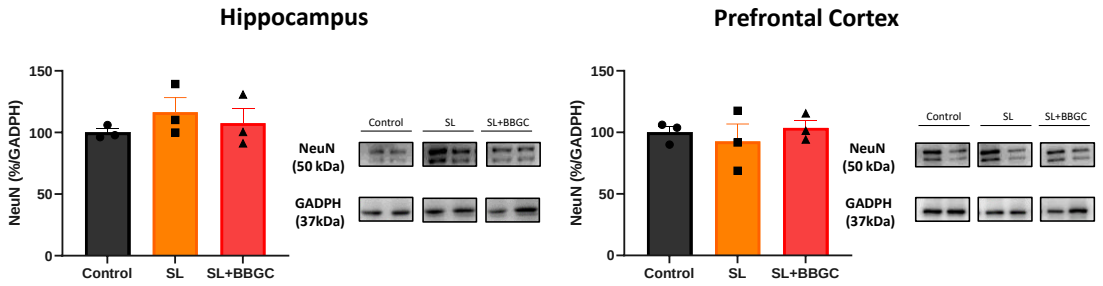

Figure 43 – Postnatal hyperphagia and maternal glycation did not induce neuronal loss offspring hippocampus and PFC. The results are shown as mean ± SEM and one-way ANOVA comparisons were conducted to compare among the groups.

Discussion

According to DoaHD, perinatal stressors and nutritional cues can shape neuronal plasticity during development and consequently increases the risk of chronic disease later in life [11, 172]. In recent years, the higher consumption of highly processed and formulated foods has led to a profound modification in the population's dietary and food preferences. In turn, Westernized diets induces a higher intake of dietary AGEs that impact metabolic status of an individual and may also contribute to the onset of neurobehavioral alterations in the progeny.

Thus, in this work we explored how maternal glycation per se, and maternal glycation in the context of hyperphagia-induced obesity, could impact offspring neurodevelopment and behaviour. Furthermore, we disclose how these conditions may modulate the levels of key protein involved in neurometabolism at hippocampus and PFC during lactation period and its behavioural consequences at the adolescence period.

I. Effect of maternal glycation on offspring brain and sex-specificities

Milk is a major source of nutrition for infant's development and growth; however, its composition is not uniform in its nutritional content and quantity, being dependent on several factors [11]. Indeed, maternal diet and metabolic status are crucial in its composition. Our results demonstrate that maternal glycation decreases the levels of triglycerides and its total antioxidant capacity. Human studies have reported that the levels of total fat circulation in milk can change depending on the maternal diet [46]. Furthermore, higher circulation of AGEs could also be linked to lower levels of antioxidant defences [72].

Concerning the metabolic state of male offspring no alterations were observed in body weight during and after the breastfeeding period. In fact, some studies, have reported that body weight and/or body mass index did not change in response to a diet rich in AGEs. However, it is important to take into consideration the duration of the diet. Several studies have demonstrated that a long-term (6 weeks) diet could result in body weight gain, whereas a short-term (3 weeks) diet intake does not significantly affect weight gain [92-93]. Since dams were treated with BBGC for only six days, after birth, the short-term treatment could be an explanation for the fact that no alterations were observed in offspring body weight. Moreover, they were fed a standard diet during all the experimental period, so no major changes on body weight were envisioned.

Regarding metabolic status male offspring from BBGC, had a significant decay in glucose decay rate – kITT – although no alterations on insulin plasmatic levels, suggesting that maternal

glycation can cause a reduction in insulin sensitivity. In fact, studies in rodent models have demonstrated that chronic AGEs diet consumption could affect insulin sensitivity and signalling, leading to insulin resistance which increases the risk of type 2 diabetes development [173]. Moreover, offspring exposed to maternal glycation induced by MG consumption develop a similar phenotype being hyperinsulinemic and glucose intolerant, suggesting defective insulin sensitivity accompanied by a decreased function of β-pancreatic cells and, indicating a higher susceptibility for the development of a type 2 diabetes phenotype [52].

Regarding female offspring, and similarly to males, BBGC treatment did not affect body weight. Although, plasmatic insulin levels were decreased, the same effect was observed in the offspring whose dams were treated with DMSO – vehicle group, suggesting a vehicle effect rather than a BBGC effect. Thus, this suggest that female offspring seems to be protected from alterations in insulin sensitivity, which is consistent with studies that showed that females are less prone to develop insulin resistance due to hormonal gender sex differences [108].

Considering offspring behaviour, we observed that male offspring exposed to maternal glycation have better performance in Cliff aversion test and an early maturation of the vestibular system, also presenting expedited auditory ability and opening eyes earlier than the vehicle group. These findings are shown to be in concordance with studies that demonstrated that offspring from AGEs-rich maternal diet manifests earlier sensory reflexes compared with offspring descendent from dams that consume a standard diet [108, 109]. Thus, earlier maturation of reflexes could mirror earlier maturation of CNS [108].

However, developmental maturation of reflexes in new-born's has been shown to have different outcomes according to dietary intervention during the perinatal period. For instance, maternal HFD could induce a delayed physical maturation and a delayed manifestation of physiological reflexes. Notably, locomotor ability was observed to be transitorily impaired, at PND11 and PND12. Due to the lack of studies that reported the effect of maternal glycation on offspring neurodevelopment, no correlation was found with this finding. Studies with MG injection have demonstrated no alterations in locomotor activity. Only a study performed by *Distler et al.* has observed in adult rodent models that higher doses of MG could cause locomotor depression and ataxia [167]. Furthermore, maternal diabetes has been reported to cause a delay in offspring neurodevelopment, vestibular system maturation as well as locomotor activity [86].

Surprisingly, during the adolescence period – PND43 – male offspring from BBGC group were shown to travel a higher distance at OPF test, accompanied by a higher mean speed denoting, hyperlocomotion. Moreover, offspring pups were shown to spent more time in open arms in

EPM test, suggesting that maternal glycation has an anxiolytic effect in the offspring – comparing with the vehicle. Studies in adult rodent models have demonstrated that MG administration developed a similar behaviour with a significant reduction in anxious behaviour accompanied by an increased locomotor activity [175, 176]. Contrarily to that, maternal high-fat diet consumption has been shown to trigger in the offspring an anxious-like behaviour without alterations in locomotor activity [104-105]. Importantly, how does glycation suppress anxiety-like behaviour? Studies have pointed to MG as an agonist of $GABA_A$ receptor, which leads to the suppression of the anxious-like behaviour in mice [167]. These findings suggest that MG can act as an anxiolytic through $GABA_A$ receptor activation, similar to benzodiazepines. On the other hand, high GLO1 levels can reduce MG concentrations in the brain, thereby decreasing the activity of $GABA_A$ resulting in enhanced of anxiety-like behaviour [167]. Our results seem to be in concordance with these findings, since we observed significantly lower levels of GLO1 in the hippocampus of male offspring from BBGC group, and consequently higher accumulation of MG-derived AGEs – MGH1 and Arg-P. Furthermore, higher levels of $GABA_A$ receptors are noted, as well as increased levels of GABA vesicular transporters (that may suggest increased number of vesicles with GABA that could be released at the synaptic cleft), which can be linked with an anxiolytic effect. Additionally, no significantly alterations are found in the PFC region, suggesting that the hippocampus is more sensitive to biochemical alterations induced by glycation [175].

Regarding the neurodevelopment of female offspring, similarly to males, females demonstrate to have a higher strength in their upper limbs, as well as an anticipation of their auditory capacity and eye-opening day. Some studies in maternal diabetes have demonstrated that female offspring have a delayed in their neurodevelopment namely an impairment of discriminatory ability, vestibular system maturation, balance strength, coordination, and locomotion [86]. Our results suggest that both male and female offspring are susceptible to neurodevelopmental changes induced by maternal glycation.

During the adolescence period, no alterations were found in locomotor activity, however, females develop a similar less anxious-like behaviour or more risk-taking phenotype as males, which can probably be explained due to the lower levels of GLO1 in the hippocampus. However, no alterations were observed in AGEs accumulation. Furthermore, glutaminase enzyme presented lower levels in the BBGC offspring, which could result in a decreased production of glutamate, consequently impacting glutamatergic synapse activity. As well, lower synapsin and PSD95 decreased levels were observed in the BBGC offspring. Few studies have investigated the role of PSD95 in inhibitory transmission. A recent study demonstrated

that knocked down PSD95 leads to fewer glutamatergic synaptic contact and an increased number of inhibitory GABAergic synaptic contents [177]. Our results suggest that the offspring exposed to maternal glycation may be increased risk of neurometabolic and synaptic impairments.

Regarding PFC region, female offspring presented a trend to have lower levels of GLO1, therefore are more prone to accumulate AGEs – MG-H1 and Arg-P. Even with no alterations in $GABA_A$ receptors observed, the number of vesicular transporters of GABA is higher, suggesting, a higher presence of GABA neurotransmitter in presynaptic terminals. Recently studies have pointed out that PSD95 deficits, during the adolescence period, affects PFC synaptic function, which consequently, increases $GABA_A$ receptor activation increasing the susceptibility to neuropsychiatric disorders such as autism [178-179].

Our results demonstrate that, male offspring are more susceptible to behaviour alterations than female offspring. In addition, maternal glycation differently impacts the different brain region of the offspring.

II. Impact of maternal glycation in the brain of overweight offspring induced by postnatal hyperphagia

Our second aim was to evaluate the impact of postnatal hyperphagia in the offspring's brain in conditions of maternal glycation. Our results demonstrated that milk composition presents a lower antioxidant capacity in postnatal overweight conditions. Furthermore, exposure to glycotoxins in the maternal diet could enhance this effect, as well as lower levels of triglycerides. These results demonstrated to be in concordance with alterations observed in milk analysis from dams exposed to glycation.

According to our results, postnatal hyperphagia was shown to increase body weight during the first days of life until the end of the experiment – PND45, inducing an obese phenotype. Several studies have demonstrated that postnatal hyperphagia increases the susceptibility to the developed of overweight/obese phenotype in the offspring [20-22]. Early postnatal overnutrition induced several changes in glucose homeostasis throughout life, such as early hyperinsulinemia and hyperglycemia that can increase the susceptibility to diabetes. Studies have pointed out early changes in offspring pancreatic cells that can affect insulin secretion. In adult SL offspring, isolated islets secrete more insulin in the presence of elevated glucose concentrations than islets from normal litter offspring. This insulin hypersecretion could be an adaptative and compensatory mechanism, to maintain normal glucose levels [180]. Our results demonstrate that, although SL offspring do not have alterations in insulin response during the

insulin tolerance test, they also present significantly lower plasmatic insulin levels, suggesting the presence of potential lesions in pancreatic cells. Indeed, in accordance with previous studies [180], preliminary data in our group demonstrated that SL offspring present dysfunction in their pancreatic cells (data not published).

High-fat diet exposure during perinatal period has been associated with increased birth weight, weight gain, fat mass and prevalence of type 2 diabetes in offspring [181]. However, a specific study performed by *Kruse M et al.* showed that offspring mice from high-fat diet dams have lower body weight, which can be explained due to a shorter duration of diet exposure. Similarly, in adult rodent models, MG treatment in obese conditions induced by a high-fat diet leads to a loss in male's body weight mass. Interestingly, our results demonstrated that in the context of maternal glycation – SL+BBGC – overweight induced by postnatal hyperphagia is lost similarly to what is observed in male offspring from the BBGC group. Thus, this suggests that maternal glycation could affect body weight composition, in conditions of hyperphagia. Although, the glucose levels during the insulin tolerance test do not present significant alterations there was an increase in plasmatic triglycerides levels, suggesting a dysregulation of lipid homeostasis consistent with a higher risk of developing metabolic syndrome. Studies have demonstrated that in response to a post-weaning high-fat diet, SL offspring have accelerated development of diabetes response, highlighting their susceptibility to pancreatic dysfunction [180].

Regarding neurodevelopment tests, SL offspring do not demonstrate alterations in the achievement of developmental milestones, such as vestibular system maturation, locomotion ability, and olfactory discrimination compared with control group. Until now, few studies have been addressing the impact of postnatal overweight on offspring neurodevelopment. A study performed by *Novais et al.* demonstrate that offspring from small litter procedure do not have alterations in their behaviour and locomotor activity which corroborate our findings [182].

Male offspring from BBGC group presented a trend for impaired locomotor activity at PND13 and PND14, and the exposure to maternal glycotoxins in the SL groups significantly impaired the locomotor ability suggesting that this effect could be due to early exposure to glycation.

Insulin has an important function in CNS development, being involved in biological processes such as neuronal proliferation, synaptogenesis and neuroprotection [165]. Our results demonstrate that overweight offspring – SL – present decreased levels of total insulin receptors in hippocampus which may be involved in neurobehavioral and neurocognitive anomalies. [135-136] To answer that, offspring from SL and SL+BBGCs groups were tested in behavioural tests that evaluate anxious-like behaviour and possible alterations on short-

memory retention. Offspring from SL group presented anxious phenotype when compared to the control group, suggesting that postnatal hyperphagia increases the susceptibility to this behaviour. Also, we observed higher levels of GLO1 and catalase protein, in the hippocampus, without no alterations in AGEs accumulation, suggesting that postnatal overweight could trigger mechanisms of protection against stress and inflammation. In fact, higher GLO1 levels have been linked with an increased risk of anxious-like behaviour.[176].

Surprisingly, offspring exposed to maternal glycation lost these mechanisms of protection, suggesting that early intake of glycotoxins could affect the activation of antioxidant defences, although no alterations were found in AGEs accumulation.

Regarding synaptic integrity, both overweight groups – SL and SL+BBGC, have significantly higher levels of pre- and post-synaptic proteins namely synapsin and PSD95. Interestingly, no alterations were found in glutaminase and glutamate transporters, as well as $GABA_A$ and its transporter, demonstrating that the higher levels of these synaptic proteins could be due to compensatory mechanisms and may eventually suggest increased synapse number. Some studies have demonstrated that postnatal overweight could yield an excessive number of essential synapses for the assembly of neural networks due to impair synaptic pruning [183]. Indeed, adult animal models with impaired synaptic pruning demonstrated to exhibited ASD-like phenotypes, including social interaction deficits and repetitive behaviour [184]. Interestingly, overweight offspring have been shown to present deficits in early social communication, social play behaviour, and increased repetitive and stereotyped movements which are characteristic of autism-like symptoms [182]. However, we have not performed behavioural tests to evaluate possible autism-like behaviours in offspring from postnatal hyperphagia. Regarding PFC results, although not significant, decreased levels of synapsin are observed in both overweight offspring groups, without alterations in other synaptic markers. This suggests that, in conditions of hyperphagia, the hippocampus is a more vulnerable region to neuronal alterations than the PFC.

Considering PFC results in SL+BBGC group, similar findings were observed regarding synaptic markers from pre and postsynaptic terminals. Surprisingly, decreased levels of glutamate transporters were found compared with SL group, suggesting that excitatory synapse could be compromised, however no alterations on PSD95 are noted. Indeed, a study performed by *Bocarsly et al.* found that adult obese rats fed with a high-fat diet have decreased spine density on PFC together with decreased levels of synaptic proteins such as vGLUT1 and VGAT, followed by a decline in cognitive function [185]. Although we have not observed behavioural

alterations, this decrease in synaptic marker levels could be indicative of a higher risk of offspring developing cognitive and behavioural dysfunctions later in life.

Conclusion and Future Perspectives

In recent years, lactation period has been considered a critical programming window with an important relevance in metabolic programming [11]. Indeed, several studies have demonstrated that maternal diets and her metabolic status have a deep effect in new-born's development and growth, which could increase the risk of developing metabolic syndrome later in life. Furthermore, postnatal period was demonstrated to be crucial in the shaping and maturation of CNS, and any insult could compromise its proper development, increasing the susceptibility of neuronal disorder during life [172].

Taken together, our results provide evidence for the impact of maternal glycation during lactation period. Indeed, we demonstrate that maternal glycation was able to cause alterations on milk composition and consequently modulate offspring behaviour in a sex-dependent manner, also having brain region specificities. At juvenile period, maternal glycotoxins exposure induced an anxiolytic effect in the offspring, possibly due to a modulation of GABAergic system.

We also demonstrated that postnatal hyperphagia leads to overweight and alterations on insulin sensitivity response. Although no significant alterations were found during neurodevelopment, overweight offspring develop an anxious-like behaviour with higher risk for metabolic syndrome. However, it would be interesting to evaluate the insulin signalling pathway at the different brain regions to further confirm these alterations. Furthermore, some questions need to be addressed. For instance, it would be interesting to evaluate if postnatal hyperphagia could induce behaviour alterations related with ASD. On the other hand, we hypothesize that synapse number increase in the levels of synaptic proteins, suggesting an impairment of synaptic pruning. Since microglia cells show to be crucial in this process, it could also be important to possible microglial changes as their morphology and phagocytic capacity in conditions of postnatal hyperphagia.

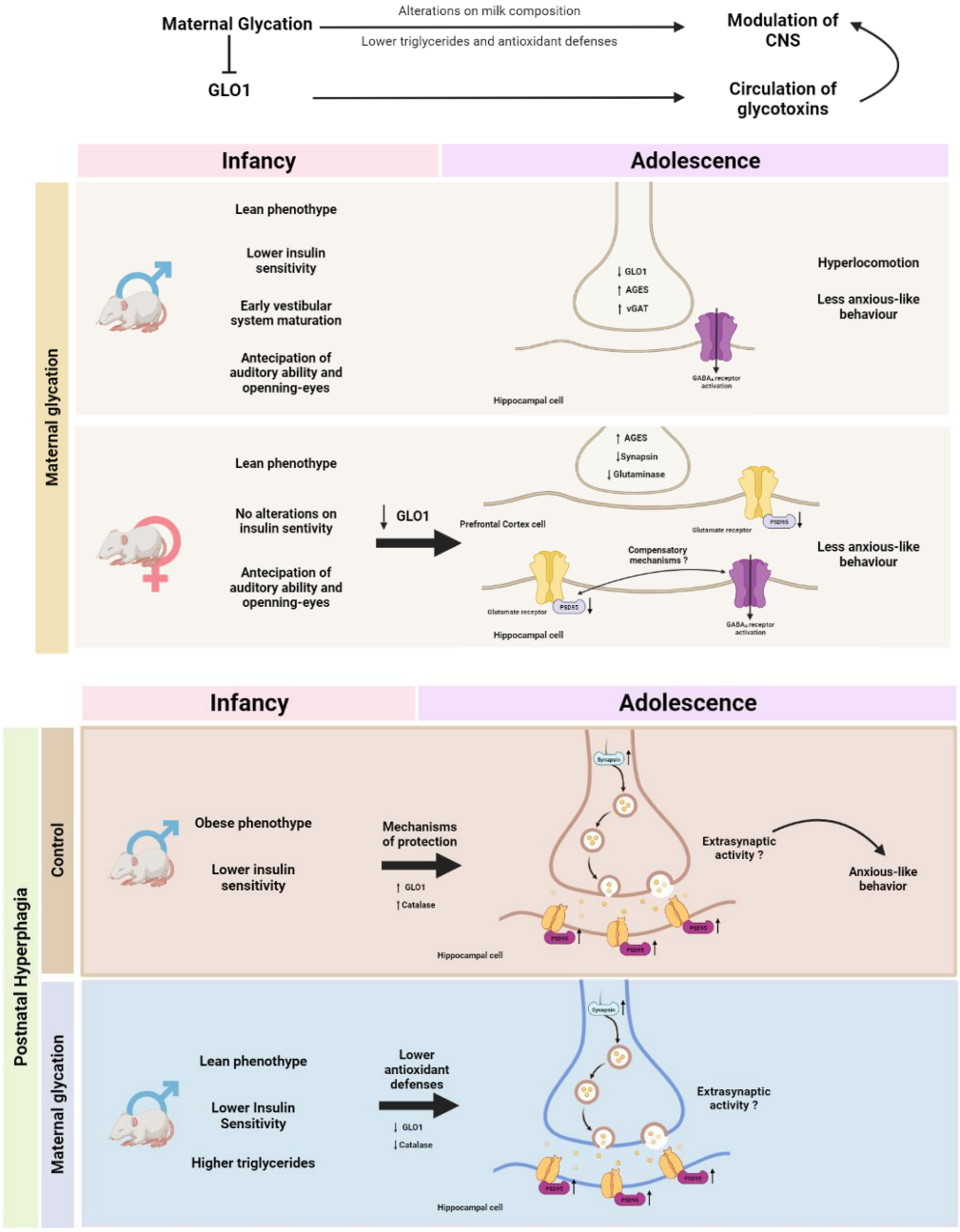

Figure 44 – An overview of the impact of maternal glycation and postnatal hyperphagia, during lactation period – attending sex- and brain region specificities. Maternal glycation induces a lean phenotype in both male and female offspring and induces neurodevelopmental alterations. Male offspring hippocampus is more susceptible to maternal glycation possibly due to lower levels of GLO1, accompanied with higher accumulation of AGEs. Consequently, there is an increased expression of $GABA_A$ receptors associated with a disinhibited phenotype. Considering female offspring, PFC and hippocampus regions are vulnerable to maternal glycation. Indeed, in female offspring hippocampus $GABA_A$ receptor levels are increased, which can result from lower levels of PSD95, as a compensatory mechanism. On the other hand, at PFC there is a higher accumulation of AGEs, in result of lower GLO1 levels. Furthermore, there is also a lower content of synaptic proteins.

Attending to the impact of postnatal hyperphagia, offspring have increased body weight gain together with lower insulin sensitivity. In response to that, offspring developed mechanisms of protection, namely higher levels of GLO1 and catalase. Furthermore, this may underlie an anxious-like behaviour. In this context, offspring when exposed to maternal glycation, lose body weight mass, as well as antioxidant defences, being more prone to stress. Nevertheless, higher levels of synaptic proteins are still observed.

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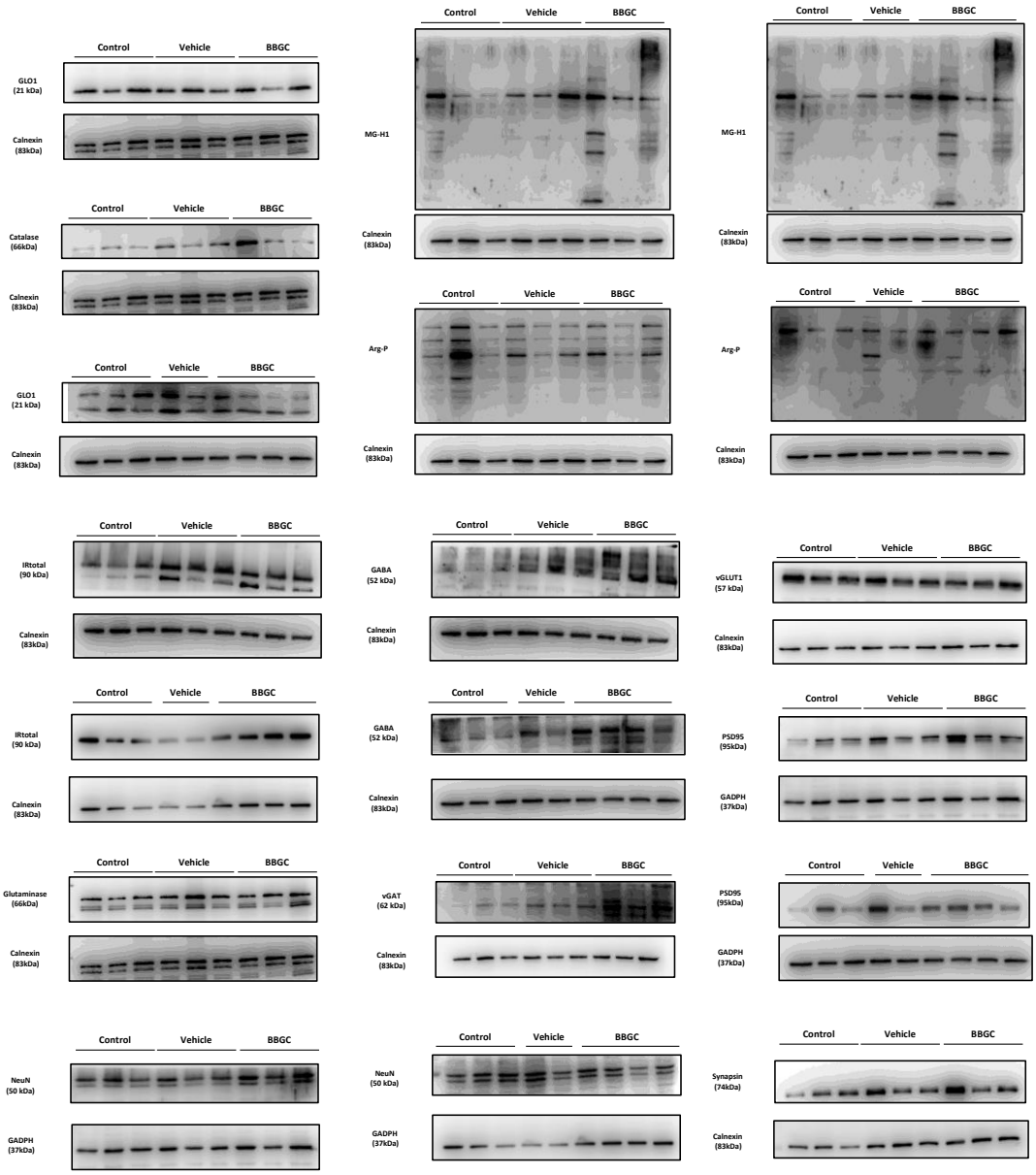
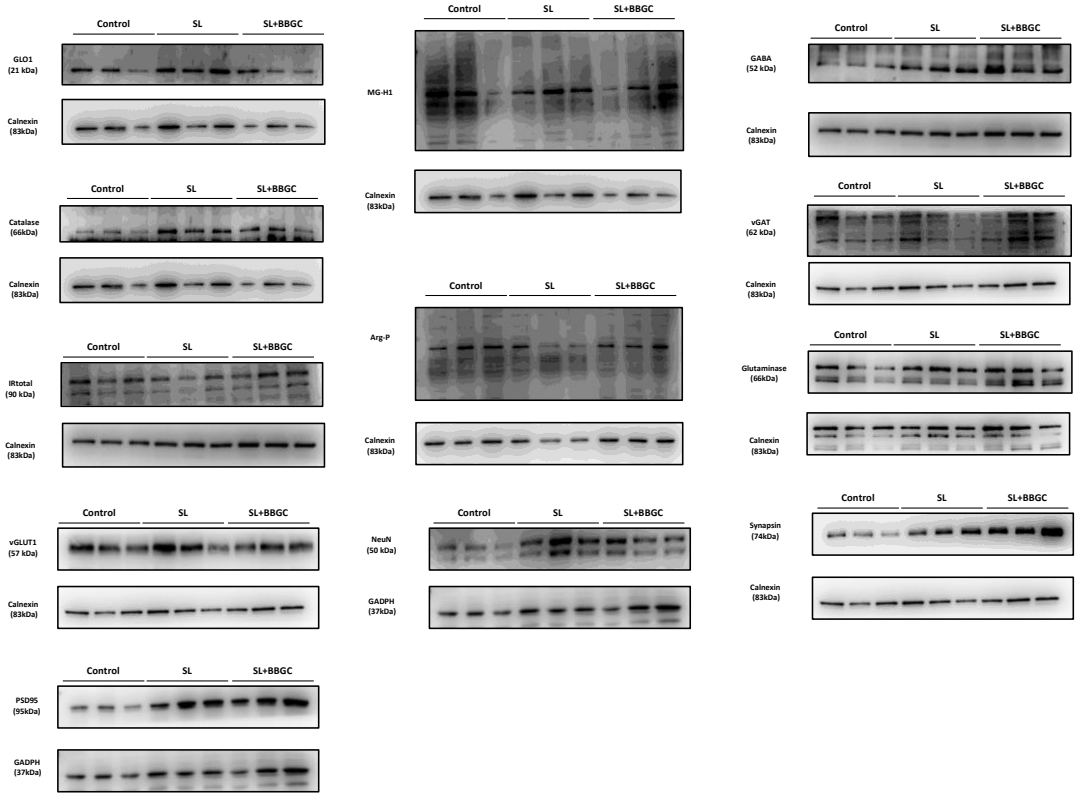
Anexx 1

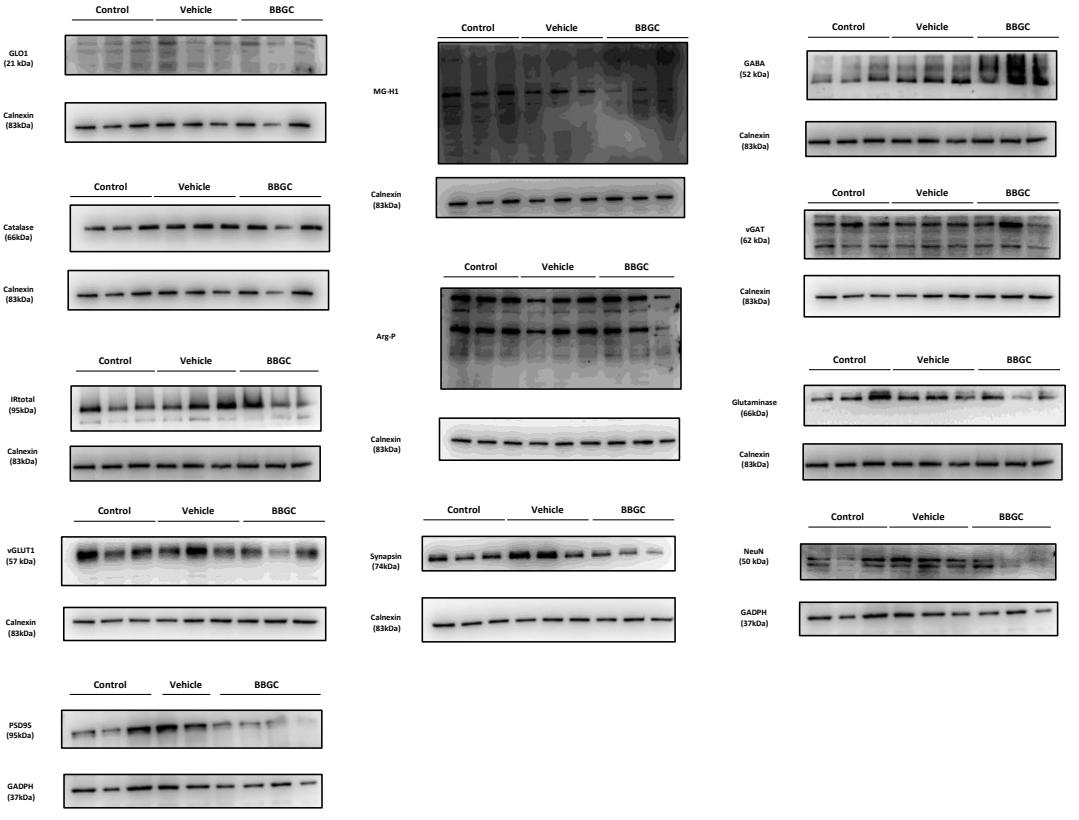
Western Blots - male offspring hippocampus exposed to maternal glycation



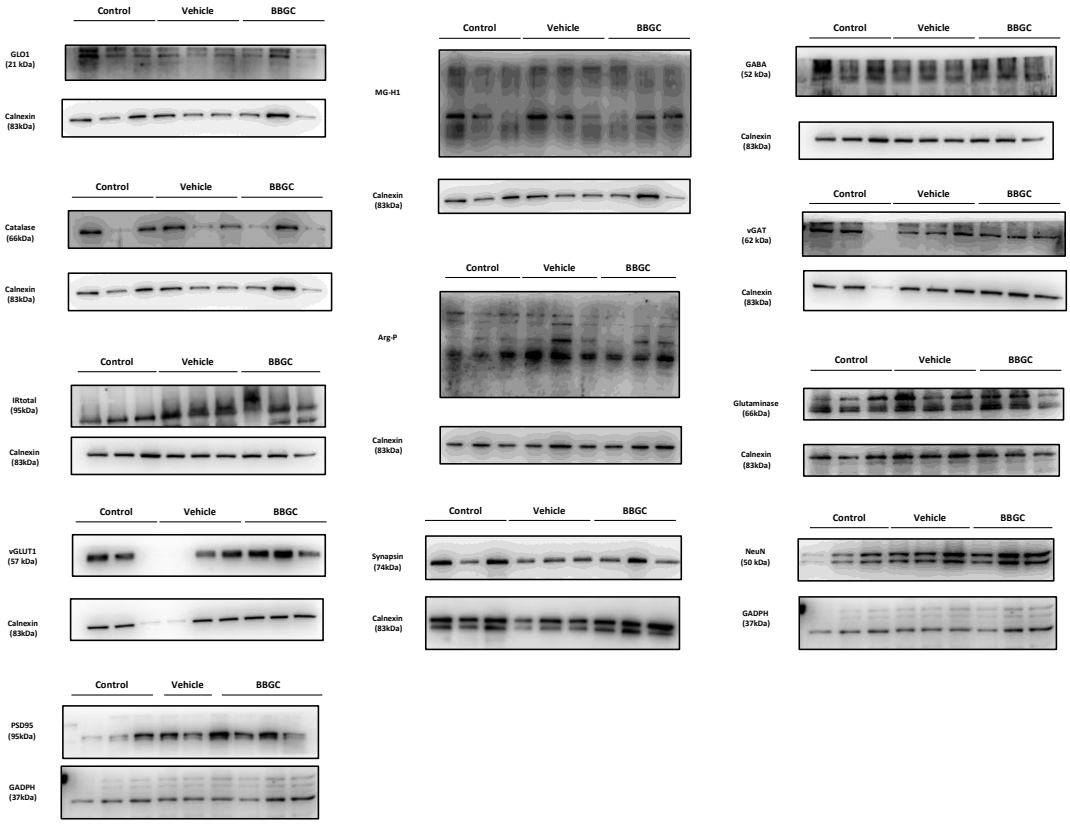
Western Blots - male offspring hippocampus exposed to postnatal hyperphagia



Western Blots - female offspring hippocampus exposed to maternal glycation



Western Blots - male offspring PFC exposed to maternal glycation



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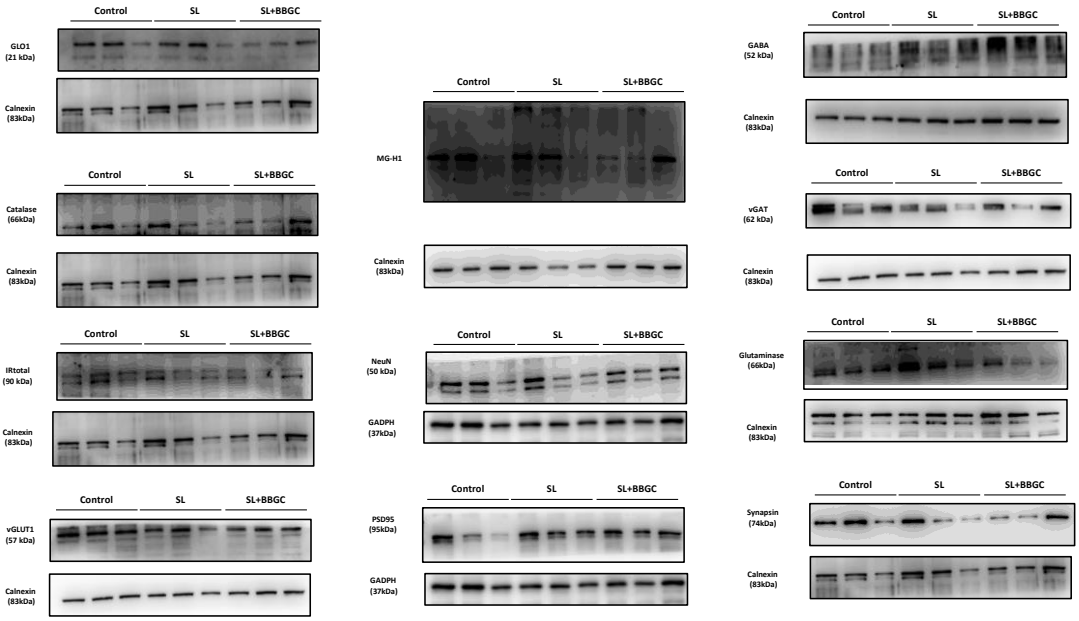
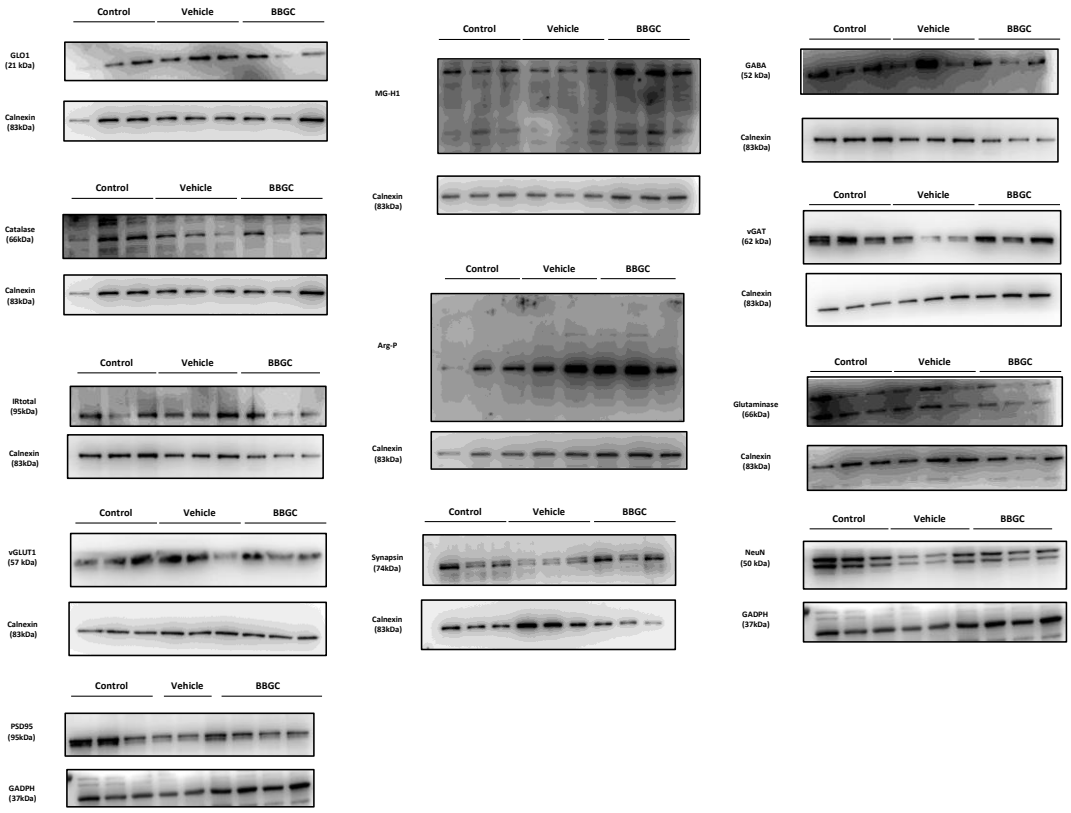


Western Blots - female offspring PFC exposed to maternal glycation



Anexx 2

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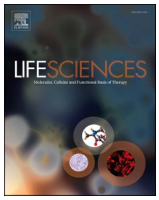

Programming of future generations during breastfeeding: The intricate relation between metabolic and neurodevelopment disorders

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ABSTRACT

Lactation is a crucial postnatal programming window which can interfere with child development and predispose to metabolic disorders later in life, as insulin resistance and obesity. Although breastfeeding is known to prevent many diseases in the newborn, changes in milk composition have been correlated with alterations in central nervous system maturation and differentiation. Changes in milk quality and quantity may predispose to metabolic disorders later in life but have also been linked to the development of neuronal diseases. Maternal metabolic condition, diet and behaviours have been considered determinant for metabolic programming in the child, although the mechanisms involved remain to be elucidated. Some of such mechanisms may also be related with the increasing prevalence of neurodevelopmental and behavioural diseases in the younger generations. This review focuses on the interconnected risks between changes of maternal metabolic status/unbalanced diets during lactation and offspring's development of metabolic and neurodevelopmental disorders. Furthermore, the present review reunites the current knowledge about the mechanisms underlying the association between these disorders and highlights the need of further exploring the impact of lactation period on neurodevelopmental and metabolic outcomes.

1. Introduction

The embryonic and postnatal periods are considered critical phases for child development, being lactation a fundamental postnatal programming window [1]. Lactation period is crucial for organ differentiation and maturation, especially for those related with glucose homeostasis, such as the brain, the pancreas, the adipose tissue, and the liver [2]. The biological active components of the milk, such as hormones, immunoglobulins, nucleotides, growth factors and antioxidant enzymes are involved in metabolic regulation and can mediate growth and development in infancy [3].

Diabetes is a pathological condition of the lactating mother that may pre-exist (type 2 diabetes or type 1 diabetes) [3], or can be developed during pregnancy, gestational diabetes mellitus (GDM), which is a common pregnancy complication. Diabetes is associated with several short- and long-term complications for the mother and the newborns. It is known that this pathology can delay the onset of lactogenesis II, which occurs during the first postpartum days in women and is involved in a programmed set of changes in milk composition and volume [7].

Maternal metabolic and nutritional changes and other types of stressors during the perinatal development (pregnancy and lactation) may induce long-term consequences on offspring metabolic status and contribute for the onset of metabolic syndrome or obesity in the offspring. As well, this increased metabolic risk of the offspring has been suggested to be associated to a higher risk of neurodevelopment disorders, both at childhood and adulthood, potentially increasing the risk to mood disorders, such as depression or anxiety at adulthood [4–6]. The negative impact of such factors on offspring's neurodevelopment may lead to the emergence or predisposition to neuronal disorders later in life [1].

According to the World Health Organization (WHO), in 2016, more than 1.9 billion adults were overweight, being 540 million of these obese. Besides that, over 340 million children and adolescents aged 5–19 were considered overweight or obese, showing an increasing prevalence

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of obesity in the world and a concern to childhood obesity growth [42].

This review aims to discuss the current knowledge regarding the influence of maternal metabolic status and behaviour during breastfeeding on offspring's risk for metabolic and neurodevelopmental alterations, as well as the connection between them, the mechanisms potentially involved and their impact at adulthood.

2. Methodology

Pubmed database was used to carry out online search using the following search strategies; PubMed: "Lactation period" OR "breastfeeding" AND "maternal metabolic condition" OR "maternal diet" OR "maternal behaviour" AND "offspring" OR "lactation period" AND "neurodevelopment" AND/OR "behaviour" AND "offspring" OR "milk composition" AND "offspring" OR "lactation" OR "breastfeeding" AND "mechanisms". All relevant articles published in English were included and priority was given to the articles published in the last 5 years. Articles that did not address the main objectives of the review were eliminated.

3. Maternal milk quality and quantity during lactation period as programming factors for offspring metabolic health

In order to understand the effects of offspring under and overnutrition and its impact at adulthood, animal models have been studied specifically during the lactation period. One common experimental model is the small litter model, in which a reduction of the number of pups per litter is made after birth, leading to a lower competition for milk between pups during the suckling period [8,9]. Therefore, there is a higher consumption of calories and an unbalance between food intake and energy expenditure, which leads to an overweight/obese phenotype [19,20]. Glavas *et al.* showed that overnutrition of the pups during breastfeeding period increases body weight gain and leptinaemia [10]. Moreover, small-litter pups were also shown to develop hyperinsulinemia, hyperphagia and glucose intolerance at adulthood, suggesting that early overnutrition can alter energy homeostasis and increase the susceptibility to obesity and insulin resistance [11]. A study performed by Junior *et al.* demonstrated that overnutrition during this postnatal window can induce oxidative stress and cardiovascular dysfunction, namely cardiac hypertrophy, reduced systolic pressure and reduced vascularization [12].

On the other hand, offspring undernutrition is defined as an insufficient caloric consumption to supply the body's energy demands. In animal models, it was shown that pups nursed by protein diet-restricted mothers had lower plasma insulin and glucose levels during the lactation period [13]. A recent study in rodents performed by Miranda *et al.*, has demonstrated that maternal 50% food restriction during breastfeeding period, although inducing a lean phenotype in adult offspring, lead to hyperphagic behaviour, hypertriglyceridemia, and hypercholesterolemia [14]. Additionally, it was observed that maternal low protein diet can induce an increased hepatic deposition of fatty acids in rodent offspring, with alterations on gene expression and protein levels of key enzymes involved in glycolysis and pathways of fatty acid oxidation in skeletal muscle and adipose tissue [15]. Of note, and according to Lizarraga-Mollinedo *et al.*, undernutrition during the suckling period induces glucagon resistance and insulin hypersensitivity in the liver. As consequence, this affects the supply of glucose and ketone bodies to the brain, affecting the synthesis of neurotransmitters and energy production, which can interfere with its proper development [16].

3.1. Influence of maternal metabolic status in milk quality

Although breastmilk is a source of bioactive molecules with benefits for the healthy development of the newborns, there is evidence showing that it is influenced by the maternal metabolic condition during pregnancy and lactation period [3]. Maternal obesity has been shown to affect breastfeeding since the excess of adipose mass impairs the normal hormonal regulation, and consequently delays the onset of lactogenesis, altering milk production [17,156].

The milk from mothers with obesity was found to be richer in pro-inflammatory omega-6 fatty acids, which may influence not only the subsequent cardiometabolic status, but also neurodevelopmental outcomes [17,156]. Accordingly, maternal obesity has been consistently associated with lower breastmilk levels of important fatty acids for proper neurodevelopment and maturation of neuron membrane, such has docosahexaenoic acid (DHA) [17,154,156]. This changes in the profile of fatty acids were shown to be associated with an alteration of immunological factors in human milk, such as C-reactive protein, leptin, IL-6, insulin, TNF-alpha, ghrelin and adiponectin [17,156].

In mice models, a recent study performed by Sellayah *et al.* has shown that maternal obesity can predispose offspring to an obese phenotype with adipocyte hypertrophy [18]. In order to disclose the specific role of breastfeeding, Gorski *et al.* has used a cross-fostering mouse model to compare the differences in pups from obese and lean mothers during lactation [19]. The study showed that the offspring from obese dams, although maintaining an obese phenotype, had improved insulin sensitivity throughout life when fostered with lean dams. On the other hand, pups fed with breast milk from diabetic mothers were shown to have lower body weight and impaired glucose tolerance, impaired insulin secretion and/or insulin intolerance later at adulthood, showing the importance of lactation period to the later metabolic condition [19]. Moreover, it was also found an upregulation of 214 and 171 genes in male and female liver offspring, respectively. Male offspring had an upregulation of FOXO and PPAR pathways, while female offspring, besides PPAR pathway activation, also demonstrated an enrichment of AMPK and fatty acids metabolism activation [21].

A human study that evaluated the impact of maternal diabetes during breastfeeding has demonstrated that women with diabetes are more likely to introduce infant formulas in the first 2 days of newborn life when compared with a healthy mother. The alternative of using infant formulas can be explained by a higher difficulty to produce enough milk to the infants, being associated with an increased risk of the descendance to develop metabolic syndrome later in life [22].

3.2. Influence of maternal diet in milk quality

During normal weaning, lipids are the main metabolic fuel during breastfeeding due its importance for the central nervous system development [151]. Therefore, an appropriated supply of fatty acids, such as n-3 and n-6 long-chain polyunsaturated acids is crucial to ensure a normal development, being involved in the synthesis of important molecules that regulated several signalling pathways [156]. Maternal diet during pregnancy and lactation is relevant for fatty acid supply during perinatal period. Besides maternal metabolic status, maternal intake of unbalanced diets during pregnancy and lactation periods can also have a negative impact on the metabolic condition of the offspring. Some human studies have demonstrated the importance of milk composition, in specific arachidonic (AA) and DHA acids, to the appropriated normal growth of the newborn and to the development of brain and visual system [152]. A study performed by Barrera *et al.*, has demonstrated that Chilean Women, who had a higher intake of n-6 and a lower intake of n-3 polyunsaturated fatty acids during last stage of pregnancy and across lactation period, had a reduction of the capacity of transferring DHA to her offspring [152], as observed in erythrocytes and breast milk [152,153]. In turn, this can affect the fluidity of neuronal plasma membrane, which is responsible to signal transduction of neuronal growth and migration, synaptogenesis and synaptic plasticity. Also, these fatty acids can modulate energetic metabolism in muscle and adipose tissue, such as insulin action and may also influence inflammatory response [154,155].

Some studies have addressed the effects of a maternal high-protein diet in animal models. The offspring were shown to present lower

postweaning weight and adiposity accompanied by a lower food intake [23]. Furthermore, the pups from mothers fed with high-protein diets, presented increased blood glucose, insulin, and glucagon levels in fasting conditions after the weaning period [23,24].

Most of the studies in this field have addressed the role of cafeteria diets (diet rich in sugars and fats) in pups' metabolic health. Several experimental studies have shown that diets rich in fat and sugars have negative impacts on offspring metabolic outcomes [1]. In animal models, maternal high-fat diets were shown to cause alterations in milk composition, namely higher insulin, and long-chain polyunsaturated fatty acids levels, being associated with increased offspring growth [25,26]. Although female offspring didn't show any differences, male offspring developed insulin resistance at early adulthood, and presented increased weight gain, insulin intolerance and hyperglycaemia later at adulthood. In addition, there is also an increased expression of inflammatory markers on adipose tissue, long-term bone loss and expansion of adiposity at bone marrow [27]. In mice, maternal consumption of cafeteria diet during lactation period has shown to induce obesity and dyslipidaemia in the offspring [28,29]. Additionally, in a rat model of maternal diet-induced obesity during lactation, offspring present lower levels of proteins involved on insulin and leptin hypothalamic signalling, as signalling transducer and activator of transcription 3 (STAT-3), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT). This was associated with increased expression of neuropeptide Y (NPY) and decreased proopiomelanocortin (POMC) levels. Such changes induced a negative unbalance between hunger/satiety, promoting higher caloric uptake, and increasing the predisposition to obesity at adulthood [30]. The offspring also developed hypertrophy of pancreatic islet cells, further potentiating the development of insulin resistance later in life [30].

Maternal metabolic syndrome induced by high-fructose diets also has several effects on milk quality and offspring health. A study performed by Nogales *et al.*, has shown that maternal diet rich in fructose (65%) during breastfeeding causes maternal hypertriglyceridemia, insulin resistance (HOMA-IR index) and compromises beta-cell function, which was associated with a lower viability index of the offspring calculated on postnatal day 7 [147,148]. In addition, it was also observed that the offspring presented a higher BMI at birth, although the opposite was observed at the end of lactation period [147]. These findings could be related with fructose-induced fatty acid synthesis and decreased gluconeogenesis, leading to a modification of milk composition [147,148]. Maternal fructose-induced metabolic syndrome has been found to alter offspring appetite profile and endocrine energy balance by increasing leptin serum levels [149]. The fructose-fed dams were observed to intake lower levels of selenium, a trace element involved in insulin-like growth factor I (IGF-1) regulation, proper thyroid hormones synthesis and oxidative balance, being also involved in endocrine regulation of appetite and energy homeostasis [149]. The offspring of the fructose-fed dams presented growth retardation, namely an underdeveloped pancreas with decreased beta-cell function and low insulin secretion [149]. Moreover, the offspring were shown to develop leptin resistance, also presenting a low amount of brown adipose tissue and muscle and bone mass [149].

Besides the changes of macronutrients consumption in hypercaloric diets, other secondary metabolites may also be involved in their negative effects in the offspring. Francisco *et al.* have shown that the treatment of lactating female rats with methylglyoxal (MG), a glucose metabolism intermediary and precursor of advanced glycation end-products (AGEs), leads to increased offspring body weight and adipose tissue at adulthood, together with glucose intolerance, dyslipidaemia, and impaired β-cell function [31]. Such effects were recently proven to be directly related to glycation, since they were observed in the adulthood of pups directly treated with MG during the suckling period [32].

3.3. Influence of maternal behaviours in milk quality

Besides nutritional cues, exposure to other factors such as stress, cigarette smoking and alcohol during lactation period have also been shown to have a negative impact on offspring metabolic condition. Postpartum stress has negative consequences for lactogenesis and lactation phases, affecting hormone production. Glucocorticoids are not only involved in stress response but also in mammary gland development, lactogenesis and milk production. Furthermore, early life stress induced by maternal separation during lactation period, has been shown to be involved in alterations on satiety point, meal duration and meal size, leading to lower food intake in female offspring [33]. The interruption of breastfeeding by exclusive intake of solid foods might contribute to the lower body weight observed on the first days of weaning, suggesting that beyond nutritional challenge, physical interaction between the mother and the offspring in late lactation period may influence body weight gain [151].

Despite many women avoid smoking during pregnancy, most of them restore this habit during lactation period. Several studies have confirmed that nicotine can be transferred to the offspring through breast milk [34]. Moreover, when evaluating infants whose mothers smoked during pregnancy and lactation period, higher AGEs levels were observed in their skin [35]. Maternal smoking during the suckling period is correlated with foetal growth retardation and impaired development of multiple organ systems, such as the kidney, by inducing injury and fibrosis [36]. A study performed by Oliveira *et al.* has shown that the rat offspring whose mothers were exposed to smoke during lactation presented increased body weight, visceral adipose tissue, and leptin levels [34]. Moreover, these animals showed thyroid dysfunction and a programming for obesity development later in life [34].

Like smoking, the consumption of alcohol during this programming window has serious consequences to the offspring, including the development of metabolic syndrome. A study performed by Chen *et al.*, has shown that rodent offspring whose mothers had a heavy alcohol consumption during gestation and lactation, have decreased insulin sensitivity and higher glucose levels, which programs to the development of insulin resistance later in life [142]. In addition, the offspring also presented elevated plasma, liver, and muscle triglycerides levels [142]. However, few studies have evaluated the effect of alcohol consumption exclusively during lactation. A study performed by Murillo Fuentes *et al.*, has shown that the maternal consumption of alcohol during lactation period induces a lower offspring body weight gain when compared with *in utero* exposure [144]. Moreover, it was also observed that ethanol consumption significantly decreases milk consumption in both prenatally and postnatally ethanol-exposed litters during breastfeeding [144]. These findings were also corroborated by a study performed by Cheslock *et al.*, who also observed a lower body weight gain accompanied by a decrease in sucking behaviour [145]. These alterations can be related with the fact that ethanol inhibits the secretion of prolactin and oxytocin hormones [144,146].

3.4. Infant formulas as an alternative to breastfeeding

Although World Health Organization (WHO) recommends exclusive breastfeeding during the first 6 months of age, a significant percentage of children receives formula feeding as an alternative. Infant formulas are mostly composed by cow's milk proteins, which a higher content of proteins and sugars per energy when compared to human milk [37]. The higher content of proteins in these formulas increases the release of urea, creatine, threonine, and valine, which indicates a higher protein intake [38]. Moreover, infant formulas have also been shown to increase serum insulin, insulin-like growth factor I (IGF-1) and plasma glucose levels, and decrease ketone bodies, indicating a decrease of β-oxidation [38]. These changes, besides contributing to accelerated growth, weight gain and visceral adipose tissue, also increase the predisposition to oxidative stress, inflammation, and insulin resistance, which may increase the risk

of obesity, and type 2 diabetes at adulthood [39]. Moreover, the confection of infant formulas involves heat treatment, which facilitate the formation of AGEs through non-enzymatic reactions [40].

4. Influence of lactation period in offspring neurodevelopment and behaviour

4.1. Maternal metabolic condition and risk of offspring neurodevelopmental diseases

The maternal metabolic condition, besides increasing the risk of metabolic diseases in the offspring, has also been suggested to contribute to a higher risk of psychiatric disorders, such attention deficit hyperactivity disorder (ADHD), autism spectrum disorders (ASD), anxiety, depression, and schizophrenia [42–44,143] (Fig. 1).

In human patients, maternal obesity was shown to account for a two-fold increased risk of having a child with ADHD [42]. Children of obese or overweight mothers have an increased risk of having more symptoms related with this disease, such as inattention and difficulty to deal with emotions [42]. Maternal diabetes has been further associated with an induction of cognitive impairments and an increased susceptibility to neuropsychiatric disorders [43]. A study involving 212 children with ADHD has shown that gestational diabetes was associated with an over two-fold increased risk of developing this neuropsychiatric disease, and a recent meta-analysis showed that pregestational diabetes could also increase the risk of ADHD in the offspring by 44% [42,44]. Moreover, maternal diabetes was also shown to be positively associated with ASD risk in offspring [45–47]. A systematic review and meta-analysis published in 2018 involving 16 studies, reported that children's ASD was positively associated, not only with GDM, but also with diabetes before pregnancy [45,46].

In rodent models, maternal high-fat diet-induced obesity has been showed to increase anxiety behaviour and reduce sociability in female offspring, which is a common feature of neurodevelopmental disorders such as ASD and ADHD [48]. Maternal obesity has been associated with increased offspring risk of disrupted emotions like fear and sadness, being also involved in increased internalizing behaviours associated with depression and anxiety [49]. These changes in development were accompanied by neuroinflammation, namely in the prefrontal cortex, due to increased expression of proinflammatory cytokines in the first days of life and latter in adulthood, together with increased hippocampal microglial activation and lipid peroxidation [49,50]. Maternal obesity has also been shown to impair neuronal plasticity in the offspring by decreasing neurogenesis, and synaptic function [41].

In animal models of diabetes during pregnancy and lactation, newborns from diabetic dams present higher levels of AGEs and inflammatory markers, hippocampal RAGE levels, neuronal activity, and oxidative stress, contributing to behavioural and memory alterations in early adulthood [51]. A study performed by Sousa *et al.* showed altered expression of neuronal markers, such as neural stem cell markers in males and neuronal precursors in both male and female offspring of diabetic mothers. Such alterations were associated with an early delay in offspring development, affecting vestibular system, balance, strength, coordination, and locomotion, as well as impaired short-term memory in female offspring at infancy [52]. Maternal diabetes, also impacts on glyoxalase-methylglyoxal (GLO-MG) pathway, increasing the circulation of maternal MG levels. This was associated to premature neurogenesis and depletion in embryonic mouse cortical neural precursor cells, which can predispose to cognitive dysfunction [53], impairments of visual reception and motor skills, and adaptative communication and socialization [54].

Importantly, the studies currently available are exclusively focused on the perinatal period, including pregnancy and lactation, and little is known about the specific role of each of these programming periods in the ethology of neurodevelopmental and psychiatric disorders.

4.2. Maternal diets and risk of offspring neurodevelopmental diseases

Maternal high-fat diets considered unbalanced or obesogenic have been shown to induce neurophysiological changes, such as impaired brain glucose regulation, changes in the levels of excitatory and inhibitory amino acids and neurotransmitters, neuroinflammation, oxidative stress and alterations in the structural integrity of the blood-brain barrier that can directly or indirectly impair hippocampal-dependent learning and memory operations (Fig. 1). As already detailed, fatty acids unbalance may trigger a proinflammatory and oxidative

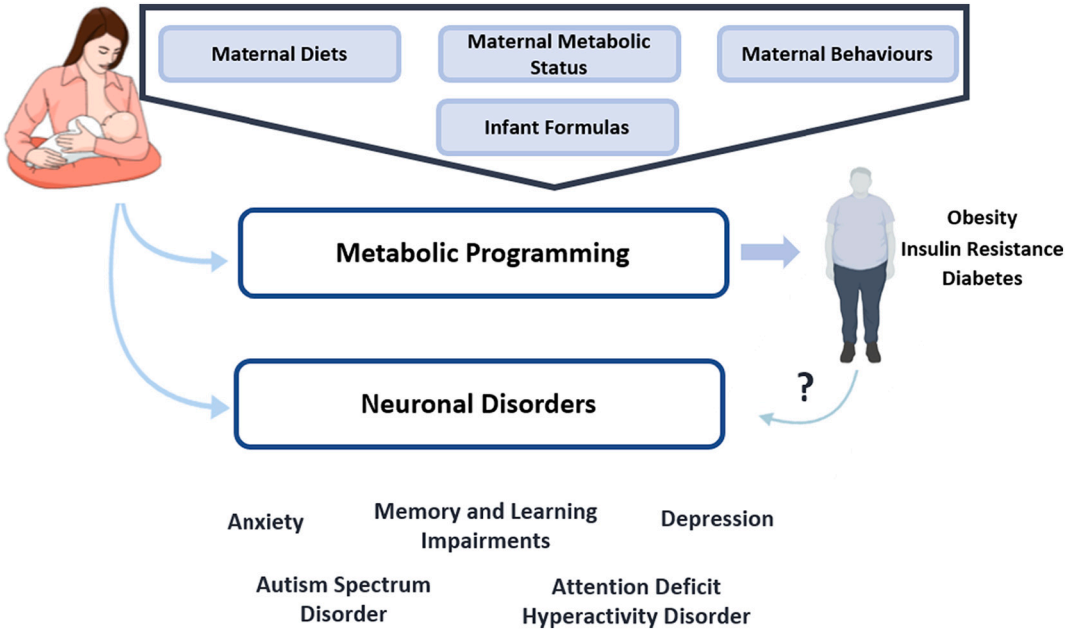

Fig. 1. Maternal metabolic condition, diet and behaviours adopted during lactation period may predispose the offspring for metabolic syndrome such as obesity, insulin resistance and diabetes, at adulthood. These conditions can also have an impact in offspring neurodevelopment and increase their susceptibility for anxiety, depression, learning and memory impairments and higher risk for autism spectrum disorder and attention deficit hyperactivity disorder.

environment. A recent study performed in piglets has shown that maternal diet mainly composed by fish oil (rich in long-chain n-3 polyunsaturated fatty acids) enhance the antioxidant capacity in offspring [157]. In addition, this type of diet increased the levels of IgG and IL-10 in the colostrum, which could be responsible to improve the anti-inflammatory capacity of the offspring [157]. The addition of 3–5% of fish-oil to sow feed during lactation, was shown to promote the growth of offspring piglets, due the increased secretion of milk fat and immunoglobulins [158,159]. On the other hand, a study performed by Liermann *et al.*, has shown that a diet combining linoleic acid (omega-6) and saturated fatty acids increases the concentration of IL-1β, prostaglandin E2 and interleukin 6 [160].

In humans, the analysis of different fatty acids in diets has been correlated with the risk of ASD in the offspring [55]. Higher levels of omega-6 fatty acids in mother's diet reduce by 34% the risk of ASD in the offspring, while a reduction of omega-3 fatty acids intake may increase 54% the development of this disease [55,56].

Interestingly, in animal models, maternal cafeteria diets during breastfeeding were demonstrated to have different outcomes in the offspring. Wright *et al.* demonstrated that there is an improvement on memory performance in male offspring while, but, on the opposite, female offspring have an impairment on memory tasks [63]. In addition, other studies in piglets and mouse models have shown that maternal exposure to western or high fat diets during pre and postnatal periods impairs spatial learning and memory, raising the question about the mechanisms and factors associated with neurodevelopment deficits [60,64,65].

The specific contribution of the lactation period has been addressed in animal models. Offspring of high-fat diet-induced obese rodent models present decreased sociability, and increased anxiety-like behaviour and hyperactivity, which are ADHD-like symptoms [48]. Also, maternal high-fat diet during suckling period was shown to increase the expression of pro-inflammatory cytokines and glucocorticoids receptors in the offspring hippocampus, and consequently, leading to a higher anxious behaviour [106]. In the study performed by Teixeira *et al.* in rodents, offspring hippocampal oxidative stress caused by maternal cafeteria diet, from lactation period until adulthood, was also associated with anxious-like behaviour, memory impairment and decreased social interaction, despite having a better performance in locomotion evaluation [29]. A recent study performed in minipigs during gestation and lactation periods, has demonstrated that inflammation caused by maternal western diets decrease neurogenesis activity at the hippocampus and cortex, consequently affecting learning and cognitive and memory processes [60].

Maternal cafeteria diets consumption during pre and postnatal periods, not only increase oxidative stress and inflammation, but also affect the normal function of several neurotransmitters, such as the serotonergic and dopaminergic systems, leading to a predisposition to neuropsychiatric diseases, disturbances in locomotion and anxious behaviour [57,58]. As well, maternal high-fat diet exposure for 9 weeks (including gestation and lactation periods) induced an increased expression of brain-derived neurotrophic factor (BDNF) in offspring dorsal hippocampal and an increase in $GABA_A$ α2 subunit and serotonin receptors 5-HT1A in the ventral hippocampus, which was also associated with an increase anxious-like behaviour [4]. In addition, in rodent models, it was shown that the preference of maternal high-fat-diets consumption during pregnancy and breastfeeding, instead of a low-fat diet, increases circulating plasma leptin levels in the offspring [59]. Interestingly, the cross-fostering of the pups, during lactation period, from a normal standard to a high-fat maternal diet, was shown to have similar negative outcomes by increasing the circulation of leptin levels, suggesting the importance of this period in the modulation of appetite/reward systems and the metabolic status of the offspring [59].

Regarding the effects of sugars, Erbas *et al.* showed that, in rodents, a long-term exposure to maternal fructose diet during pregnancy and lactation periods impairs sociability in male offspring. Moreover, it was observed an impairment of locomotor activity and memory performance in both male and female offspring, which may be correlated with a development of autistic social deficits [61]. The long-term exposure of fructose diet increases the circulation of TNF-α levels, in both male and female offspring rats. In addition, there was a significant reduction of neuronal markers related with neuronal growth and synaptic formation in the hippocampus [61]. In accordance, maternal consumption of a diet rich in Maillard Reaction Products during lactation period, which introduces considerable quantities of AGEs into a diet, accelerated male offspring neurodevelopment, improved working memory but induced an anxious-like behaviour [62].

Although maternal high-fat diets have consequences to the neurodevelopment of the offspring, maternal malnutrition can also have negative impacts. Some experimental studies in rodents have shown that maternal protein restriction during the perinatal period can negatively affects neurogenesis, cell migration, myelination, and plasticity, which can have consequences in exploratory behaviour, anxiety, and learning and memory abilities [66]. A study performed by Batista *et al.,* in rodent models, has shown that maternal protein malnutrition during pregnancy and lactation periods induces a decrease in male and female offspring vocalizations, impairs social discrimination and decreases social play behaviours, which are associated with ASD phenotype [67]. In addition, maternal protein-restricted diet during these periods has demonstrated similar negative outcomes in the offspring to a diet rich in fat, with an impairment in learning and motivation [68]. Moreover, this poor nutrition of the offspring during breastfeeding period can cause a dysregulation of gene expression in the medial prefrontal cortex, which can be associated with behavioural alterations associated with neurodevelopmental disorders [69].

4.3. Maternal behaviours and risk of offspring neurodevelopmental diseases

The exposure to stress or stress mediators, such as glucocorticoids, during critical periods of development has an impact in the neuroendocrine and behavioural systems of the brain, being a relevant risk factor to psychiatric disorders later in life, such as anxiety and depression [70–72] (Fig. 1). Infants of depressed mothers were shown to have behavioural difficulties, like socialization, and cognitive delays, with a higher risk of developing psychiatric disorders during the adolescence period [73,74]. On the other hand, studies also showed that relaxation interventions during the lactation period could have positive effects on maternal psychological state, breast milk intake, milk composition, and infant behaviour and growth [75]. Early behaviour or temperament of breastfed infants has been associated with higher maternal breast milk cortisol levels, suggesting that mothers may shape infant behaviour by the transmission of bioactive factors in milk [76–78]. Mothers who were less stressed or doing relaxation intervention during lactation period, had longer and better-quality time to physically bond with their infants [78]. Consequently, this stimulated or facilitated infant sleep [75].

In rodents, maternal exposure to social stress during lactation period depresses maternal care, increases maternal anhedonia and anxiety, and induces a mild impairment growth in the offspring [79,80]. Such effects were associated with a modulation in gene expression of the neuropeptide's orexin and its receptors (Orx1r and Orx2r), which are involved in maternal care and depressive behaviour, modulating the development of mood and anxiety disorders in the offspring [81]. Deprivation of maternal care during perinatal period can have marked effects on emotional development of the offspring [150]. Animal studies have demonstrated that maternal deprivation or neglect during lactation induce a higher risk for future psychiatric illness, accompanied by increased anxiety-like behaviour and aggression, delayed conditioned-fear extinction and decrease empathy [150]. These behavioural alterations have been linked with alterations in BDNF signalling in central nervous system which can be responsible for neuronal dysfunction. Moreover, early weaning could also promote de circulation of

corticosterone levels at the prefrontal cortex (PFC) which induces higher anxiety at adulthood [150]. In accordance, it has also been shown an anxious-like behaviour in male and female offspring after prenatal maternal glucocorticoid administration, although the role of lactation was not specifically addressed [70,71].

As mentioned above, maternal cigarette smoking during late gestation and lactation periods may have negative consequences for the offspring. Maternal nicotine exposure was shown to impair hippocampal neurogenesis, potentially affecting anxiety-like behaviour and impulsive decision-making later in life, increasing the incidence of ADHD or addictive disorders [82,83]. As well, the maternal consumption of alcohol during lactation has a negative impact in the brain function, being associated to decreased cognition and difficulties in completing tasks [84]. Exposure to alcohol through milk induces hyperactivity and deficits in learning and memory in young mice [85] and exposure to alcohol in milk formula during early postnatal period also increases offspring microglial activation in hippocampus potentially impairing offspring immune function and cognition later in life [86].

5. Mechanisms upon lactation period underlying the risk of neurodevelopmental diseases at adulthood

Although breastfeeding and maternal behaviours were shown to be crucial to determining the risk of neurodevelopment and neuropsychiatric diseases, the mechanisms involved in these diseases are still poorly understood. Several studies have demonstrated a dysregulation of insulin and leptin signalling [41,87–101], inflammation [33,96,102–107], alterations in neurogenesis [91,92,103,108–112], dysfunction of GABAergic [4,12,61,113–116,118–120], serotoninergic [4,33,61,129–131], and dopaminergic systems [118,132–140], which may negatively influence offspring outcomes (Fig. 2).

5.1. Dysregulation of hormones controlling metabolic processes

The metabolic condition and diet of the mother during lactation are crucial for the correct development of the offspring, being insulin an important growth factor for brain development. In pregnancy, alterations of mother's insulin sensitivity due to obesity and/or diabetes could influence the neurodevelopmental state of the offspring. Lactation period is particularly important to provide not only nutrients to the growth of the offspring, but also hormones like insulin, leptin, insulin growth-factors (IGFs) and their binding proteins [87–89] which are not only absorbed at the intestine but can also cross the blood-brain barrier [90].

Rodent models have disclosed this issue. Reduction of neuronal plasticity, decreasing neurogenesis, and synaptic function in the hyperinsulinemic offspring of diet-induced dams was shown to be associated to an impairment of hippocampal insulin resistance [41]. The exposure to maternal high-fat diets during lactation was shown to activate mitogen-activated protein kinases (MAPK) in hippocampus and central insulin resistance [91,92]. Lower insulin signalling in the hippocampus could be linked to chronic inflammation, oxidative stress, and impaired of growth and neuronal transmission, which may impact learning and memory processes [91,92]. AKT is a downstream molecule of IRS and plays a crucial role in insulin signalling. Maternal high-fat diet or obesity decreases AKT phosphorylation in adult rat male offspring dorsal hippocampus, which is associated with poorer cognitive function [93,94].

Leptin has receptors located in hippocampus, cortex, amygdala, thalamus, and hypothalamus, being involved in behavioural control [95]. Besides passing through the placenta to regulate nutrient handling during pregnancy, leptin can also be found in milk [96,97]. Leptin signalling may help to regulate synaptic plasticity and trafficking of neurotransmitter receptors [98]. Furthermore, leptin administration has been shown to improve cognitive performance of wild type mice at adulthood, while rodent models of leptin deficiency have impaired spatial memory and long-term potentiation [99,100]. However, higher leptin levels may have negative outcomes and increased leptinaemia in the offspring may increase the risk of leptin resistance. In rodent models, the offspring of obese mothers were shown to have increased plasma leptin levels, possibly resulting from leptin resistance, which was correlated with higher risk of depression and anxiety behaviour [95,97]. Accordingly, maternal high-fat diet was associated with a decreased hippocampal gene expression of leptin receptor in offspring later in life, resulting in cognitive impairment [101]. Thus, it is possible that leptin may serve as a signal of energy reserves and induce proper nervous system development. However, in conditions of leptin resistance such mechanisms may be lost. Given the existence of common cellular mechanisms between leptin and insulin resistance, the increase of leptin in the milk and mother's insulin resistance may be interconnected and orchestrate a metabolic imprinting in the offspring towards impaired energy balance.

5.2. Inflammation and oxidative stress

Maternal obesity and diabetes are associated with chronic inflammatory processes, resulting in higher secretion of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, inducing inflammation in the offspring hippocampus, and being associated with neurodevelopmental and neuropsychiatric disorders [96,102].

Maternal high-fat diets during lactation period can also stimulate the activation of inflammatory pathways, such as JNK, and increased TNF-α mRNA expression in adolescent mice hippocampus [103]. In addition, the exposure to these diets during the postnatal period can reprogram the hippocampal neurogenesis later at adolescence period, increasing the levels of inflammatory markers, such as TNF-α, IL-1β and IL-6 in hippocampus, hypothalamus, and frontal cortex. This inflammatory environment can induce oxidative stress and increase reactive oxygen species (ROS) in the offspring, while reducing the levels of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, being also associated with an impairment of spatial memory [102,104].

The upregulation of pro-inflammatory cytokines in the first months of life can influence the development of neural pathways regulating behaviour, such as the hypothalamic-pituitary-adrenal (HPA) axis, serotonergic system and BDNF levels [33]. The upregulation of HPA axis, impairs glucocorticoid receptor (GR) leading to higher susceptibility to stress, depression, and anxious behaviours [105]. HPA stimulation was also observed in rodent models after early weaning, being associated with persistent anxiety. Higher circulating levels of corticosterone can trigger anxious-like behaviours through modulation of GR-expressing neurons and downregulation of BDNF in the PFC [150]. Moreover, early weaning could also increase the accumulation of ROS species, at hypothalamus, especially in female offspring [151]. Consequently, this stress environment can also interfere with the activity of antioxidant enzymes in hippocampus and hypothalamus offspring. More specifically, it was observed a decrease in superoxide dismutase activity in the hippocampus of female offspring, while in males the effects were more pronounced at the hypothalamus, promoting a dysregulation in the ratio of SOD/CAT activity [151]. In addition, GLO1 activity has been shown to be upregulated at female offspring's hippocampus, while in male offspring this effect was observed in the hypothalamus [151].

The consumption of maternal trans-fat diets during pregnancy and lactation periods, besides increasing the production of proinflammatory cytokines, activates glucocorticoid receptors in the hippocampus of adult offspring, which could also lead to an anxious behaviour [106]. Moreover, maternal high-fat diet exclusively during lactation can lead to instability and loss of spines in cerebral cortex at juvenile offspring, even when there is a shift of the diet after weaning. This can cause a synaptic impairment and brain dysfunction at adulthood, which is associated with increase oxidative stress and lipid peroxidation during this period [107].

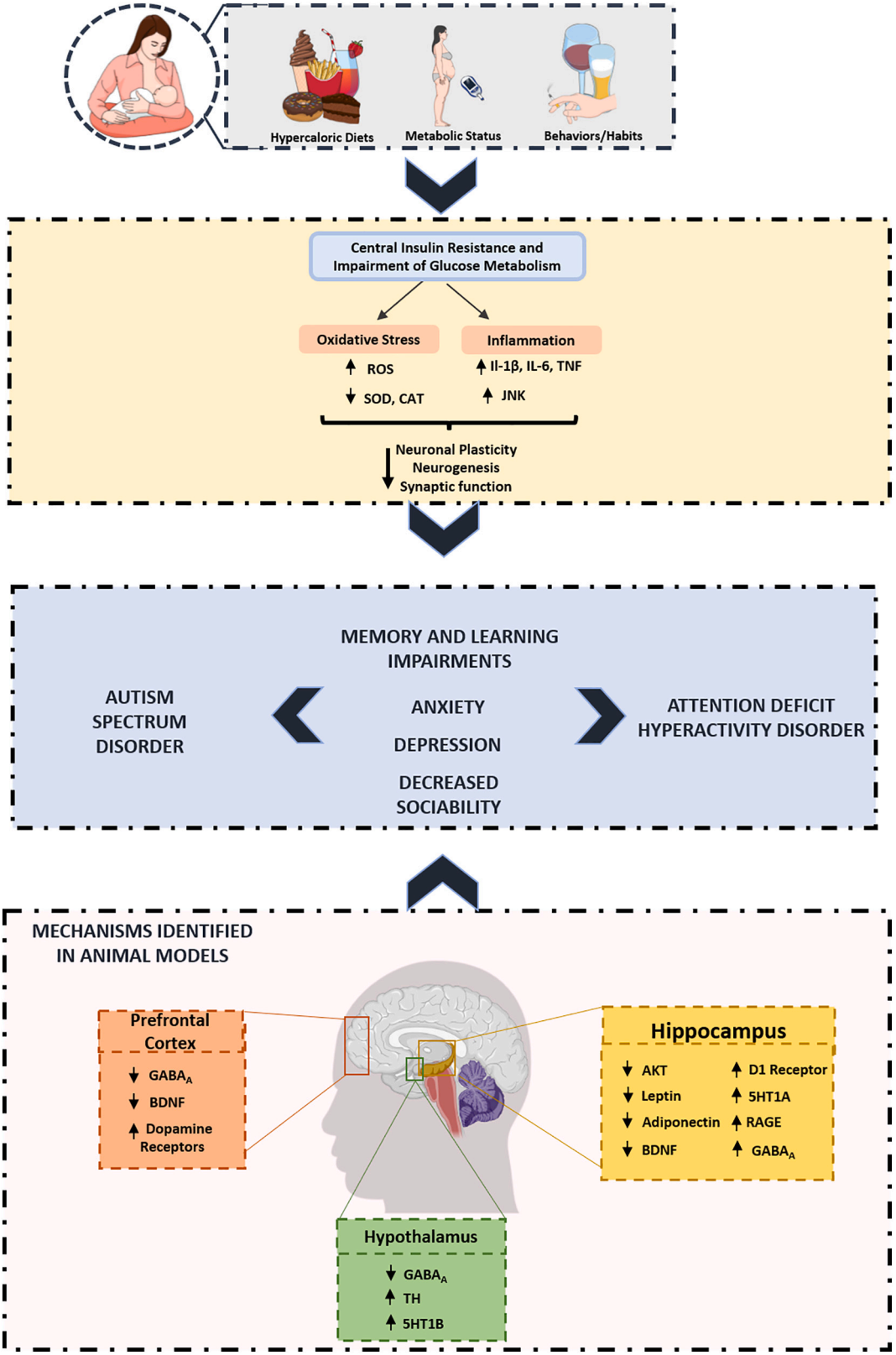

Fig. 2. The possible mechanisms underlying neurodevelopmental and behaviour alterations are still poorly understood. Nevertheless, the consumption of hypercaloric diets, the metabolic status and the behaviours adopted by the mother during breastfeeding period, may predispose the offspring for the development of central insulin resistance and impairment of glucose metabolism. As consequence, this can potentiate alterations in BNDF signalling, GABAergic, dopaminergic, and serotoninergic systems, in different central brain regions, affecting the proper neurodevelopment of the offspring.

5.3. Alteration of brain-derived neurotrophic factor

The last week of gestation and the first 2 weeks of postnatal development encompass a critical period for hippocampal neurogenesis. BDNF is considered an important regulator of brain synaptic transmission, plasticity, and growth playing a critical role in hippocampal long-term potentiation, which is a long-term enhancement of synaptic efficacy underlying learning and memory [108]. Since spatial learning and memory are dependent on alterations in synaptic plasticity and dendritic spine maturation, nutritional manipulation during development is likely to affect key neurotrophins and synaptic proteins which could be important in determining behaviour.

In adult rodent models, it has been demonstrated a correlation between the decreased expression of BDNF in hippocampus and cortex and negative outcomes in learning and memory processes [91]. Cellular actions of BDNF are mediated by binding to its receptor, and by activating cytoplasmic signalling pathways of kinases such as MAPK. MAPK have a regulatory role in neuroplasticity, proliferation, and cell differentiation [109]. The exposure to cafeteria diets during pregnancy or lactation periods potentiates an increase of MAPK expression in hippocampal region. This MAPK upregulation may suggest that the BDNF intracellular signalling could be upregulated to increase long-term potentiation via positive feedback. Consequently, this could affect memory performance at adolescence and adulthood phases [92,103]. Interestingly, although the exposure to an obese environment during perinatal period may promote the expression of genes related with synaptic plasticity like BDNF, it is accompanied by a decreased ratio of synaptophysin-to-synaptotagmin, a marker of impaired synaptic plasticity and cognitive function, suggesting that BDNF upregulation beyond normal levels may also be deleterious for synaptic plasticity [110].

The consumption of maternal high-fat diet may decrease the expression of BDNF in early phases of brain development. The exposure to this obesogenic environment during lactation period leads to a persistent BDNF decrease expression in male offspring hippocampus resulting in an impairment of spatial memory [94]. In young animals from diet-induced obese dams, the lipid peroxidation rate was found elevated in the hippocampus, suggesting that the reduced levels of BDNF could be due to oxidative stress [111]. The consumption of high-fat diet during postnatal period, has been shown to induce similar negative outcomes, suggesting that this period is crucial in the modulation and expression of BDNF in offspring [112].

5.4. Gabaergic system

GABA receptors mediate inhibitory neurotransmission, which regulates excitatory glutamatergic neurons. $GABA_A$ receptor, in particular, is a ligand-dependent Cl^- channel that, when activated, increases intracellular uptake of Cl^-, leading to membrane hyperpolarization and consequently, reduction of neuronal excitability. $GABA_A$ receptors have been linked to learning, memory, and mental development, and GABA serum and plasma levels have been considered as a potential peripheral marker for depression and other stress-related disorders at adulthood [113,114]. Maternal separation during lactation period was shown to originate a decrease in the expression of $GABA_A$ receptors in prefrontal cortex and evoked anxiety and depressive-like behaviour in adulthood [115,116].

Maternal high-fat diet exposure in perinatal period was shown to induce a downregulation of GABAergic neurons in prefrontal cortex of the offspring and consequently behavioural alterations, such as anhedonia, depressive- and anxiety-like behaviour, and memory impairment [118]. The consumption of maternal high fat-diets during pregnancy and lactation periods can be associated with a lower hypothalamic expression of genes involved in GABA system, which can interfere with brain modification and plasticity [117]. Interestingly, similar animal studies of maternal high-fat diets during lactation have also demonstrated that increased expression of $GABA_A$ receptor in the dorsal hippocampus could be involved in the risk of anxiety-like behaviour at adulthood, although the mechanisms of its increased expression were not addressed [4].

It has also been suggested that maternal high-fat diet enhances offspring hedonic feeding [119]. Since GABA neurons can mediate the reward system, changes in offspring GABAergic system may alter offspring reward system and influence feeding behaviour later in life [120]. Nevertheless, further studies on the remodeling of the reward system during early life are needed.

GABA synthesis is regulated by glutamic acid decarboxylase, which in adult brain exist in two isoforms (GAD65 and GAD67). A decreased expression of GAD67 has been associated with individuals with schizophrenia and ASD [121,122]. Therefore, a decreased GABA synthesis can negatively affect several physiological functions such as locomotor activity and cognitive expression, which are characteristic of schizophrenia and autism diseases [123,124]. A long-term exposure of maternal diet during lactation period has shown to cause a significant decrease level of GAD67 in brain male offspring, which it was accompanied by behavioural alterations characteristic of autism disease [61].

Specific glucose metabolites have also been shown to modulate GABAergic activity, namely the methylglyoxal (MG) and its detoxifying enzyme Glioxalase-1 [125]. However, MG was suggested to function as a $GABA_A$ agonist in physiological concentrations, modulating the anxiolytic behaviour. It was demonstrated that the low-dose treatment with MG can activate $GABA_A$ receptors in primary neurons promoting a decrease in anxious-like behaviour [127]. At adulthood mouse models, Glioxalase-1 (GLO1) expression was associated with an increase of anxious-like behaviour [126]. GLO1 overexpression increases the metabolization of MG and consequently leads to an increased anxious behaviour due a decreased $GABA_A$ receptor activation [127]. Nevertheless, such studies were performed in adult mice and using physiological methylglyoxal concentrations. Further studies on maternal glycation during perinatal period could be important to address the importance of these mechanisms during the suckling period and the risk of anxiety at adulthood.

5.5. Serotoninergic system

Serotoninergic system is involved in the neural regulation of mood and anxiety, being a target to the treatment of depression and anxiety disorders. The levels of serotonin increase in the hippocampus in the first 2 weeks of life in rodents and the proper serotonin receptor expression during the perinatal period is critical to establish a normal anxiety-like behaviour later at adulthood [128]. Therefore, early life insults can induce alterations in the neurodevelopment of the offspring. Stress exposure through maternal separation during breastfeeding was shown to cause an overexpression of serotonin receptor – 5HT1B - in the hypothalamus of the offspring, affecting eating behaviour [33]. In addition, stress exposure decreased offspring locomotor activity and the time spent in the center of the open field test, in female offspring, which is as indicator of altered emotionality and increased anxiety-behaviour [129].

In mouse models, maternal high-fat diet consumption during lactation promotes an increased expression of serotonin receptor and activation of serotonin pathway in the ventral hippocampus, together with increased $GABA_A$ activation in dorsal hippocampus in offspring, which was associated with an anxiety-like behaviour [4]. Additionally, there is an increased expression of tryptophan hydroxylase 2 (THP2) in hippocampus, the rate limiting enzyme in serotonin synthesis, while its mRNA expression is downregulated, suggesting post-transcriptional interference [103]. Interestingly, the exposure to the same type of diet in non-human primates, was also shown to increase the mRNA levels of THP2 in median raphe and decrease in dorsal raphe promoting an increased anxiety-like behaviour [130]. These findings suggest that maternal high-fat diet can have different effects upon expression and activation of serotonin system in specific cerebral regions.

Interestingly, the long-term maternal exposure of fructose diet induces decreased brain levels of 5-HT1A (end-metabolite of serotonin), with alteration in sociability and learning behaviour as in ASD [61]. These finding are similar to previous studies showing a significant lower serotonin turnover in patients with ASD [131]. The different diet composition adopted by the mother during the perinatal period can therefore induce different alterations in serotonin system, with an impact in offspring behavioural outcomes. Nevertheless, more studies assessing modulation of serotonin system during early life are needed.

5.6. Dopaminergic system

Dopamine is involved in the regulation of hunger, satiety, and motivation. Diet modifications such as high- and low-calorie diets are known to alter the expression of dopaminergic system-related genes in different brain areas, especially in hypothalamus and ventral tegmental area (VTA) [132]. In humans, impaired dopaminergic signalling was shown to be associated with ASD and ADHD [133,134].

An exposure to high-fat diets during the perinatal period can modulate the development and function of mesocorticolimbic dopamine system. After the consumption of maternal high-fat diets, adult offspring present increased tyrosine hydroxylase expression in VTA and in nucleus accumbens (NAc), and also a significant increase in dopamine and DOPAC content in NAc which can increase the risk of behavioural alterations [132]. Although the mechanisms involved are still to address, the increase of plasma leptin levels following a perinatal high-fat diet consumption was recently suggested as a modulator of dopamine system in adult offspring [132].

As mentioned, maternal high-fat diets during lactation can reprogram the dopamine system in mice. Recent studies have demonstrated that maternal high-fat diet produced a sexually dimorphic behaviour in offspring. In males, it was observed an hyperlocomotion, while in female an increased preference by palatable and sucrose food was found. Interestingly, these behavioural alterations were shown to result from an excessive dopaminergic action in target regions, despite the robust reduction of dopamine neurons firing, projection and dopamine release. Moreover, increased firing and excitability of postsynaptic dopamine 1 receptors, together with a decrease of dopamine 2 receptors activity, were observed in medium spiny neurons [135]. Interestingly, hyperactivation of D1 receptors and/or suppression of D2 receptors at the dorsal striatum region has been shown to be linked with autistic behaviours [136]. Furthermore, the analysis of frontal cortex RNA-seq of the offspring shows an increase expression of genes involved in dopaminergic and glutamatergic synapses [118,132].

The consumption of restrictive or hyperlipidic diets during pregnancy and lactation was associated with a reduced expression of enzymes responsible for dopamine synthesis, in particular tyrosine hydroxylase (TH) and dopamine decarboxylase in VTA of the offspring, with no behavioural alterations. Interestingly, in dams, it was observed an increased expression of both enzymes in VTA associated with a hyperactivity when exposed to open field test, suggesting a compensatory mechanism [137].

The dam-pup relationship is essential for the neuronal development of neonatal rats. The disruption of the neural process can affect the regions that control feeding behaviour. Maternal separation has been associated with eating behaviour and dopaminergic system alterations [139]. Maternal separation promoted higher palatable diet intake in the offspring and sexual dimorphism with an increased gene expression of dopaminergic receptors D1 and D2 only in the midbrain region of male offspring [138].

The combination of maternal high-fat diet consumption and postnatal stress can induce alteration increased the motivation to the consumption for palatable food in the offspring [140]. Additionally, the alteration in food-motivated behaviour shows to be correlated with alteration in mesolimbic dopamine system, which plays a key role in reward-related processes. In NAc of the offspring, it was observed a higher expression of a short D2 receptor isoform and dopamine transporter after the induction of both maternal insults [140,141]. As consequence, the modulation of dopamine system could increase the vulnerability to an obesogenic environment at adulthood, due to an increased food-motivated behaviour [140].

6. Concluding remarks and future perspectives

Factors such as maternal obesity, metabolic state, diet, and behaviour are not only important during pregnancy period but are also crucial during lactation. It has been shown that these factors can directly affect the metabolic state of the offspring, increasing the predisposition to develop insulin resistance, obesity, adipose tissue dysfunction, and, consequently, metabolic syndrome at adulthood. Additionally, lactation was shown to be a sensible metabolic window important to modulate neurogenesis and neurotransmission pathways in the offspring. Disturbances in these systems could predispose to the appearance of neurodevelopmental and neuropsychiatric diseases such as, ADHD, ASD, anxiety, and depression. Importantly, although some light has already been shed over the factors influencing programming of neurodevelopment and psychiatric diseases, the mechanism governing their development and the association with metabolic disturbances are still to disclose and need further investigation. Most of the experimental studies exploring this relation between maternal metabolic condition and diet with offspring metabolic and neurodevelopmental diseases were based on associations and are difficult to prove. The implication of the different mechanisms in such offspring's outcomes is difficult to understand, as well as the role of specific brain regions for such outcomes. On the other hand, loss-of or gain-of-function experiments have the risk of compensatory mechanisms and must be analysed carefully. Although this relation is apparently stronger than initially thought, the pathway for its disclosure is still long and difficult, and more mechanistic studies are necessary to address it. Given the common increasing incidence of metabolic, neurodevelopmental and psychiatric diseases, the identification of the intricate relation between them will certainly have a huge impact in human health, both at the levels of metabolic diseases monitoring and nutrition during pregnancy and lactation. It is crucial to increase the public awareness regarding the impact of maternal metabolism, diet and behaviours in the future generations and how can that imprint a long-lasting and harmful impact in offspring physiology.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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