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PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

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Evaluation of binge eating behaviour induced by intermittent access to High Sugar and Butter (HSB) diet in C57BL/6

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"Evaluation of binge eating behaviour induced by intermittent access to High Sugar and Butter (HSB) diet in C57BL/6"

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ABREVIATIONS

AC – Adenylyl Cyclase.

AN – Anorexia Nervosa.

Bdnf – Brain-derived Neurotrophic Factor.

BED – Binge Eating Disorder.

BN – Bulimia Nervosa.

cAMP – Cyclic adenosine monophosphate.

cDNA – complementary DNA.

D1– Type 1family of Dopamine Receptors.

D2 – Type 2 family of Dopamine Receptors.

DARPP-32 – Dopamine- and cAMP-regulated phosphoprotein.

DI – Discrimination Index.

dNTP – deoxyribonucleotide triphosphate.

DRD (1-5) – Dopamine receptor (1-5).

EDTA – Etilenodiamino acid.

Fri – Friday.

GABA – γ-Aminobutyric Acid.

HSB – High Sugar and Butter.

Kcal – kilocalorie.

Kg – Kilogram.

MC – Mesocortical Pathway.

ML – Mesolimbic Pathway.

Mon – Monday.

N. Accumbens – Nucleus Accumbens.

NOR – Novel Object Recognition.

NS – Nigro-Striatal Pathway.

PFC – Prefrontal Cortex.

PKA – Protein Kinase A.

PMSF – Fenilmetanosufonil flurorete.

PP-1 – protein phosphatase-1.

PYY – Peptide YY.

RS – Reward System.

Sat – Saturday.

SDS – sodium dodecyl sulfate.

SDS-PAGE – dodecyl sulfate polyacrylamide gel electrophoresis.

Subst. Nigra – Substantia Nigra.

Sun – Sunday.

TBS – Tris-Buffered Saline.

TBST – Tris-Buffered Saline + Tween.

TEMED – Tetramethylethylenediamine.

Thu – Thursday.

Tue – Tuesday.

UFMG – *Universidade Federal de Minas Gerais.*

VTA – Ventral Tegmental Area.

Wed – Wednesday.

ABSTRACT

It is known that high palatable food interacts with the complex systemic and neural systems that regulate hunger and satiety, and that it can culminate in the loss of control over food consumption, the core diagnose of binge eating disorder. Despite this fact, little is known about the neurobiology of this phenotype. The prefrontal cortex, which is part of the reward system of the brain, plays important roles in the control of impulsivity and goal-directed actions, which places it as an important target that could potentiate food seeking and contribute to the establishment of this disorder. The present work aimed to evaluate the binge eating behaviour induced by intermittent access to High Sugar and Butter (HSB) diet in C57BL/6 and also to evaluate how this phenotype affects anxiety and memory of mice as well as the transcriptional regulation of dopaminergic, GABAergic receptors in the prefrontal cortex of animals. To address these issues, two experiments were set (one of 4 weeks and one of 8 weeks). Upon arrival, male mice were divided in 3 groups: CHOW-d (that had daily access to maintenance diet), HSB-i (that had daily access to maintenance diet and 3 times a week had access to HSB) and HSB-d (that had daily access to HSB). In the last week of each experiment, animals were submitted to three behavioural tests: marble-burying test, light dark box and a repetition of the light dark box with the exception of the presence of food pellets in the light zone. In addition, in the 6th week of the 8 week experiment, animals were submitted to the novel object recognition test. Food consumption and bodyweight were measured throughout the experiment. The Prefrontal Cortex was collected for transcriptional analysis of *Drd1*, *Drd2*, *Gabbr1*, *Gabbr2* and *Bdnf* by qPCR and western blot analysis. The results showed that the HSB-i group established a pattern of food intake in which mice consumed significantly more kilocalories than the other groups on the days that HSB was available, and significantly fewer kilocalories on the complementary days. In all accesses days, this group also ingested significantly higher quantities of kilocalories in comparison with the other groups in the period of 2h and this consumption represented most of total 24h energy consumption (i.e. last access day, it represented 91%). Over the course of the time, binge episodes intensified and got more severe, which could be an indicative of reduced sensitivity and tolerance. Despite this large consumption by the HSB-i group, only the HSB-d group gained weight and increased adipose index throughout the experiment. In the 4 week experiment, HSB-i group showed a high motivational drive to obtain food, while chronic consumption of this diet induced an anxiolytic effect in HSB-d group, but these behaviors did not repeat after 4 more weeks of experiment, which could be related with environmental influences. No differences were found in the novel object recognition test. Despite the clear and severe binge eating phenotype achieved, only a downregulation of *Drd1* gene in prefrontal cortex of animals after 8 weeks of experiment was found. This result could be an indicative of a dopaminergic hypoactivity in this region, a reflex of other reward regions dopaminergic modulation, or also that in this binge eating protocol, the phenotype is not driven by dopaminergic and GABAergic alterations in the prefrontal cortex. Nevertheless, a replication of this study is mandatory to confirm these assumptions.

1. INTRODUCTION

1.1. Eating Disorders

Eating is one of the most essential behaviours for survival as it provides different substrates necessary for physiological maintenance and energy homeostasis. Inappropriate food intake can have severe neurological and physiological consequences as poor nutrition, developmental problems, obesity and eating disorders (Gahagan, 2012). Among these consequences, eating disorders encompasses a heterogeneous group of psychiatry diseases characterized by disturbances in the person's eating behavior and diverse negative outcomes, including impairments in cognitive, emotional and social functioning (Culbert, Racine and Klump, 2015; Volpe *et al.*, 2016). Different integrative personality, social, cultural and genetic aspects can induce eating disorders and lead to maladaptive fasting or overeating (Culbert, Racine and Klump, 2015).

Bulimia Nervosa (BN), Anorexia Nervosa (AN) and Binge Eating Disorder (BED) are the most common types of eating disorders diagnosed. While AN is characterized by food intake restriction leading to a significantly low body weight, BN is characterized by episodes of eating large amounts of food in a discrete period of time (binge eating) followed by compensatory behaviors, as purging and exercising (DSM V, 2013; Ham, Iorio and Sovinsky, 2015). In both cases individuals tend to prevent weight gain, present nutritional deficits and intense disturbances of self perception of their bodies (DSM V, 2013). BED, in another hand, is characterized by recurrent episodes of binge eating without inappropriate compensatory behaviours (as purging, fasting or exercising) and at the same time, is the core diagnostic feature of AN and BN (Mcelroy *et al.,* 2012; Brownell and Walsh, 2018; Burton *et al.*, 2018). Although all these disorders have major individual and social impacts, BED stands out for presenting the highest specific eating disorder prevalence, which is estimated to be between 2-5% of general adult population (Balodis, Grilo and Potenza, 2015; Hutson, Balodis and Potenza, 2018) and also to present a heritability ranging between 41 to 57% (Kessler *et al.,* 2013).

1.2. Binge Eating Disorder

Binge eating is characterized by recurrent episodes of eating unusual large amount of food in a brief period of time without compensatory behaviours (Goodman *et al.,* 2018). These episodes are associated with a behavior impairment known as

eating compulsion, in which individuals feel an uncontrollable internal drive to eat despite feelings as shame and guilt or even the feeling of being satiated (Berner *et al.*, 2017; Hutson, Balodis and Potenza, 2018; Moore *et al.*, 2018). Individuals who suffer from BED present wide functional consequences, such as impairments in quality of life and social well-being, increase risk of weight gain and development of obesity as well as psychiatric comorbidities (DSMV, 2013; Blasio *et al.* 2013; Lardeux, Kim and Nicola, 2015). Among these psychiatry disabilities, BED has been strongly associated with anxiety (Rosenbaum and White, 2013) and also suggested to impair different types of memory (Svaldi *et al.*, 2014; Eneva, Murray and Chen, 2017).

For a long time, BED was classified as "Eating Disorder not Otherwise Specified" (Wilfley *et al.*, 2007). It was only recently (2013) that this disorder was introduced as a distinct eating disorder in the 5th edition of Diagnostic and Statistical Manual of Mental Disorders (DSM) (DSM V, 2013, p.350-353). This enabled a better knowledge of BED's features, diagnose criteria, levels of severity and functional consequences (DSM V, 2013, p.350-353; Smink *et al.*, 2014; Kessler *et al.*, 2016). According to this manual, along with the binge episode, some characteristics of this disorder include: eating faster than normal, eating without feeling hungry and until feeling uncomfortably full, eating privately due to shame, guilt or self repulsion and feelings of loss of control (Leehr *et al.*, 2015). In addition, to be characterized as BED, it is expected at least one binge episode a week during the period of at least three months (DSMV, 2013).

The clinical assessment of binge eating can be challenging, once it evaluate private and personal aspects that, sometimes, are difficult to declare (e.g. guilt, shame) and recall (e.g. severity and recurrence of episodes) (Duarte, Pinto-gouveia and Ferreira, 2015). After the addition of BED in the DSM V, different screening tools, as self report questionnaires and structured interviews (e.g. Questionnaire on Eating and Weight Patterns Revised and Eating Disorder Assessment for DSM-5), have been updated to help BED's diagnose and improve the accuracy of the assessment (Yanovski *et al.*, 2014; Brownell and Walsh, 2018; Burton *et al.*, 2018). Despite the progress in the clinical diagnostic of BED, the neurobiological features of this disorder are still little understood, which has encouraged researchers to elucidate the genetic, neurochemical and physiological targets implicated in this behavior (Lardeux, Kim and Nicola, 2015; Kessler *et al.*, 2016; Novelle and Diéguez, 2018).

1.3. Neurobiology of BED

The homeostatic control of food intake is processed by a complex network encompassing the central nervous system, gastrointestinal organs, neuropeptides and hormones signals that together control hunger and satiety and maintain an energetic homeostasis (Morton *et al.*, 2006; Ferrario *et al.*, 2016). The non-homeostatic control of food intake involves the influence of food's hedonic properties on brain areas related to preference and wanting (motivated feeding), which consequently affects meal size and frequency (Ferrario *et al.*, 2016; Beaulieu *et al.*, 2018). Signals from the cognitive and reward brain areas can override homeostatic regulation, which consequently can lead to the loss of control over food consumption and maladaptive overeating (Novelle and Diéguez, 2018).

The communication that leads to this disturbance in eating behaviour is complex. Signals from the digestion and energy expenditure processes modulate external sensory, cortical and limbic structures involved in learning, consolidation of habits and executive control of decision-making, affecting sensitivity to reward (Berthoud, 2012; Perello *et al.*, 2014; Cassidy and Tong, 2017) (Figure 1). Concomitantly, inputs from the Pre-Frontal Cortex (PFC), limbic structures and regions of the Reward System (RS), and external sensory machinery influence directly the integrative energy sensor and also the motor cortex, which guides behavior (Berthoud, 2012) (Figure 1). Taken together, this bidirectional interconnection affects the sense of hunger and satiety, the interpretation of food cues and motivation, enabling the establishment of the loss of control over food consumption. Palatable foods, rich in fat and sugar, act as powerful reinforcements in this scenario, and repeated exposure to it consolidates learning, memory and motivational drive to ingest food, which contributes to overconsumption, the core diagnose of BED (Nestler, 2005; Alonso-alonso *et al.*, 2015; Schulte, Avena and Gearhardt, 2015).

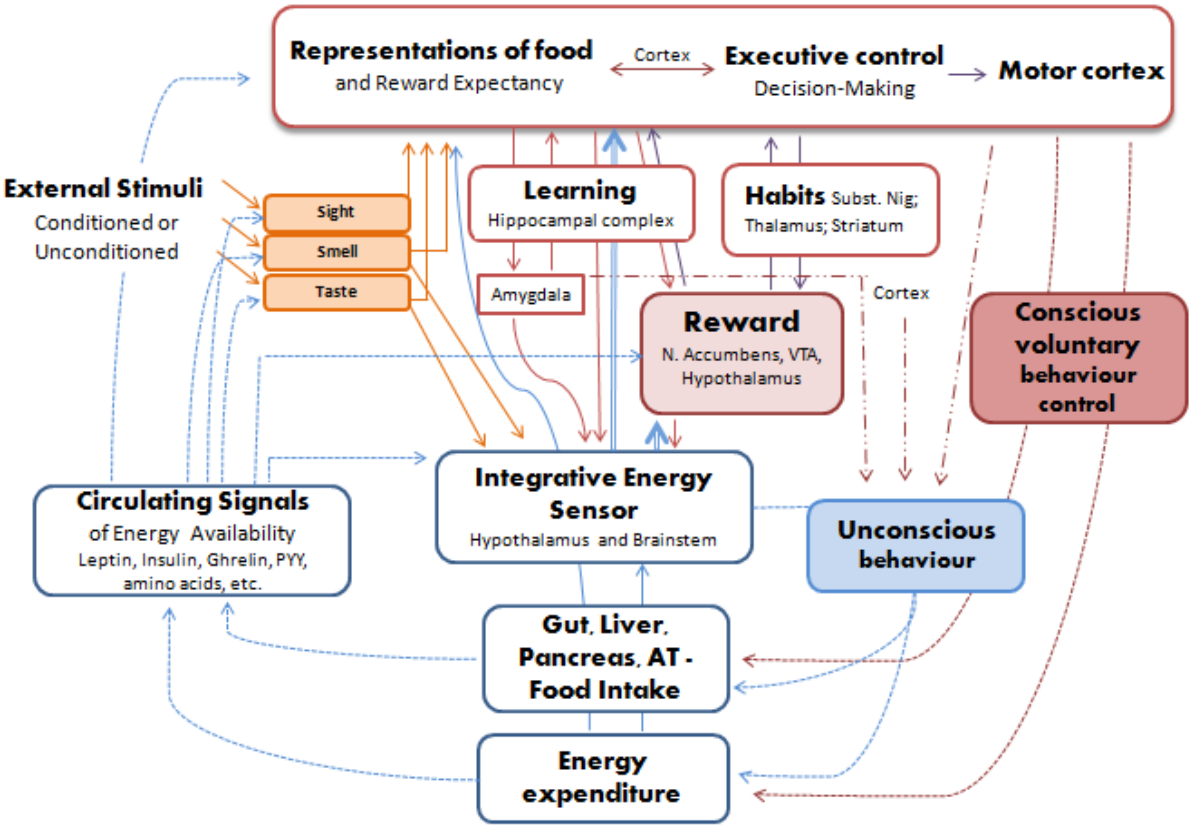
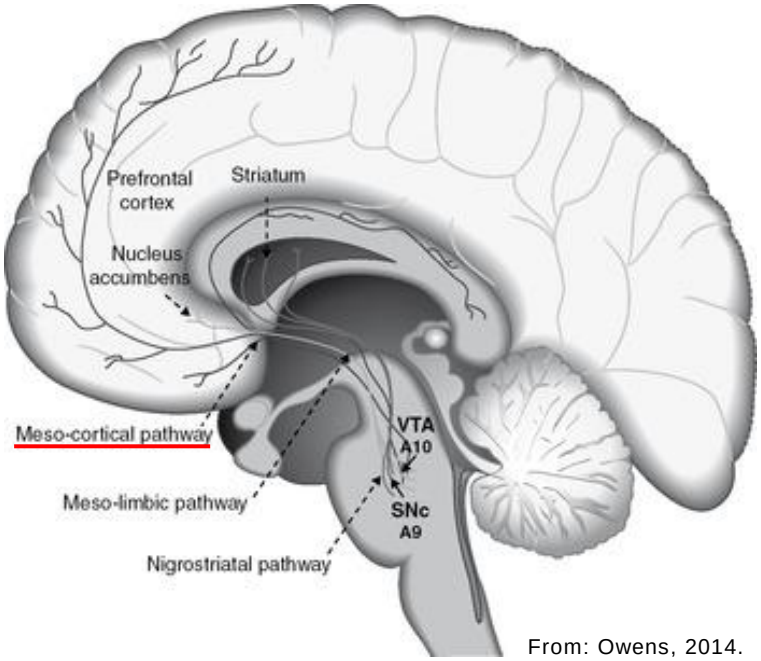


Figure 1. Major neural and integrative systems of the homeostatic (blue boxes and arrows) and non-homeostatic/ emotional (red boxes and arrows) control of the ingestive behavior. Bottom-up regulation of cognitive emotional processes by metabolic signals and their derivates is accomplished by: (i) circulating hormones and metabolites acting in the integrative energy sensor, but also in the external sensory processing system and in te cortical and limbic structures (blue arrows with broken lines), (ii) food intake information from vagal and spinal sensory neurons to all levels of neuroaxis, including cortex (full blue lines and arrows), (iii) neural signals generated from the hypothalamus distributed to areas involved in the reward and learning (duplicated blue line with full arrow). Taken together, these ascending modulatory mechanisms influence in part the reward directed to certain nutrients. Top-down modulation of food intake and energy expenditure by cognitive/emotional reward systems is accomplished by (i) direct external sensory input (sight, smell and taste) to the hypothalamus (dark yellow lines and arrows), (ii) input from cortex, amygdale and reward processing systems to hypothalamus (full red lines and arrows), (iii) inputs from cortex, amygdale and motor pathways that drives involuntary behavior (irregularly broken red lines and arrows) and inputs from motor pathways that drive voluntary behavior (evenly broken red lines in right). Taken together, these modulations affects the homeostatic control of food intake and influences meal size, pattern of ingestion and loss of control. N. Accumbens, Nucleus Accumbens; VTA, ventral tegmental area; Subst. Nigra, Substantia Nigra; PYY, peptide YY. Adapted from Berthoud, 2012.

Different circuitries encompasses the RS of the brain, a highly evolutionary conserved neuronal organization responsible for attributing hedonic values to different stimuli and responsible to override homeostatic control of food ingestion (Kelley, 2004; Hyman, Malenka and Nestler 2006; Volkow, Koob and Mclellan, 2016; Novelle and Diéguez, 2018). Among them, the dopaminergic projections in the (i) Nigro-Striatal Pathway (NS) plays important roles in control of movements and sensory stimuli, (ii) Mesolimbic Pathway (ML) are significant for cognition and emotional response to reward and (iii) Mesocortical Pathway (MC) are important for

cognition, memory, execution and motivated behaviour (Ayano, 2016) (Figure 2). Because BED, as other loss of control disorders, is highly associated with poor impulsivity control, impaired decision making and high reward sensitivity, a key participation of the MC and PFC in this process has been proposed (Volkow, 2002; Berthoud, 2012; Moore *et al.*, 2017; Miller and Cummings, 2017). Indeed, PFC interconnections with limbic structures provide biological substrates of motivational value guiding behavior accordingly with internal states and intention (Miller and Cohen, 2001; Pistillo *et al.*, 2015; Baldo, 2016).



From: Owens, 2014.

Figure 2. Dopaminergic circuitries in the Reward System. The mesocortical pathway is comprised by dopaminergic projections from the Ventral Tegmental Area (VTA) to the prefrontal cortex (PFC). The mesolimbic pathway is composed by dopaminergic projections from VTA to Nucleus Accumbens. Nigroestriatal pathway is composed by dopaminergic projections Substantia Nigra (SNc) to the Dorsal Striatum.

The PFC consists in the medial, lateral and orbital cortex region of the anterior portion of the mammalian brain, and is directly implicated in different cognitive functions, as execution of organized and goal-directed actions, memory, impulsivity control and decision-making processes (Goyal *et al.*, 2008; Kim and Lee, 2011; Fuster, 2015). Signaling in this pathway occurs via different neurotransmitters, and γ-Aminobutyric Acid (GABA) and dopamine are among the most acknowledged neurotransmitters with important roles in PFC function (Fuster, 2015).

GABA is the main inhibitory neurotransmitter of the nervous system and its proper inhibitory signaling is necessary for normal function of the nervous organization (Braat and Kooy, 2015; Fuster, 2015). Its inhibitory information is transmitted through two types of receptors: ionotropic $GABA_A$ and metabotropic $GABA_B$. $GABA_A$ receptors are ligand-gated chloride channels that results in fast inhibition when activated by GABA (Gauthier and Nuss, 2015). In contrast, $GABA_B$ is composed by the heterodimers $GABA_{B1}$ and $GABA_{B2}$ subunits, which are coupled to G-protein, and when activated by GABA induces the activation of potassium channels and results to a slow membrane hyperpolarization (Kasten and Boehm, 2015; Schwenk *et al.*, 2015; Breton and Stuart, 2017). This last receptor, responsible for the neuromodulatory effect of GABA, has been implicated in a wide spectrum of neuropsychiatry behaviors, including the development of substance abuse, anxiety and depression (Kumar *et al.*, 2013; Kasten and Boehm, 2015). Regarding its participation in diseases involving loss of control over a stimulus, it is has been proposed that different addictive compounds interact with G-coupled proteins expressed on inhibitory interneurons suppressing its spontaneous activity (Cruz *et al.*, 2004). In addition, transcriptional modulation of the gene encoding this receptor has also been reported in the PFC of animal protocols of addictive compounds, (Ribeiro *et al.*, 2012; Wearne *et al.*, 2016), and GABAergic inactivation in PFC of rats under a binge protocol increased binge size (Corwin *et al.*, 2016), corroborating that, indeed, neural inhibitory control deregulation in important reward areas, as PFC, is an important feature to be considered in the study of loss of control disorders.

Dopamine is a catecholamine with important roles in the reward-guided learning, motivation and also feeding and strongly important for PFC function (Walton and Bouret, 2018). Postsynaptic signaling occurs through dopamine activation of the G-protein coupled dopamine receptors, which are divided in two major groups: D1 type (excitatory) and D2 type (inhibitory) (Mizuta *et al.*, 2012; Beaulieu, Espinoza and Gainetdinov, 2014). D1-type (DRD1 and DRD5) are associated with $G\alpha_{s/olf}$ subunit and activates Adenylyl Cyclase (AC), while D2-type (DRD2, DRD3 and DRD4), coupled with $G\alpha_{i/o}$ subunit prevents or have no effect in AC activation (Kline *et al.*, 2018). Activity of AC via D1 receptors stimulation, induces the activation of Protein Kinase A (PKA) which phosphorylates several targets, including Dopamine- and cAMP-regulated phosphoprotein (DARPP-32) in Thr^{34}, which enables DARPP-32 to inhibit protein phosphatase-1 (PP-1) (Figure 3)

(Gould and Manji, 2005; Kline *et al.*, 2018). Dopamine D-2 receptor activation inhibits this downstream cascade (Figure 3).

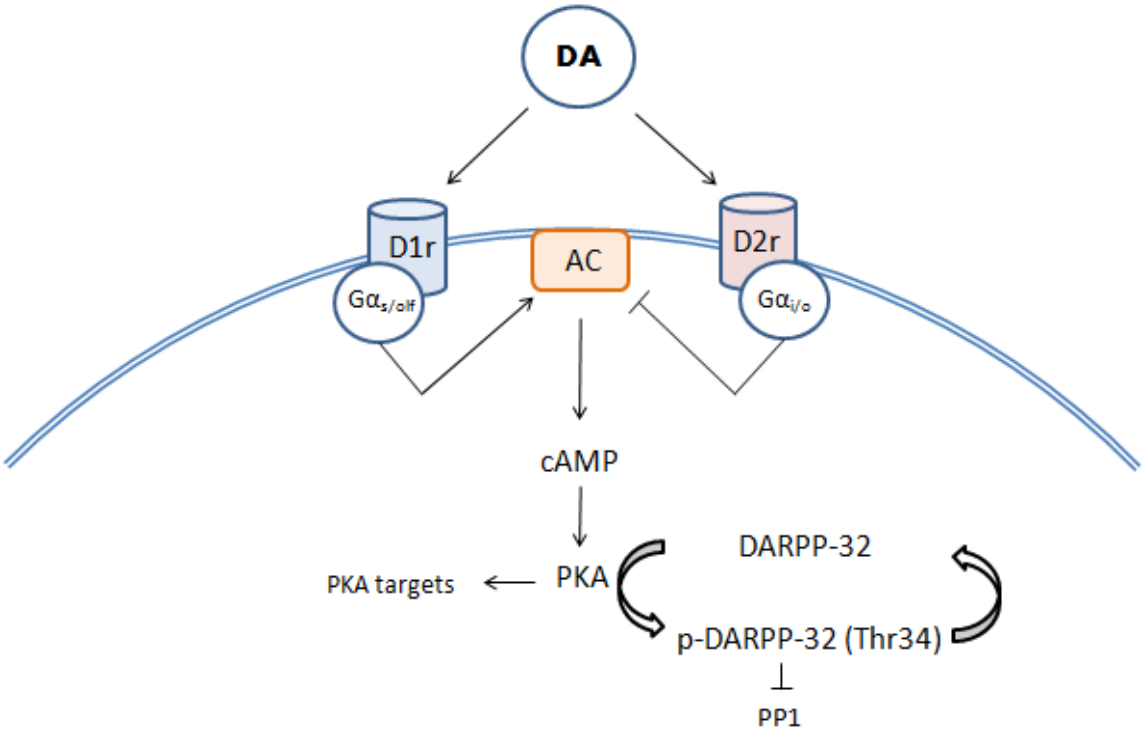


Figure 3. Dopamine receptors 1 and 2 activation. When Dopamine binds to D1-type receptors (DRD1 and DRD5), Adenylyl cyclase is activated, and through ATP expenditure, Cyclic adenosine monophosphate (cAMP) is formed and induces the activation of PKA. PKA phosporylates several targets, including DARPP-32 in Thr^{34}, which, in this form, has a key role to inhibit PP-1. D2-type (DRD2, DRD3 and DRD4) receptors activation inhibits this downstream cascade. DA, dopamine; D1r, D1-type dopamine receptors; D2r, D2-type dopamine receptors; PKA, Protein Kinase A; DARPP-32, cAMP-regulated phosphoprotein; PP1, protein phosphatase-1.

PP1 is widely expressed in the brain, and differently from PKA, it has no substrate specificity, being able to dephosphorylate a wide range of molecules, including PKA targets (Leslie and Nairn, 2018). Hence, dopamine response will occur accordingly to the balance of its receptors activation, and availability of p-DARPP-32 Thr^{34} could be an indicative of D1 or D2 receptors type activation. Both receptors type are widely expressed in the PFC interneurons and pyramidal cells allowing dopamine to influence in executive function, self-control and consequently ingestion behavior and motivation (Corwin *et al.*, 2016). Moreover, dopamine modulation in PFC has also impact in some of the psychiatry disabilities seen in BED, as anxiety (Zarrindast and Khakpai, 2015; Park and Moghaddam, 2017) and memory deficits (Wimber *et al.*, 2011; Puig *et al.*, 2014), placing the understanding of its modulation an important feature to be considered in the study of BED.

1.4. The Limited Access Model

Animal models are an important tool to access and understand different phenotypes. Despite the fact that no animal model can reproduce all characteristic and social influences on human disorders, it is unquestionable that they help to uncover some of the molecular mechanisms behind the different phenotypes. In the BED scenario, to be classified as binge, animals have to meet the criteria of this disorder, which is to consume large amounts of food in a brief period of time, and this quantity should exceed the one consumed by the control group in the same circumstances and period of time (Mathes *et al.*, 2009). With this precedent, there are different mouse models available, like the food restriction model, stress-induced hyperphagia model and limited access model (Turton, Chami and Treasure, 2017). This last one stands out for accessing the binge eating phenotype without promoting stresses or food deprivation that could cause neuronal and biochemical changes that affect animal's physiology and behavior and mask the intrinsic binge eating behavior neurobiological causes and consequences. To stimulate binge eating in this model, animals have access to a high palatable food for a limited period of time (usually 2h) and in an intermittent manner (3 times a week) (Corwin, Avena and Boggiano, 2011). As animals are never food deprived, this model transcribes the human behavior of overeating even in the absence of hungry or the biological need for nutrients and energy (Lardeux, Kim and Nicola, 2015).

Recent studies have shown the chronic consumption of High Sugar and Butter (HSB) diet have severe metabolic effects in C57BL/6, affects transcriptional regulation of some genes in PFC and when withdrawn, also plays a role in ethanol preference (Maioli *et al.*, 2015; Carvalho *et al.*, 2018). As this diet has never been tested to binge-eating and seems to modulate important brain regions that drive this disorder, the present work aimed to evaluate the binge-eating-like behavior in C57BL/6 via intermittent access to HSB. We also investigated the effects of the phenotype achieved by this source of palatable food in anxiety and memory of mice as well as the transcriptional regulation of dopaminergic, GABAergic receptors in the PFC of animals.

2. AIMS

2.1. Global

- Evaluate the binge eating behaviour induced by intermittent access to High Sugar and Butter (HSB) diet in C57BL/6.

2.2. Specifics

- Induce binge eating behaviour in C57BL/6 through intermittent access to High Sugar and Butter (HSB) diet in a 4 and 8 weeks protocol.
- Discriminate if binge eating behaviour is induced by hypercaloric diet or the intermittent protocol.
- Evaluate anxiety and memory deficits in mice.
- Investigate if the binge eating behaviour is correlated with differential transcriptional profile of dopaminergic and GABAergic receptor genes in PFC of mice.
- Evaluate if the availability of activated of p-DARPP-32^{Thr34} and PKA in the PFC of mice is associated with the phenotype and the transcriptional regulation pattern of the target genes.

3. MATERIAL AND METHODS

3.1. Animals

Two experiments, with different animals, were performed in order to better understand the impact of time over the establishment of binge eating behaviour: Experiment 1 lasted 4 weeks and Experiment 2 lasted 8 weeks. Both of them were undertaken in six weeks old C57BL/6 male mice (fourty for each experiment) provided by the Animal Facility of the *Universidade Federal de Minas Gerais* (UFMG). Upon arrival, mice were housed individually in standard cages in a room under 12/12 hour light/dark cycle with free access to water and standard maintenance chow (Nuvilab CR-1) for 1 week to acclimatise to the new room. This study was approved by the ethics committee of CEUA-UFMG (protocol: 333/2017) and all efforts were made to minimize animal suffering.

3.2. Dietary Composition

It is known that food rich in sugar and fat can induce loss of control over food consumption (Schulte, Avena e Gearhardt, 2015). Two types of diet were used in both experiments: (1) Standard chow diet (29,5% protein, 58,5% carbohydrate, 12% fat by energy, 3.00 kcal/g; Nuvilab®) is the commercial food for lab mice, and it is composed by cereal flour, animal protein flour, soy oil and vitamins and mineral (Maioli *et al.*, 2015), and (2) High Sugar and Butter diet (HSB) (16% protein, 36% carbohydrate, 48% fat by energy, 4.90 kcal/g) is a high fat and palatable diet that is efficient in inducing metabolic alterations in mice chronically fed with it (Maioli *et al.*, 2015). The HSB diet was manually prepared a day prior the start of the experiment and stored as pellet in order to reduce spillage. Preparation followed a strict protocol and was performed under appropriate conditions of cleaning and storage.

3.3. Limited Access to High Fat Protocol

The protocol applied was an adaptation of a previously model described by Corwin and Wojnicki (2006) and widely adapted in different studies and animal models of binge eating behavior (Berner, Avena and Hoebel, 2008; Bake, Morgan and Mercer, 2014; Bake, Hellgren and Dickson, 2017). In our study, two experiments were performed: Experiment 1 lasted 4 weeks and Experiment 2 lasted 8 weeks (Figure 4).

After acclimatization period, mice were randomly divided in three groups: CHOW-daily (CHOW-d), HSB-daily (HSB-d) and HSB-intermittent (HSB-i) as follows:

- CHOW-d: animals that had access to standard chow everyday throughout the experiment (n=20; 10 for each experiment).
- HSB-i: animals that had access to chow everyday and 3 times a week had access to HSB for 2 hours (n=20; 10 for each experiment).
- HSB-d: animals that had access to HSB diet everyday throughout the experiment (n=20; 10 for each experiment).

In order to confirm that binge eating behavior is established by high calorie and high palatable foods and not by a routine of exposure to an alternative diet a fourth group was determined:

- CHOW-i: animals that had access to HSB diet everyday and 3 times a week had access to chow for 2 hours (n=20; 10 for each experiment).

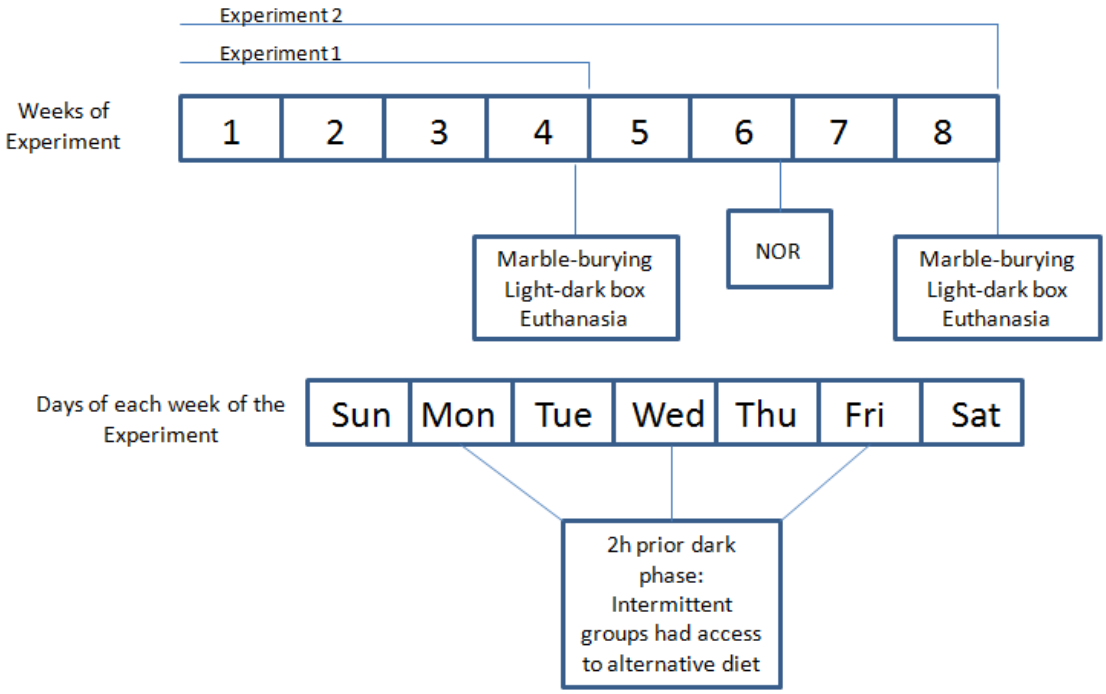


Figure 4 Experimental design. Experiment 1 lasted 4 weeks and experiment 2 lasted 8 weeks. In both experiments, acclimatization lasted 1 week and preceded the experiment. In the end of 4th and 8th week animals were submitted to marble-burying and light-dark box test and were euthanized after light-dark box test. In the end of 6th week of experiment 2, animals were submitted to the novel object recognition (NOR) test. In all days of the week, animals had access to their dietary treatments and water. Three days a week (Monday, Wednesday and Friday) intermittent groups (HSB-i and CHOW-i) had a 2h access to the alternative diet of their treatments together with their previous diets. These accesses occurred 2h prior dark phase. Mice were never food deprived and had always access to water. Sun - Sunday; Mon - Monday; Tue - Tuesday; Wed - Wednesday; Thu - Thursday; Fri - Friday and Sat - Saturday.

All accesses periods occurred in the 2h prior the dark phase on Mondays, Wednesdays and Fridays of each week and, during these periods, mice from intermittent groups (HSB-i) had access to both diets, so that they could choose between them. Mice were never food deprived and had always access to water. Body weights were recorded weekly. Food intake was measured daily by weighing pellets given in the day (or in the 2h access period) and pellets after consumption. The weight of food intake in grams were converted to energy (kcal) and normalized by the mouse body weight of the week in order to better compare energy consumption between groups.

In the 6th week and in the three last access days of 4th and 8th week, animals were submitted to behavioural tests (Figure 4). In the last day of each experiment, and after the 2h access period, animals were euthanized and PFC was collected and store in -80°C until processing.

3.4. Behavioural Tests

On the last three days of access (4th and 8th weeks) period and prior these accesses time, three behavioural tests were performed, one in each day, in order to avoid interaction between tests (Figure 4). The first of them was the marble-burying test which establishes an index of obsessive-compulsive-like behaviour (Deacon, 2006). In this test, each mouse was individually placed in a standard cage with 5-cm thick sawdust bedding, and 18 glass marbles were arranged in three rows of six units evenly distributed (Figure 5A). Mice were observed for the burying behavior and after 15 minutes the number of marbles buried (that had 2/3 of their depth covered by sawdust) was counted.

The second test was the light-dark box, which assess anxiety-like behavior in rodents (Bourin and Hascoët, 2003). The apparatus consisted in a box divided in two compartments: 2/3 of the box was white and brightened and 1/3 was black and covered. Both sides were connected through a small door. Each mouse was placed in the dark compartment, and after the door that connected both sides were opened, the mouse had 5 minutes to explore both compartments (Figure 5B).

The third test consisted in an adaptation of the light dark-box. The apparatus was the same box explained previously with the exception of the introduction of pellets of food in the middle of the light and bright zone (Figure 5C). The food

placed in this zone corresponded to the food that the animal would have access in the access period. As the light zone represents an aversive environment to the mouse, this methodology allows us to assess risk-taking and motivation to obtain food in these animals right prior an access period. In both light-dark box tests the apparatus was cleaned with 70% ethanol after each test and only after it was dried the next subject was tested. Tests took place in an environment free of sounds and experimenter presence. All tests were recorded and analyzed using Xplorat Software (Tejada, Chaim and Morato, 2018). The latency to enter for the first time the light compartment, the number of transitions between compartments and the time spent in the light zone were used to evaluate anxiety-like behavior and motivation.

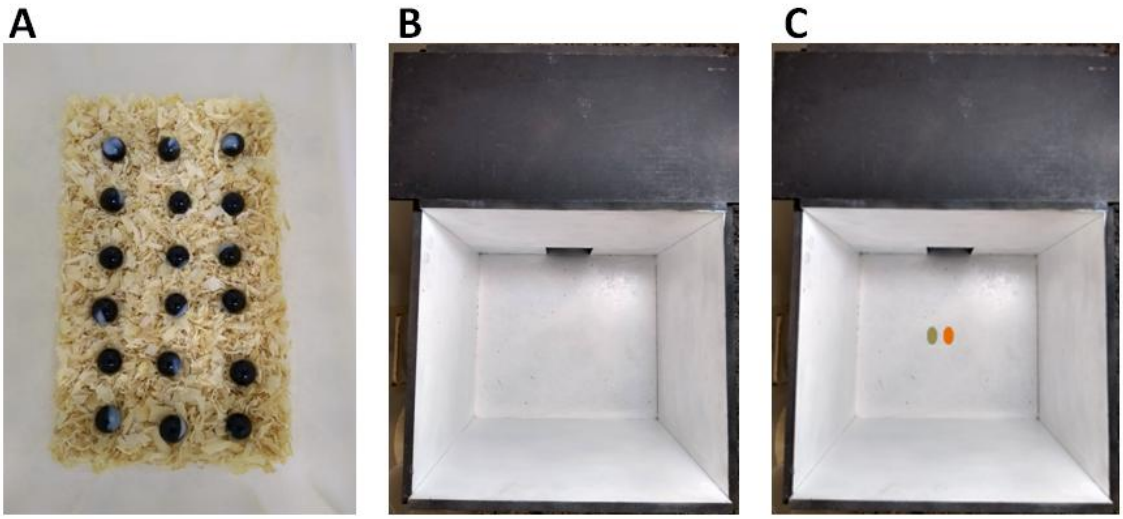


Figure 5 Top-view of marble-burying and light-dark behavioural test apparatus. The marble-burying test was performed in a cage with 5cm thick sawdust bedding and 18 glass marbles evenly distributed in three rows of six units (**A**). The light-dark test was performed in a wooden box divided in two compartments: 2/3 of the box was white and brightened and 1/3 was black and covered. Both sides were connected through a small door (**B**). An adaptation of the light-dark test was performed in the same apparatus of the traditional test with the introduction of the access pellets in the center of the white zone (**C**). All images are displayed as the view from the top of the apparatus.

On the 6th week of the experiment, in non-accesses days, mice were subjected Novel Object Recognition (NOR) test to evaluate general hippocampal and cortical dependent contextual learning (Chawla *et al.*, 2017). The apparatus consisted of a clear wooden box (30 cm^2 square chamber and 18.5 cm height). A day prior the start of the test, animals had 5 minutes to free explore the box and acclimatized with the new environment. After 24 hours, in the training trials, two

identical objects were placed in opposite sides of the box, and animals had 5 minutes to free explore the box and objects (Acquisition) (Figure 6). Animals that did not reach the criterion of 20 seconds exploration of both objects were excluded from analysis (Leger *et al.*, 2013). Object exploration was defined as head and nose orientation to at least 2 cm around the object. After 2 hours, one of the familiar objects was changed for a new one (second object) and animals were placed again in the box and had again 5 minutes to free explore the box and objects (2h Recall) (Figure 6). After 24 hours, the previously new object was replaced with a different one (third object) and mice were tested again for 5 minutes (24h recall) (Figure 6). Arena and objects were cleaned with ethanol 70% between each mouse trial in order to avoid odor cues.

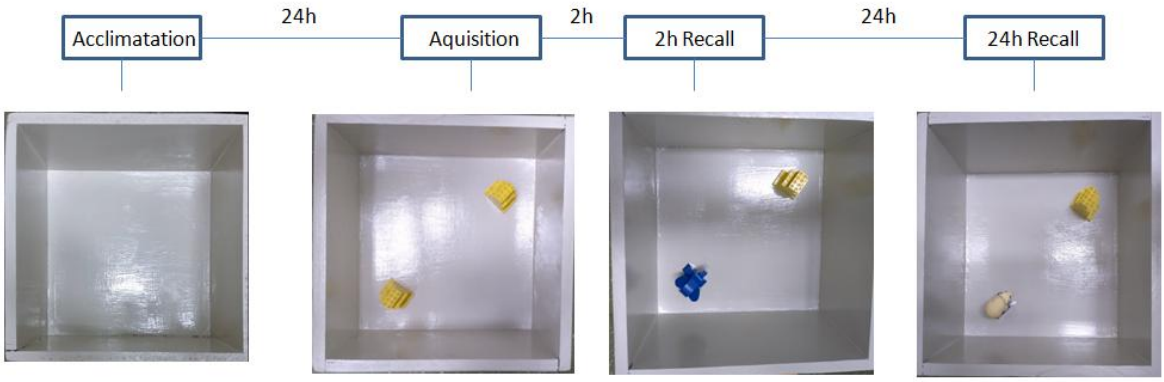


Figure 6 Novel object recognition test. First, animals freely explored the apparatus for 5 minutes in order to acclimatize to the new environment. After 24h, two identical objects were placed in opposite sides of the chamber and animals had 5 minutes to explore (acquisition). After 2h, one of the previously object was replaced to a new one, and animals were tested for 5 minutes (2h recall). After 24h, the different object from the previous test was replaced to a new one, and animals had 5 minutes to free explore the box and objects (24h recall).

NOR test was recorded and analyzed with Ethovision XT 14 (Noldus, Netherlands) in 1 minute temporal intervals. An area of 2cm around the object was defined as exploration arena (Figure 7A). Multiple body point was selected and exploration was considered when head and nose were inside the exploration arena (Figure 7B). The exploration time in each object (familiar and novel) was considered for analysis, and the Discrimination Index (DI) of the novel object was calculated as DI = (Novel Object Exploration Time/Total Exploration Time)–(Familiar Object Exploration Time/Total Exploration Time) × 100) (Arqué *et al.*, 2008).

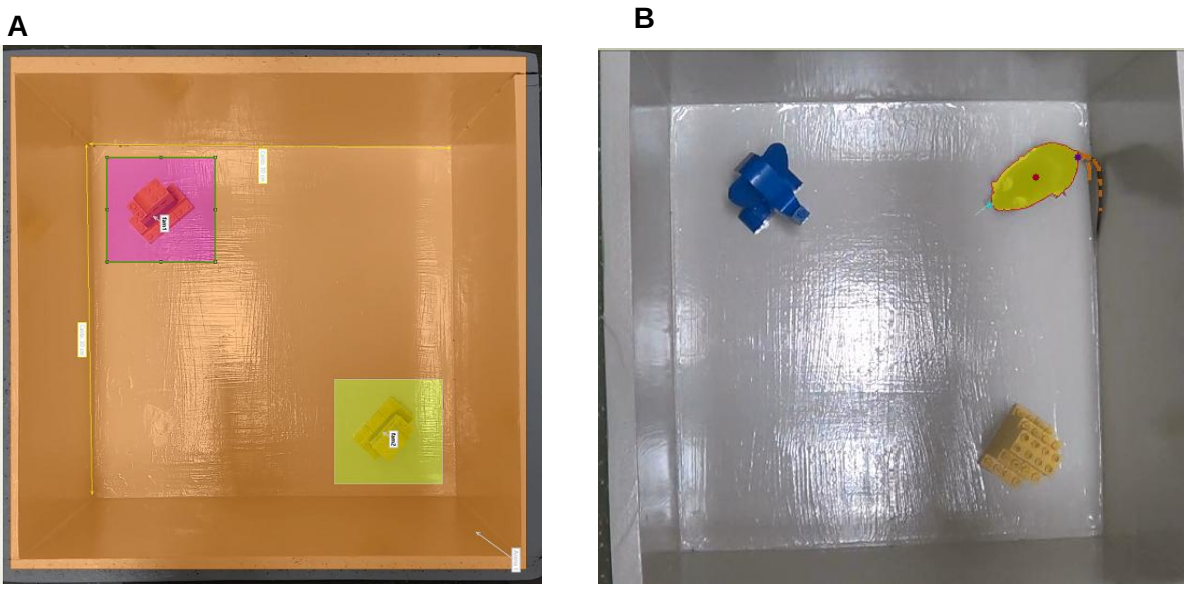


Figure 7 Novel object recognition test parameters. (**A**) An arena of 2cm distance from each corner of the object was selected to define the exploration area (yellow and pink square) inside the total box arena (orange square). (**B**) Multiple body point was selected in the software in order to consider exploration only when head and nose was pointing and inside the exploration arena. Nose point is indicated in the blue spot; body centre in the red spot and tail in the purple spot as indicated in the mouse.

3.5. Total RNA isolation

Brains were dissected on ice and the PFC removed and stored at -80°C. Total RNA was extracted using ReliaPrep^{TM} RNA Tissue Miniprep System (Promega, São Paulo, Brazil), according to manufacturer's instructions. Samples were quantified using DeNovix DS-11 (DeNovix, Delaware, EUA) and the integrity of RNA was tested.

3.6. Reversal transcription and real-time PCR (qPCR)

The complementary DNA synthesis (cDNA) was performed with oligo (dT20) primers (Prodimol Biotecnology, Belo Horizonte, Brazil), dNTP mix (10mM), Reaction Buffer 5X (Thermo Fisher Scientific, São Paulo, Brazil), Ribolock RNase Inhibitor (Thermo Fisher Scientific) and RevertAID® Reverse Transcriptase (Thermo Fisher Scientific), according to manufacturer's instructions.

Dopamine and GABAergic primer sequences were obtained from Carvalho *et al.* (2018) and *Bdnf* primer sequences were obtained from Magalhães *et al.* (2017).

The transcript expression levels of target genes were measured using the CFX 96TM Real-Time system thermocycler. The real-time PCR reactions for each gene were performed using 10µL of Kappa SYBR® Fast qPCR Kit Master Mix (Kappa Biosystems, São Paulo, Brazil), 1µL of cDNA (10ng/µL), 0.4µL of primer solution (10 pM) and 8.2µL of ultra-pure water (Thermo Fisher Scientific, São Paulo, Brazil). In all reactions, a negative control, without cDNA template (NTC), was tested, and the final reaction volume was kept as 20µL. For each pair of primer used, a standard curve with six concentrations (40ng, 20ng, 10ng, 5ng, 2.5ng and 1.25ng/µl) was performed. Reactions were pipetted in triplicate for the standard curves and in duplicate for quantification. PCR amplification was performed without the extension step (95°C for 3 min, followed by 40 cycles of 95°C for 3 secs and 60°C for 20 sec). Fluorescence levels were measured during the last step of each cycle (60°C). Efficiency was calculated by the software Bio-Rad CFX Manager® (Biorad), and only values within the interval of 95-105% were accepted. The dissociation curves for each primer pair were analyzed, and no spurious products or primer dimers have been detected. The relative quantities of the transcripts were calculated by the delta–delta Ct method using the geometric average of the two reference genes (*Gapdh* and *Bact),* according to Vandesompele *et al.*(2002).

3.7. Western Blot Analysis

Animals PFC were sonicated in 100µl of lysis buffer containing: 0.5µl Triton X100, 0.2 µl EDTA (Etilenodiamino acid, 10mM), 1µl of E64 (10µM), 1 µl of Pepstatin A (1µM), 0.2µl of Sodium Ortovanate (0.5mM), 1µl of PMSF (Fenilmetanosufonil flurorete, 2mM), 1 µl of HALT^{TM} Phosphatase Inhibitor Cocktail (Thermo Scientific) and 96µl of TBS 1X (pH 7.4). Samples were always maintained in ice, and after sonication, samples were centrifuged at 14000 x g, in 4°C for 20 minutes and the supernatant was pippeted to a clean tube. Protein content was quantified through Qubit® Protein Assay Kit (Molecular Probes, Life Technologies). 40µg of protein (100 µg for p-DARPP assay), diluted in lysis buffer, were denaturated at 100°C for 5 minutes and then, 20µl of this solution was applied in the SDS/PAGE gel (made mixing the 12% with 20% gel). For the 12% gel, 4.0335mL of solution A; 2.5mL of Solution B; 0.1mL of SDS 10%; 3.35mL of distilled water; 70 µl of PSA 10% and 7µl of TEMED were used. For the 20% gel, 6.67mL of solution A; 2.5mL of Solution B; 0.1mL of SDS 10%; 670µL of distilled water; 70 µl of PSA 10% and 7µl of TEMED were used (Table 1).

Table 1. SDS/PAGE gel components. All solutions are diluted in distilled water and pH adjusted with HCl.

Solution A	11.1g of Acrylamide; 0.4g of N'N' Bis Acrylamide for 50mL solution.
Solution B	9.075g of Trisbase for 50mL solution; pH 8.8.
SDS 10%	1g of Sodiumduodecysulfate for 10mL solution.
PSA 10%	0.2g of Ammonium Persulfate for 2mL solution.
TEMED	Tetramethylethylenediamine

Running buffer was prepared with 25mM Tris, 25mM Glicine (pH 8.3) and 0.1% SDS, and proteins were separated after running 2 hours and 45 minutes at 180V. After running, samples were transferred to a nitrocellulose 300mm width membrane (Sartorius Stedim, Biotech, Germany) using a transfer buffer with 14.42g glicine, 3.03g tris base, 200mL methanol, 800mL of distilled water, for 1 hour and 10 minutes at 100V. Membranes were incubated with Blocking Solution 3% (10mL TBST, 0.3g BSA –fraction V) for 10 minutes at room temperature in the shaker. After blocking step, membranes were incubated with the following antibodies: β-actin (41.7kDa; Antibodies), PKA (phosphor Ser99, GeneTex, 45kDa) and p-DARPP-32 (phosphorylated in Thr34, 23kDa, GeneTex). Antibodies were diluted in TSB 1X (pH 7.4) in the concentration of: 1:1000 for PKA and β-actin and 1:100 for p-DARPP-32. Samples were incubated overnight at 4°C. After this step, membranes were washed 3 times for 5 minutes with TBST in shaker. Posteriorly, membranes were incubated with peroxidase secondary antibody – rabbit IgG (Vectastain ABC kit- #PK-4001) for 30 minutes in room temperature and shaker. Membranes were then washed 3 times for 5 minutes with TBST. ABC reagent (Vectastain ABC kit- #PK-4001) was pipetted over the membrane, which was incubated in this reagent for 30 minutes in the shaker. Both secondary antibody and ABC reagent were diluted in blocking solution as suggested by manufacturer's instructions. Finally, after the last washing step, DAB (ImmPACT TM DAB – Peroxidase Substrate kit- SK4105, VECTOR) was diluted according to manufacturer's instructions and applied in the membrane for 2 minutes in shaker. After this brief period, membranes were washed in running water to stop DAB's reaction and membranes were dried until posterior scanning. Bands were analyzed through ImageJ software (US National Institutes of Health).

3.8. Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 6.0 software. All data were analyzed for Gaussian distribution using the Shapiro-Wilk normality test. Food intake (24h, 2h and percentage of intake in access days) and weight data were analyzed by two-way analysis of variance with repeated measures (ANOVA; groups x day of study) followed by Bonferroni post-hoc test. Adiposity index, marble burying and light-dark box behavioral data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. The comparison in the light-dark box between paired groups from 4 and 8 weeks experiments was analyzed by unpaired t-student test. Novel object recognition test was analyzed by paired t-student test. Transcription data were analyzed by one-way ANOVA followed by Bonferroni post-hoc test for 4 weeks experiment and by Kruskal-Wallis followed by Dunn's multiple comparison test for 8 weeks experiment. All data are presented as mean ± SEM. $p<0.05$ was considered statistically significant. ANOVA data are represented as F (DFn, DFd) followed by post-hoc test value and p value. t-student data are represented as t, df followed by p value.

4. RESULTS

4.1. Daily food intake

No differences were found between HSB-d and CHOW-i, indicating that, indeed, high palatable food is necessary to promote binge eating behaviour and not simply a routine to an alternative diet (Appendix A). Therefore, CHOW-i group was excluded from further analysis and discussion.

Food intake was measured daily, converted into kilocalories and normalized by mice body weight of the week. Total daily 24-h energy intake was significant influenced by the schedule of diet exposure ($F(2, 26) = 82.38$, $p<0.0001$), time ($F(55, 1430) = 69.89$, $p<0.0001$) and the interaction between them ($F(110, 1430) = 19.31$, $p< 0.0001$). The CHOW-d group presented relatively constant kilocalorie ingestion throughout the fifty six days of experiment. In contrast, HSB-d group revealed a fluctuation in energy ingestion. This group either consumed higher kilocalories than the CHOW-d group (which occurred in most days) or equal, but never less. HSB-i group, in another hand, established a pattern of ingestion in which mice consumed significantly more kilocalories than the other groups on the days that HSB was available (Mondays, Wednesdays and Fridays), and significantly fewer kilocalories on the complementary days in which HSB was not available (Tuesdays, Thursdays, Saturdays and Sundays). This pattern started in the 14th day and remained through the rest of the experiment. Moreover, in the second month of the experiment, in the non-accesses days, HSB-i group ingested fewer kilocalories than the basal ingestion of the control group (Figure 8).

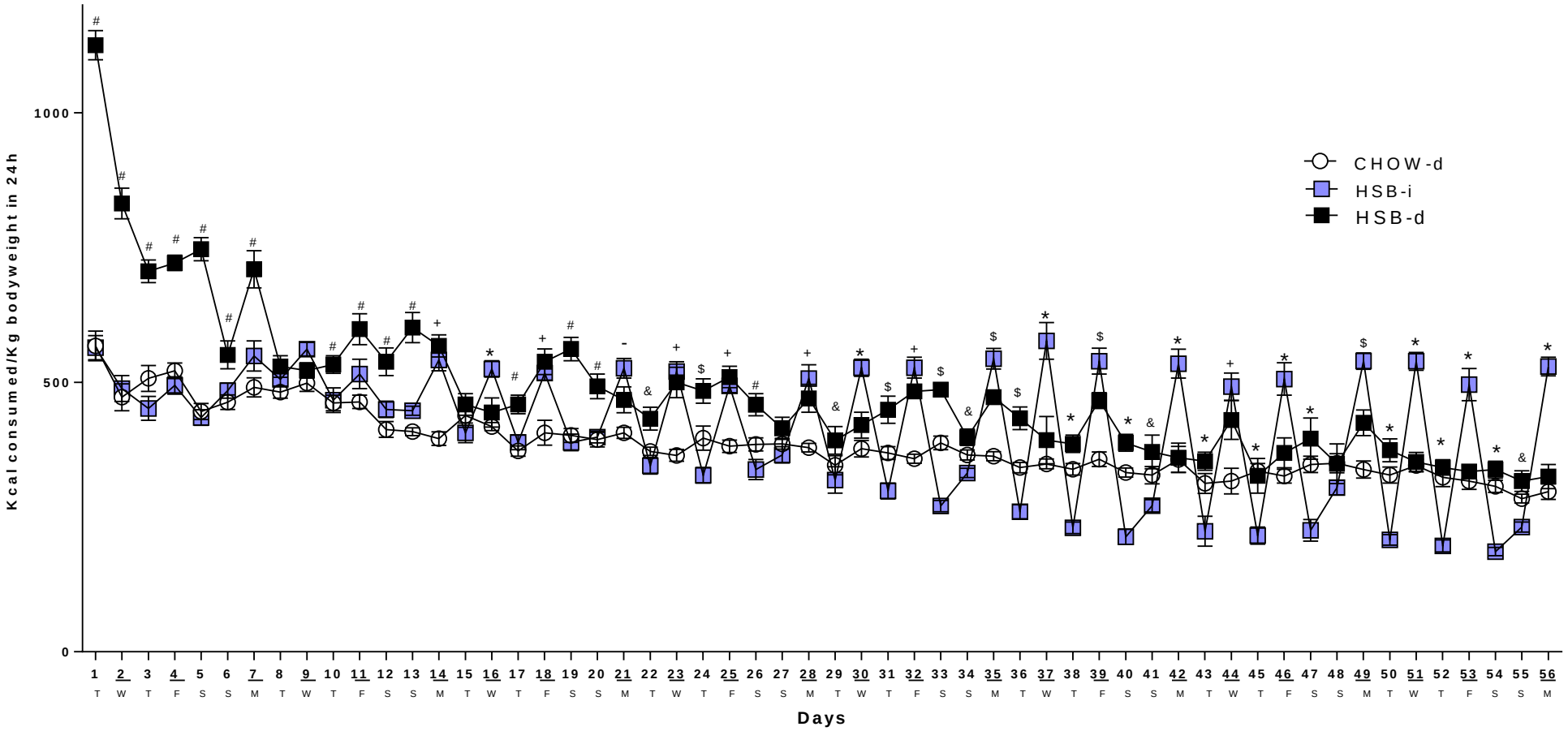
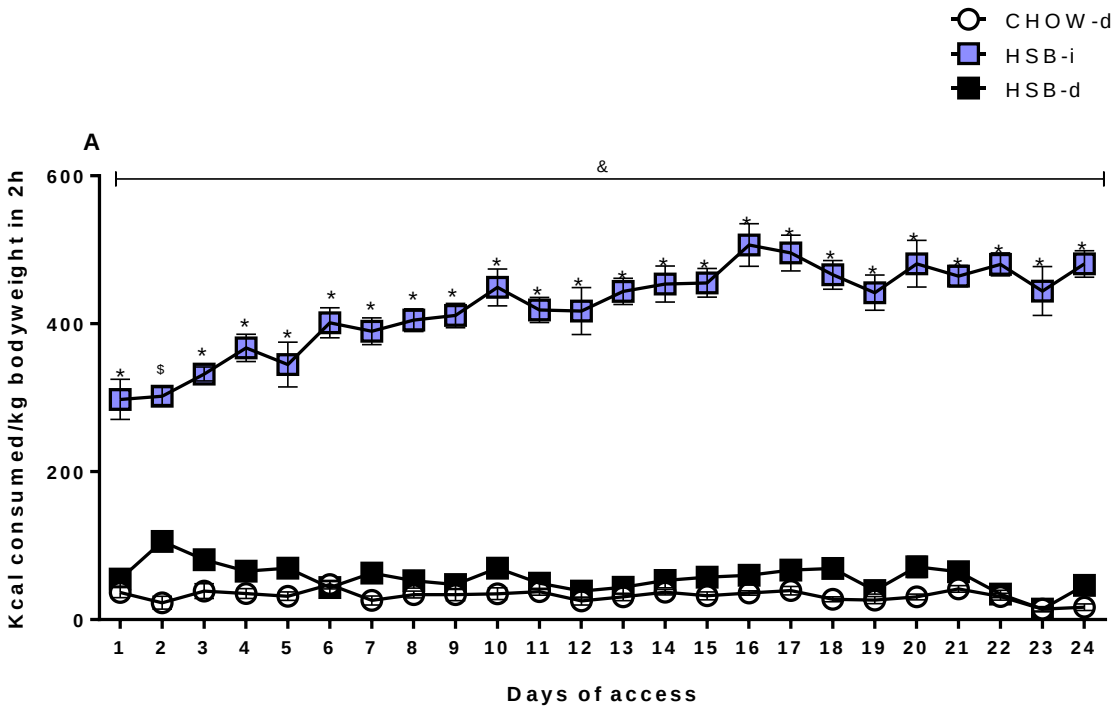


Figure 8 Total daily 24h energy consumption (kcal/kg of body weight). Days 1-7, 10, 11, 12, 13, 17, 19, 20 and 26 (#) $p<0.05$ for HSB-d vs CHOW-d and HSB-i. Days 14, 8, 23, 25, 28 and 32 (+) $p<0.05$ for CHOW-d vs HSB-i and HSB-d. Days 16, 30, 37, 38, 40, 42, 43, 45, 46, 47, 50, 51, 52, 53, 54 and 56 (*) $p<0.05$ for HSB-i vs CHOW-d and HSB-d. Day 21 (-) $p<0.05$ for HSB-i vs CHOW-d. Days 22, 29, 34, 41 and 55 (&) $p<0.05$ for HSB-i vs HSB-d. Days 24, 31, 33, 35, 36, 39 and 49 ($) <0.05 for differences between all groups. Underlined days correspond to the accesses days (Mondays, Wednesdays and Fridays) as indicated. Data are represented by mean±SEM. Two-way ANOVA with repeated measures followed by Bonferroni post-hoc test.

4.2. Accesses days food intake

Food intake on accesses days were measured and normalized by the week body weight. The schedule of diet exposure (F (2, 26) = 680.8 p< 0.0001), time (F (23,598) = 6.623 p< 0.0001) and the interaction of both (F (46,598) = 9.483 p< 0.0001) significantly influenced the kilocalorie consumption on the groups. In all accesses days, HSB-i group ingested significantly higher quantities of kilocalories in comparison with the other groups (p< 0.05 for HSB-i vs CHOW-d and HSB-d) (Figure 9A). In addition, when comparing the first access day with the last one in HSB-i group, a significant increase in consumption was evidenced (t=5,389 df=8, p=0.0007 for first day vs last day). This high consumption in 2h accounted for most of the total 24h access day energy consumption, which was influenced by the diet (F (1, 432) = 8521 p<0.0001) (Figure 9B). In the first access day, mice from HSB-i group ingested 60% of their total 24h energy from HSB diet in 2h (t= 6.401, p<0.05). Over the accesses days, this consumption increased, achieving 95% (t=27.90, p<0.05) on the twentieth day and 91% (t=25.39, p<0.05) on the last access day showing the progression of the binge eating behaviour (Figure 9B).



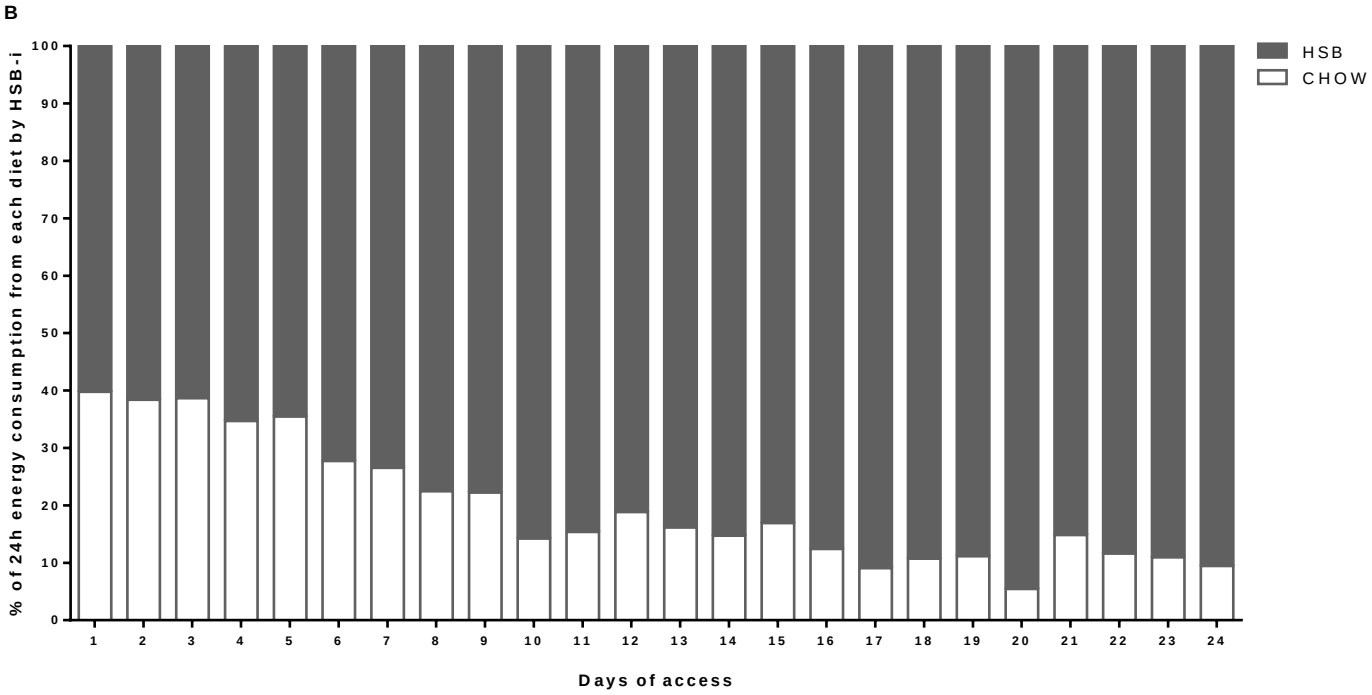


Figure 9 Access day's food intake. (**A**) Kilocalories consumed in 2h normalized by body weight. In all days, HSB-i consumed significantly higher kilocalories than the other groups (*, p< 0.0001 for HSB-i vs chow-d e HSB-d). In day 2, all groups presented differences in consumption ($, p< 0.05 for CHOW-d vs HSB-i vs HSB-d). In addition, in the last access day, animals from HSB-i group ingested significantly higher calories when compared with HSB-i in the first access day (p<0.05 for last day vs first day). (**B**) Percentage of 24h energy consumption from each diet by HSB-i on accesses days. In all accesses days, HSB-i group ingested more kilocalories from the HSB diet than the chow diet (p<0.001). Data are represented by mean±SEM for (**A**) and mean for (**B**). Two-way ANOVA with repeated measures followed by Bonferroni post-hoc test for both A and B, and t-student test for the comparison between the first and last access day in A.

4.3. Weight gain and Adiposity index

Animal body weight was measured weekly. The schedule of diet exposure ($F(2,26) = 32.47$ $p<0.0001$), time ($F(7, 182) = 295.9$ $p<0.0001$ and the interaction between them ($F(14,182) = 58.14$ $p< 0.0001$) affected weight gain. Post-hoc analysis revealed that from the second week forth, HSB-d group gain significantly more weight than the other groups (p<0.05 for HSB-d vs CHOW-d and HSB-i) (Figure 10). At the end of 4 weeks and the end of 8 weeks, adiposity index was calculated as the ratio of perigonadal adipose tissue weight and body weight of the animal. ANOVA indicated that the diet schedule significantly affected adipose index between groups ($F(5, 54) = 103.6$ $p<0.0001$). Post-hoc test revealed that HSB-d

group presented higher adipose index in 4 weeks than CHOW-d (t=8.882, p<0.05) and HSB-i (t=8.113, p<0.05), and that this pattern remained in 8 weeks (t=17.35 and t=16.61 for HSB-d vs CHOW-d and HSB-i respectively, p<0.05). Moreover, HSB-d group increased adiposity index from four to eight weeks of experiment (t=9.119, p<0.05) (Figure 11).

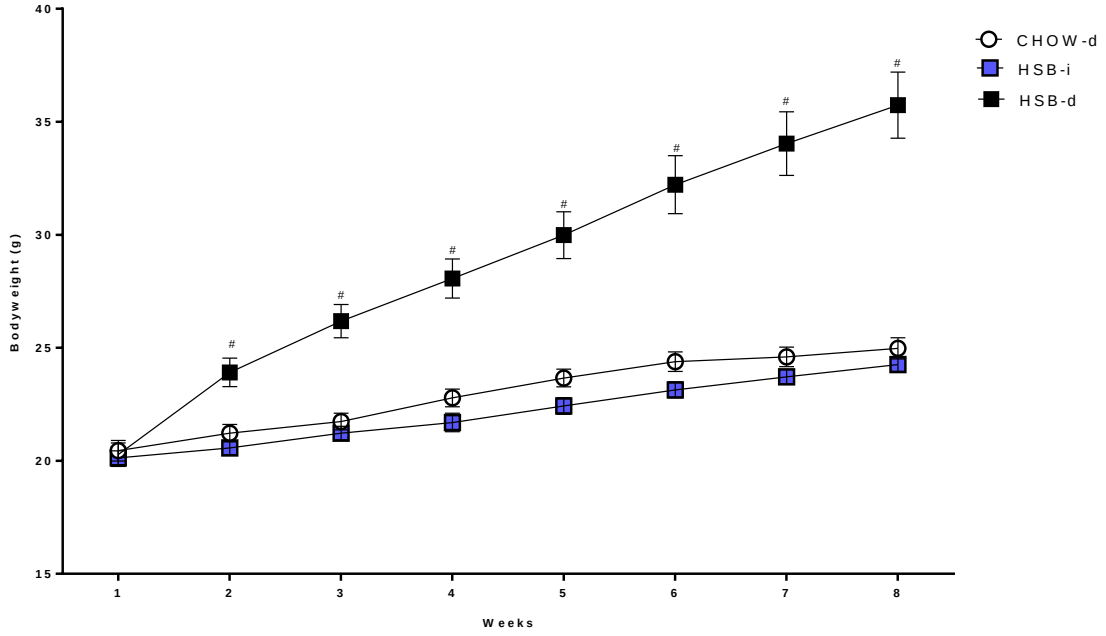


Figure 10 Body weight. From the second week forth, HSB-d group presented significantly higher body weight than the other groups (# p<0.05 for HSB-d group vs CHOW-d and HSB-i). Data are shown as mean ± SEM. Two-way ANOVA with repeated measure followed by Bonferroni post-hoc test.

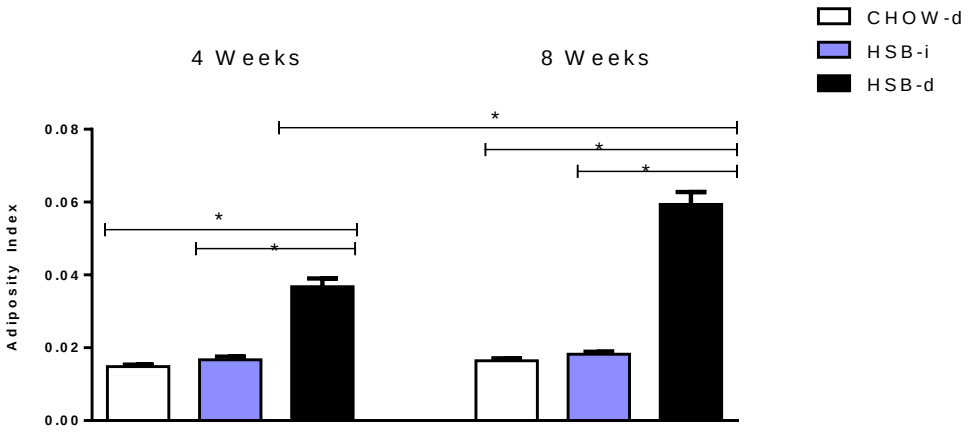


Figure 11 Adiposity index. In both four and eight weeks experiment, HSB-d group presented higher adiposity index than the other groups (* p<0.05). In addition, HSB-d group from eight weeks experiment presented higher adiposity index than this group in four week experiment (* p<0.05). Data are shown as mean ± SEM. One-way ANOVA followed by Bonferroni post-hoc test.

4.4. Behavioural tests

4.4.1. Marble-burying test

In the first access day of the last week (both 4 and 8 weeks experiment) marble-burying test was performed. No differences in the marbles buried were found between groups (Figure 12).

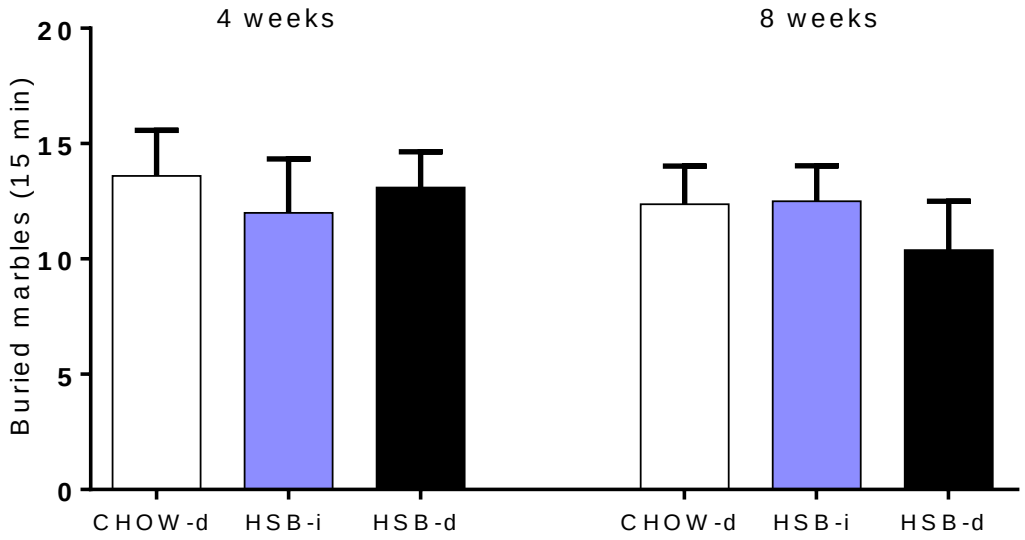


Figure 12.Marble-burying test. No differences were found between groups (p>0.05). Data are shown as mean ± SEM. One way ANOVA followed by Bonferroni post-hoc test.

4.4.2 Light-dark box – Food stimulus analysis

In the last week of each experiment, on the last two days of access and prior these accesses time the light-dark box behavioral test was performed. The first test consisted in the light-dark box with no stimuli in the light zone. For the second test, the food relative to the access time was introduced in the center of light zone. In the four week experiment, no difference was found in the latency time between groups in the test with no food stimuli ($p>0.05$). The introduction of the food in the white zone, however, changed some parameters ($F(5, 51) = 6.473$, $p<0.05$). The post-hoc test revealed that HSB-i group presented reduced latency when compared with themselves in the test with no food stimuli ($t= 3.241$, $p<0.05$ for HSB-i/no pellet vs HSB-i/with pellet) and the same occurred for HSB-d ($t=3.297$, $p<0.05$ for HSB-d/no no pellet vs HSB-d/with pellet) (Figure 13A). No differences were found in the

latency time in the 8 weeks experiment (Figure 13B). In both 4 and 8 weeks experiments, there were no differences in the number of transitions between chambers between groups (Figure 13C and D). Regarding the time spent in the white zone, in the 4 week experiment, ANOVA identified differences both in the test with pellet and the test with no pellet ($F(5, 51) = 10.22$, $p < 0.0001$). In the test with no food stimuli, HSB-d group spent more time in the light zone than CHOW-d group ($t = 3.363$, $p<0.05$ for HSB-d/no pellet vs CHOW-d/no pellet). The introduction of the food in the white zone altered HSB-i behaviour, which spent more time in the white zone with this stimuli when compared itself in the no pellet test ($t = 4.372$, $p<0.05$ for HSB-i/with food vs HSB-i/ no food), and also when compared with CHOW in the food stimuli environment ($t=5.115$, $p<0.05$ for HSB-i/with pellet vs CHOW-d/with pellet). HSB-d group remained with the same behaviour and again spent more time in the white zone than the CHOW-d group with food stimuli in the light zone ($t=3.886$, $p<0.05$ for HSB-d/with pellet vs CHOW-d/with pellet) (Figure 13E). In the 8 weeks experiment no differences were found between groups in the traditional test ($p>0.05$). The introduction of the pellet in the white zone, however, induced alterations ($F(5, 45) = 3.042$, $p<0.05$) in the HSB-d group that spent more time in this zone when compared with CHOW-d group ($t=3.199$, $p<0.05$ for HSB-d/with pellet vs CHOW-d/with pellet) (Figure 13F).

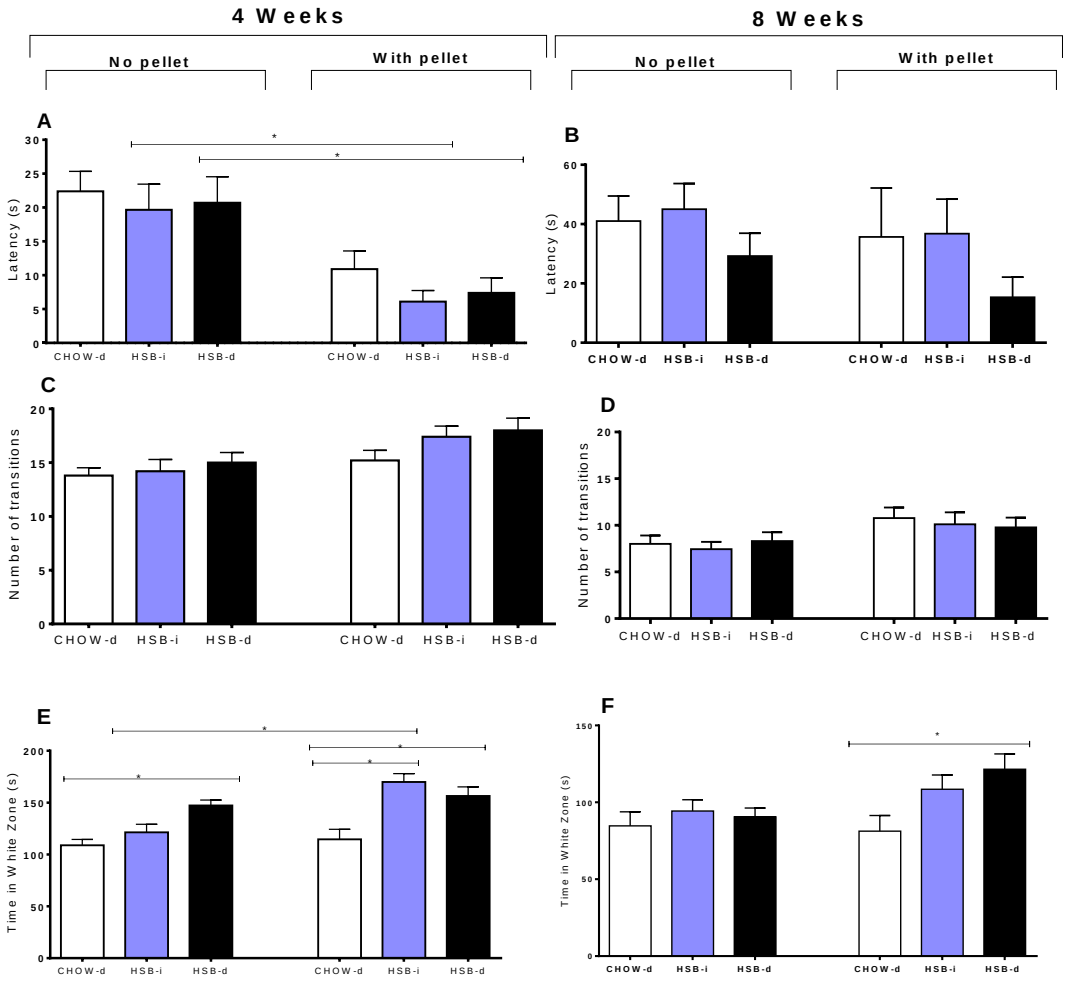


Figure 13. Light-dark box behavioural test without and with food stimulus in the 4 (left) and 8 weeks (right) experiment. Graphs in the left represent data from the 4 weeks experiment and graphs from the right represent 8 weeks experiment. In both experiments two tests were performed with an interval of 2 days between them. The first one corresponded to the traditional light-dark box (no pellet) and the second one was the same test with an introduction of the access period corresponding food in the white zone (with pellet). (**A**) and (**B**) latency time to first entry the bright zone. The presence of food stimuli reduced the latency time for the HSB-i and HSB-d group when compared to these groups without food stimuli ($p< 0.05$ for HSB-i with food stimulus vs HSB-i without food stimulus; $p<0.05$ for HSB-d with food stimulus vs HSB-d without food stimulus(**A**). No differences were found in the latency time between groups in the 8 weeks experiment ($p>0.05$) (**B**). (**C**) and (**D**) number of transitions between chambers. No difference was found in this parameter in both experiments ($p>0.05$). (**E**) and (**F**) Time spent in the white zone. The chronic consumption of HSB by the HSB-d group reduced anxiety shown by the significant higher time spent in the white zone by this group ($p<0.05$ for HSB-d vs CHOW-d in the no pellet test). This behaviour remained with the food stimuli ($p<0.05$ for HSB-d group with food stimuli vs CHOW-d with food stimuli) (**E**). When the food was introduced in the white zone, HSB-i reverted its behaviour and spent more time in the white zone ($p<0.05$ for HSB-i with food stimuli vs CHOW-d with food stimuli and HSB-i with no food stimuli) (**E**). The introduction of pellet induced HSB-d group to spend more time in the white zone when compared with CHOW-d group ($p<0.05$ for HSB-d vs CHOW-d in the test with food stimuli) (**F**). Data are shown as mean ± SEM. One way ANOVA followed by Bonferroni post-hoc test.

4.4.3 Light-dark box – Time analysis

Since 8 weeks light-dark box data seemed discrepant from 4 weeks data, it interested us to evaluate if 4 additional weeks experiment significantly altered mice behavior in this test. To address this issue we compared by t-student test both with and without pellet data from each group with the same group 8 weeks later. Indeed, in all parameters differences were found. In the traditional test (with no food stimuli), HSB-i from eight weeks took longer to first explore the white chamber ($t=2,682$ $df=16$, $p<0.05$ for HSB-i 8 vs HSB-i 4) (Figure 14A). When comparing data with food stimuli, again, only HSB-i from 8 differed from HSB-i from 4 weeks experiment, taking longer to first explore the white arena ($t=3.453$ $df=16$, $p<0.05$ for HSB-i 8 vs HSB 4) (Figure 14B).

In both with and without food stimuli, all 8 weeks groups transited less between chambers when compared with their respective groups from 4 weeks experiment (No food stimuli: $t=4,992$ $df=16$, $p<0.05$ for CHOW-d 4 vs CHOW-d 8 $t=4,908$ $df=16$, $p<0.05$ for HSB-i 4 vs HSB-i 8; $t=4,648$ $df=16$, $p<0.05$ for HSB-d 4 vs HSB-d 8) (Figure 14C) (With food stimuli: $t=2,869$ $df=16$, $p<0.05$ for CHOW-d 4 vs CHOW-d 8; $t=3,986$ $df=16$, $p<0.05$ for HSB-i 4 vs HSB-i 8; $t=5,185$ $df=16$, $p<0.05$ for HSB-d 4 vs HSB-d 8) (Figure 14D).

Regarding the time spent in the white zone, in both traditional test and with food stimuli test, all groups from 8 weeks experiment spent less time in this zone when compared with their respective group from 4 weeks experiment (No food stimuli: $t=2,252$ $df=16$, $p<0.05$ for CHOW-d 4 vs CHOW-d 8; $t=2,524$ $df=16$, $p<0.05$ for HSB-i 4 vs HSB-i 8 and $t=7,269$ $df=16$, $p<0.05$ for HSB-d 4 vs HSB-d 8) (Figure 14E). (With food stimuli: $t=2,360$ $df=16$, $p<0.03$ for CHOW-d 4 vs CHOW-d 8; $t=5,009$ $df=16$, $p<0.05$ for HSB-i 4 vs HSB-i 8 and $t=2,635$ $df=16$, for HSB-d 4 vs HSB-d 8) (Figure 14F).

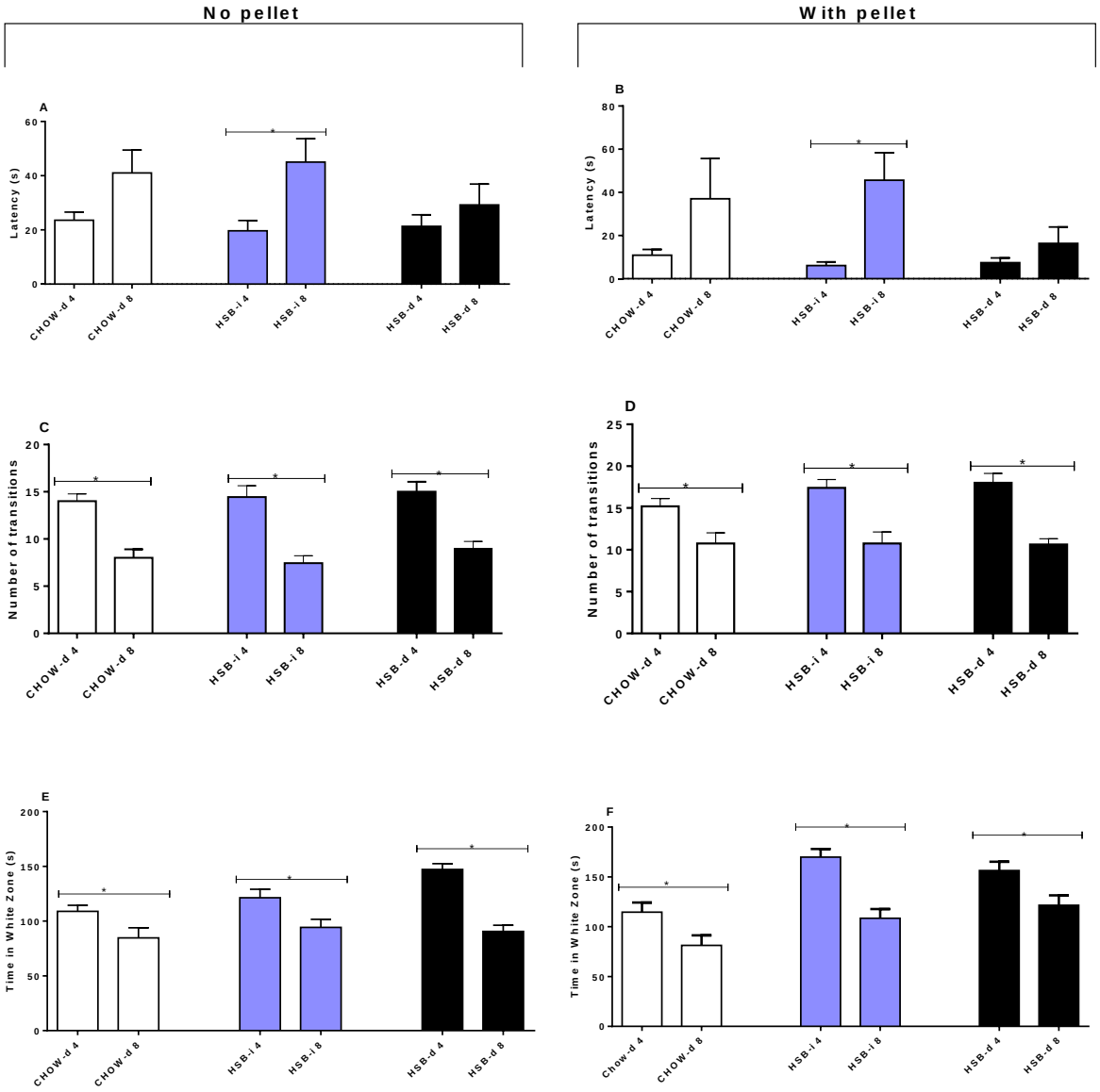


Figure 14 Light-dark box behavioural test between groups from 4 with 8 weeks. Graphs in the left represent the traditional test (with no food stimuli) and graphs in the right represent data from the with food stimuli test. (**A**) and (**B**) Latency time to first entry the bright zone. Animals from 8 weeks HSB-i group took longer to first explore the white chamber ($p<0.05$ for HSB-i 4 vs HSB-i 8) (**A**), and this behaviour remained regardless the presence of food stimuli in the white zone ($p<0.05$ for HSB-i 4 vs HSB-i 8) (**B**). (**C**) and (**D**) number of transitions between chambers. In both traditional (**C**) and with food stimuli (**D**), groups from 8 week experiment transited less between chambers ($p<0.05$ for 8 weeks groups vs their respectives 4 weeks group). (**E**) and (**F**) Time spent in the white zone. Similar to the pattern seen in number of transitions, in both traditional (**E**) and with food stimuli (**F**), groups from 8 weeks spent less time in the white zone when compared with their respective 4 week group ($p<0.05$ for each 8 week group vs its respective 4 week group). Data are shown as mean ± SEM. T-student test.

4.5. Novel Object Recognition

In the end of 6th week and in non-access days, animals were submitted to the NOR test. Animals that did not reach the minimum 20 seconds exploration of both objects were excluded from analysis (n=7). No differences were found between the exploration time from of the familiar object and the novel object and neither in the discrimination index in all groups in both 2h and 24h recall test (Figure 15).

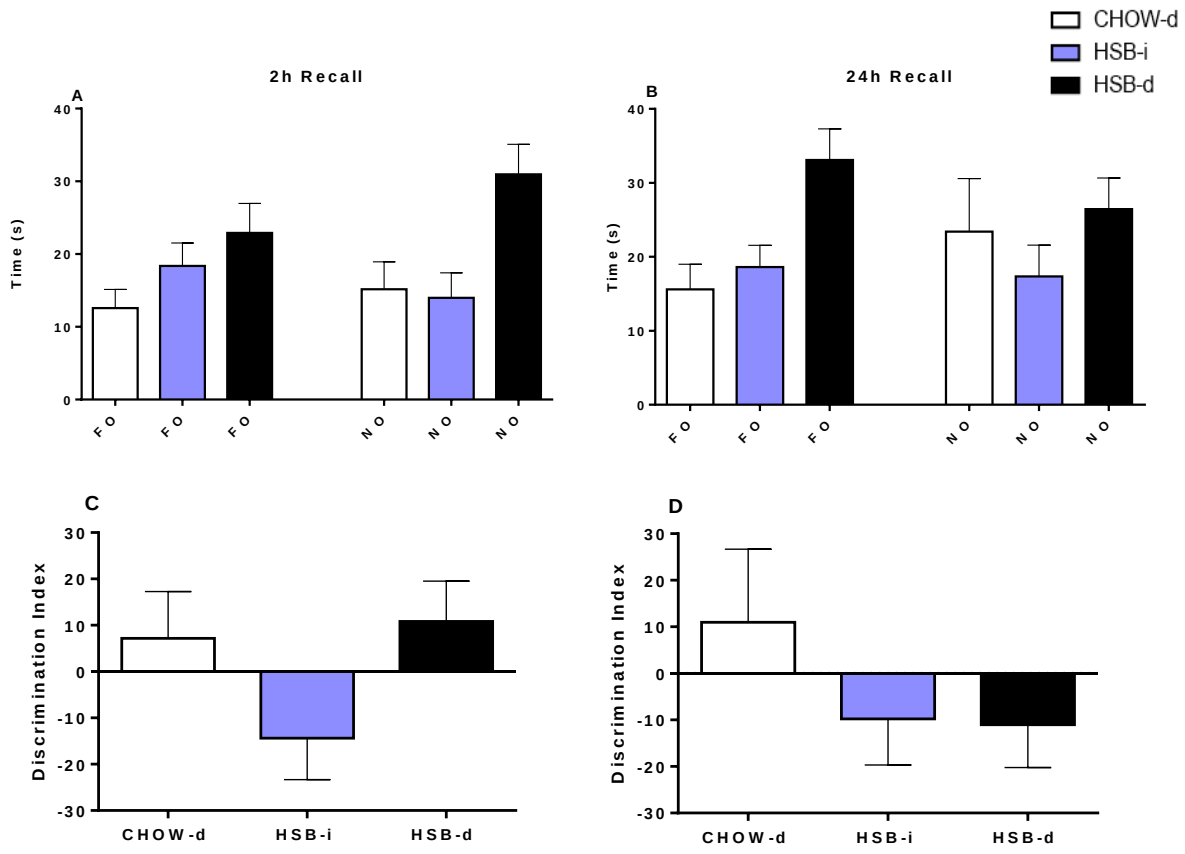


Figure 15. Exploration time of the familiar object and novel object and discrimination index in 2h and 24h recall in the NOR test. No differences were found between the exploration time of the familiar object in comparison with the exploration time in of the novel object between each group in the 2h and 24h recall test (**A** and **B** respectively) and neither in the discrimination index in both 2h and 24h recall tests (**C** and **D** respectively) . Data are shown as mean ± SEM. Paired t-student test.

4.6. Transcriptional Expression of Dopaminergic and GABAergic Receptors and Brain-derived Neurotrophic Factor

After 4 and 8 weeks, PFC was collected for qPCR analysis of dopaminergic, GABAergic and *Bdnf* genes. No differences were found in *Drd1* relative transcription in 4 weeks (Figure 16A). However a statistical difference was obtained in 8 weeks of experiment (Kruskal-Wallis statistic = 7.340, $p<0.05$). Dunn's multiple comparisons test revealed a downregulation of this receptor transcript in HSB-i group compared with CHOW-d group (Figure16B). Neither 4 nor 8 weeks of dieting protocol affected *Drd2* and *Gabbr2* transcription (Figure 16 C and D; G and H, respectively). In contrast, both 4 and 8 weeks experiment affected *Gabbr1* transcription ($F(2, 20) = 4.929$, $p<0.05$ for 4 weeks experiment, and Kruskal-Wallis statistic = 6.260, $p<0.05$ for 8 weeks experiment). Post hoc analysis revealed a downregulation of this receptor in the HSB-d group when compared with CHOW-d group ($t = 3.055$ for HSB-d vs CHOW-d, $p<0.05$ in 4 week experiment, and Dunn's multiple comparisons test with $p<0.05$ for HSB-d vs CHOW-d in 8 weeks experiment) (Figure 16 E and F respectively).

Brain-derived neurotrophic factor (*Bdnf*) transcript was also evaluated. Although no differences were found in 4 weeks experiment (Figure 17A), 8 weeks of dieting protocol affected its transcription (Kruskal-Wallis statistic = 6.320, $p< 0.05$). Dunn's *post-hoc* multiple comparisons test revealed a downregulation of this transcript in the HSB-d group when compared with CHOW-d group ($p<0.05$ for HSB-d vs CHOW-d) (Figure 17 B).

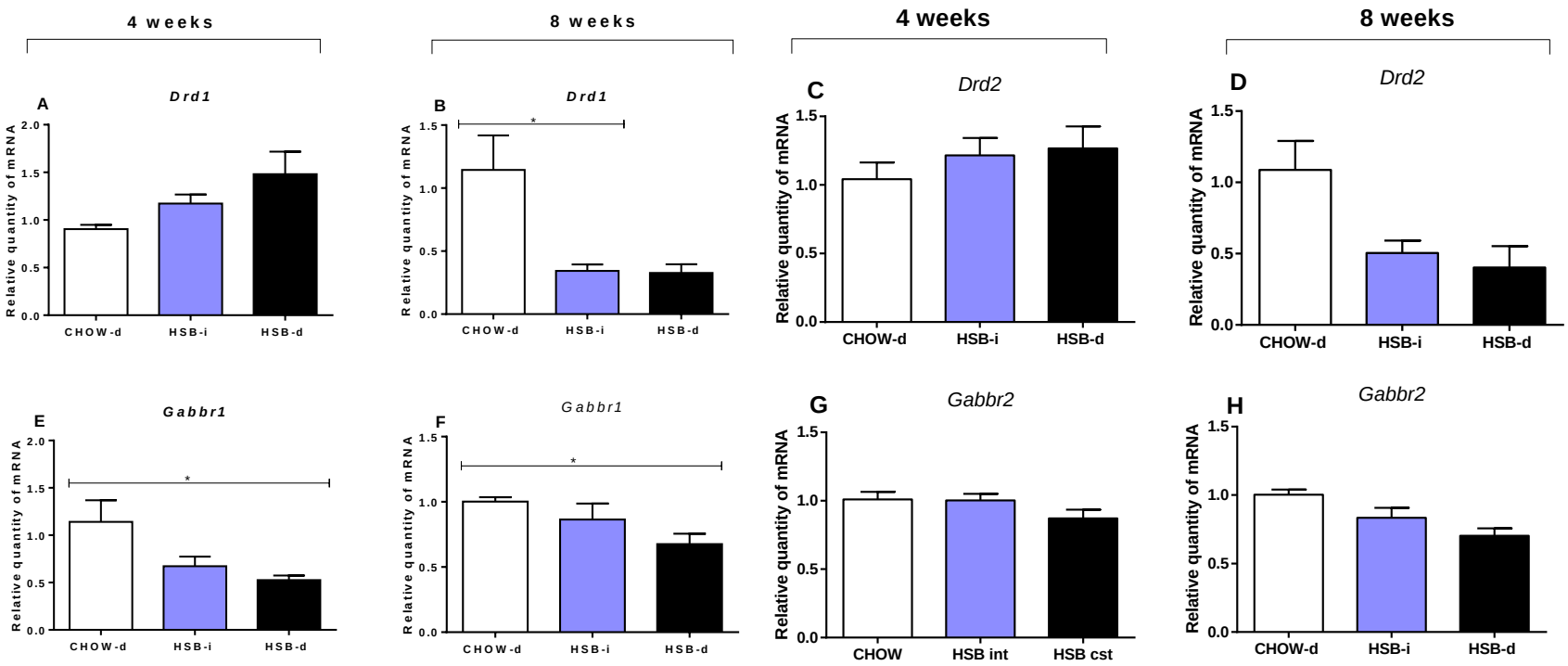


Figure 16. Relative transcript expression of dopaminergic receptors (*Drd1* and *Drd2*) and gabaergic receptors (*Gabbr1* and *Gabbr2*) in the prefrontal cortex of animal in 4 and 8 weeks of experiment. No differences were found in the *Drd1* relative expression mRNA in 4 weeks (**A**), but a downregulation of *Drd1* was found in the HSB-i group when compared with CHOW-d group in 8 weeks (* p<0.05 for HSB-i vs CHOW-d) (**B**). Neither the 4 weeks nor the 8 weeks treatment affected *Drd2* expression (**C** and **D**). mRNA of subunit 1 of $GABA_b$ receptor (*Gabbr1*) was downregulated in HSB-d group when compared with CHOW-d group in both 4 and 8 weeks (* p<0.05 for HSB-d vs CHOW-d) (**E** and **F**). No differences were found for G*abbr2* subunit gene (**G** and **H**). Data are shown as mean ± SEM. In 4 weeks data, one way ANOVA followed by Bonferroni post-hoc test was performed. In 8 weeks data, Kruskal-Wallis followed by Dunn's multiple comparison test was performed.

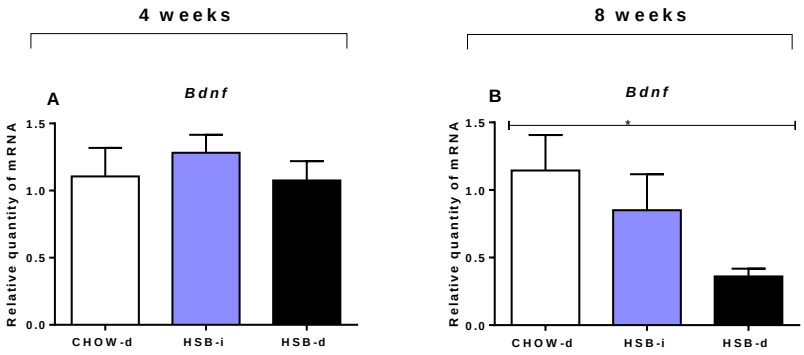


Figure 17. Relative transcript expression of Brain-Derived Neurotrophic factor (*Bdnf*) in the prefrontal cortex of animals in 4 and 8 weeks of experiment. . No differences were found in the *Bdnf* relative expression mRNA in 4 weeks (**A**), but a downregulation of *Bdnf* was found in the HSB-d group when compared with CHOW-d group in 8 weeks (* $p<0.05$ for HSB-d vs CHOW-d) (**B**). Data are shown as mean ± SEM. In 4 weeks data, one way ANOVA followed by Bonferroni post-hoc test was performed. In 8 weeks data, Kruskal-Wallis followed by Dunn's multiple comparison test was performed.

4.7. Western Blot

In the end of the 8th week, proteins were extracted from animal's PFC and western blots of phosphorylated PKA^{Ser99} and phosphorylated DARPP-32^{Thr34} were performed. Although Kruskal-Wallis test indicated statistical differences between groups regarding PKA expression (Kruskal-Wallis statistic: 5,956, p=0.025), Dunn's multiple comparisons test did not identify which group(s) was different from the rest (Figure 18).

Regarding the p-DARPP-32 protein data, no bands were evident with input of 40 and 60µg of protein. In a final attempt to capture p-DARPP-32 expression, we used 100µg of protein, changed the antibody concentration to 1:100 and decreased 5 minutes of the blocking step. Bright bands were form, and unfortunately, only one band was formed in the control group, making it impossible to analyze and plot a graph (Figure 19). As we had no more proteins to try again with a higher protein concentration, a repetition of the experiment is mandatory to confirm this result.

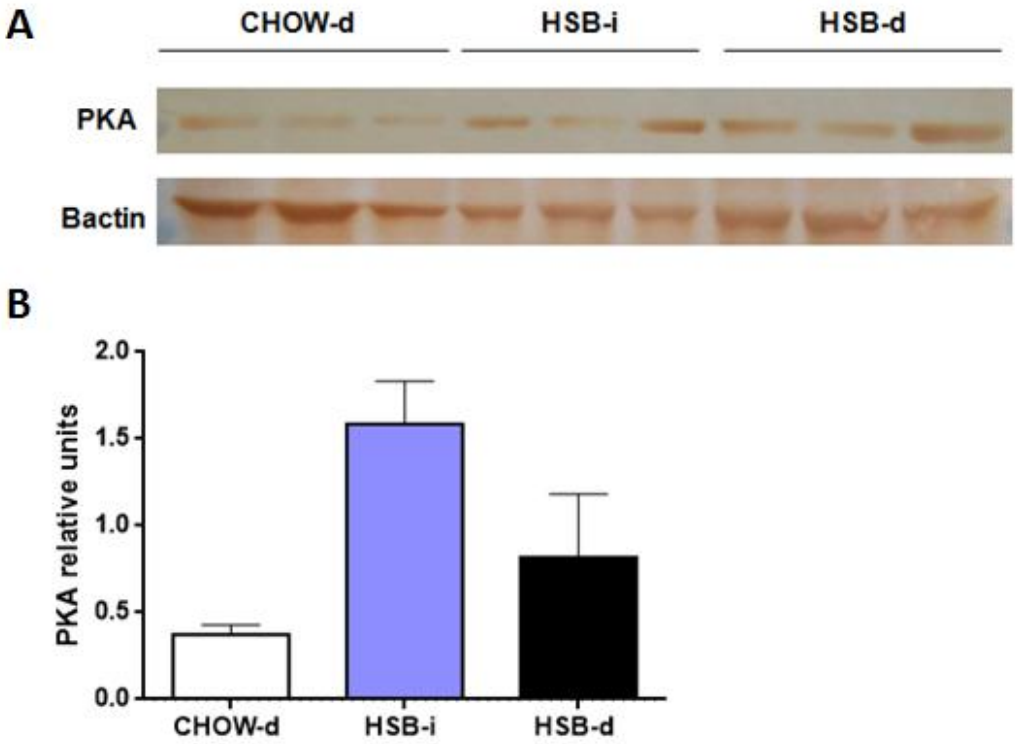


Figure 18. Relative expression of phosphorilated PKA in PFC of animals after 8 weeks. No differences were found between groups. Data are shown as mean ± SEM. Kruskal-Wallis followed by Dunn's comparison test was performed.

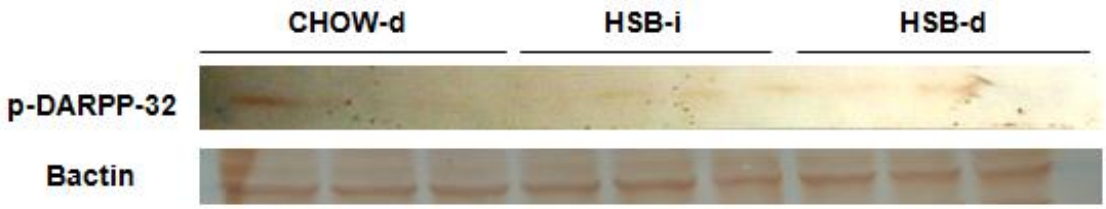


Figure 19. Membrane of p-DARPP-32^{thr34} in western blot experiment.

5. DISCUSSION

Binge eating is characterized by recurrent episodes of hyperfagia in a brief period of time accompanied by a sense of loss of control (Goodman *et al.,* 2018). When translated to an animal model, different features should be taken into account in an attempt to resemble a reasonable model for the human condition. Among them, it can be considered the amount of calories eaten, the duration of the event, escalation of intake over time, if the level of hyperphagia is significant and evidence that bingeing occurred even in the presence of aversive consequences (Perello *et al.*, 2014).

In our experiment, the 24h kilocalorie intake data showed that from the 14th day of the experiment, animals from the HSB-i group ingested significantly more kilocalories in the accesses days and significantly less on the non-accesses days, and this pattern remained throughout the experiment (Figure 8). This profile of ingestion corroborates with other studies that applied intermittent access protocol to achieve binge eating behavior in rodents (Corwin, 2004; King *et al.*, 2016; Kreisler *et al.*, 2017). Interestingly, after a month, the variation in the energy consumption between access and non-access days increased significantly, in a way that in the non-accesses days, HSB-i ingested even lower kilocalories than the basal consumption of CHOW-d group, indicating a more significantly voluntary fasting in the absence of the high palatable food. Concomitantly, in the accesses days, after a month, this group ingested higher kilocalories than all the other groups (Figure 8). This indicates that over the time, the extended intermittent access to the palatable food increased the severity of binge behavior, which is important take into account once studies vary in protocol time, being some of them of 4 weeks (Corwin, 2004; King *et al.,* 2016), 5weeks (Lardeux, Kim and Nicola, 2015) and 6 weeks (Sirohi, van Cleef and Davis, 2017). It is important to state, that this overeating and undereating pattern was developed in a voluntary manner, as animals were never food deprived. This is relevant for two reasons: food deprivation increases the rewarding value of palatable foods, inducing hyperphagia, and in humans, binge episodes are not usually driven by hunger (Perello *et al.,* 2014; Lardeux, Kim and Nicola, 2015). Therefore, this is a good model to represent the human condition and as well, to understand the reward properties of palatable foods in behavior without forced fasting interferences.

In addition, in all accesses periods, HSB-i group ingested significantly higher quantities of kilocalories when compared to the other groups, and this behavior presented a significantly escalation over time (Figure 9A). This high consumption in 2h corresponded to the majority of the 24h energy consumption of the access day, which started around 60% in the first access day, achieved 95% in the 20th day, and corresponded to 91% in the last day (Figure 9B). In general, mice are more active in the dark phase and also eat the majority of their diet during night (Ellacott *et al.*, 2010). In our study, mice ate up to 95% of their daily consumption in only 2h and in the light phase, corroborating with the increase in severity of binge episodes seen in 24h data. This escalation of intake over time can be correlated with tolerance.

Tolerance is defined by an increase in an activity (e.g. frequency, duration, intensity) in order to achieve the same initial effect produced by the stimulus, which is decreased, once repeated exposure reduces sensitivity (Starcevic, 2016). It arises as a homeostatic adaptation of the body to fit into novel situations (Glass *et al.*, 2016). Interestingly, HSB-d group had also repeated exposure to HSB diet, but it did not developed any comparable pattern, suggesting that the HSB diet and its reward properties withdrawal in non-accesses days in the HSB-i group had a key role in the escalation of consumption over time. Withdrawal induces a negative emotional state, which consequently reduces motivation for ordinary reward, increasing the reinforcement properties of palatable foods and increasing consumption of it to alleviate negative emotional states (Moore *et al.*, 2018). Previous studies from our group revealed that after 8 weeks of chronic consumption, HSB withdrawal induced a higher consumption of ethanol, indicating that the withdrawal of HSB positive reinforcement property, increased animal's seeking behavior for another source of reinforcement (Carvalho *et al.*, 2018). Although in this previous study we could not prove that HSB could induce loss of control over food consumption, taken together, these studies show that HSB have reinforcement properties, is capable of inducing loss of control and its withdrawal induces seeking behavior for other positive reinforcements (in our case the next exposure for HSB, and in the previous study case, ethanol). These findings place HSB as a good hypercaloric diet to study food addiction.

Despite the fact that HSB-i overate in all accesses days, this group did not gain weight over the experiment. Only HSB-d group increased body weight (Figure 10) from the second week forth and presented higher adiposity index at the end of 4 and 8 weeks (Figure 11). Indeed, the fluctuation of overeating and fasting in the

intermittent group resulted in a stable weight, similar to the control, which does also corroborates with body weight data from other groups studying binge eating through intermittent access (Corwin, 2004; King *et al.,* 2016; Chawla *et al.*, 2017). In addition, even though binge eating can co-occur and also increase risks of obesity, one does not predict the other (Zwaan, 2001; Leehr *et al.*, 2015; Hankey, 2017).

Besides the maladaptive eating pattern, binge eating is also correlated with anxiety and memory deficits and behavioral impairments (Rosenbaum e White, 2013; Svaldi *et al.*, 2014; Chawla *et al.*, 2017; Eneva, Murray and Chen, 2017).

In the end of each experiment, animals were submitted to the marble-burying behavioral test. The basis of this test lies on rodent's natural digging behavior, which is comparable with a type of human compulsion behavior: the obsessive-compulsive disorder (Angoa-pérez *et al.*, 2013). Despite the fact that some studies point this test as an index of anxiety (Kedia and Chattarji, 2014), there are controversial thoughts regarding this aspect (Thomas *et al.*, 2009; Angoa-pérez *et al.*, 2013). In any case, in our experiment, neither the chronic treatment with HSB nor the intermittent access affected mice natural digging behavior in both experiments (Figure 12).

In the end of the fourth and eighth week mice were tested for the light-dark box. The principle of this test lies on rodent's innate exploratory behavior in new areas and aversion to brightly and opened places (Bourin e Hascoët, 2003). In the present study two different behaviors profiles was observed, one after 4 weeks and a different one after 8 weeks. After four weeks, once animals were introduced in the chamber, there were no differences in latency time between groups, but once the pellet was introduced in the bright zone, both HSB-i and HSB-d group reduced the time spent to first explore the white zone (Figure 13A). Since the pellet placed in the bright zone corresponded to the one the animal would receive in the access time, this result indicates that the presence of the high palatable food, HSB, but not chow, induced animals to more rapidly explore the white chamber, even it being an aversive environment. In addition, HSB-d group, in both traditional and with pellet test, spent more time in the bright zone than the other groups, indicating that chronic consumption of HSB was sufficient to induce an anxiolytic effect in these animals (Figure 13E). There have been contradictory findings addressing this issue. While in Sivanathan and collaborators' work (2015) female rats exposed for 10 weeks to high fat diet spent less time in the bright zone than the low fat control group, no differences were found in both male and female C57BL/6J mice exposed

to high fat diet for 18 weeks in the light-dark box when compared to control groups in Gelineau and collaborators work (2017) and neither in male C57BL/6J mice chronically fed with high fat diet for 8 weeks in Hassan and collaborators study (2018). This suggests that the relation of high fat diet chronic consumption and anxiety-like behavior in rodents could be influenced more by the type of diet (nutrients) than the hypercaloric fact per se, as proposed by Bocarsly and collaborators (2011). Nevertheless, in the present study, 4 weeks chronic exposure to HSB diet increased time spent in the bright zone.

Interestingly, HSB-i group, in the traditional test, spent more time in the dark zone, but when the food was introduced in the white zone, this group changed its previous behavior and spent more time in the bright chamber (Figure 13E). Since it happened only in the presence of the food, this behavior did not assessed a basal anxiety-like behavior, but rather, risk-taking and motivation drive to obtain food, which was expected, as test occurred prior the access time, period in which mice were with high expectancy of HSB. Indeed, forced fasting and injection of ghrelin, two mechanisms that enhance motivation to obtain food, promoted an increased time spent in the white zone with food in C57BL/6 mice in Lockie and collaborators work (2017). Interestingly, in our study, motivational drive to obtained food was achieved without external stimuli as fasting or pharmacological modulation. It is possible that the voluntary fasting in the previous day could have enhanced this behavior. However, curiously, after 8 weeks this behavior did not repeat and neither did the anxiolytic effect seen in HSB-d group in the traditional test (Figure 13F).

Since data from the 8 weeks experimental mice seemed discrepant from 4 weeks data, we compared each group from 4 weeks with its respective in 8 weeks (Figure 14). Interestingly, 8 weeks HSB-i group took longer to first explore the white zone, even with the presence of the high palatable food in it (Figure 14 A and B). It is known that withdrawal-induced negative emotional state increases anxiety (Moore *et al.*, 2018) which could partly explain why this was the only group with high latency (at this time of experiment, this group was eating lower kilocalories than the basal consumption of CHOW group). However, one could expect that the presence of reward reinforcement in the white zone should be a positive stimulus to reduce this anxiety once test occurred prior access period and animals were with high expectancy of HSB diet. In fact, in all other parameters, 8 weeks animals exhibited lower results when compared with 4 weeks animals, indicating that a 4 weeks longer

experiment induced this higher anxiety-like behavior rate in all groups, and not the possible withdrawal negative emotional state in HSB-i group.

Since mice were 28 days older than the previous tested, it is plausible to think that aging could have an impact in their behavior. In our experiment, in the 4 weeks protocol, test occurred when mice were 11 weeks old (almost 3 months), and in the 8 weeks protocol mice were 15 weeks old (almost 4 months), which is a small difference, and in both cases, animals were still in their young adulthood. However, a large-scale behavioral analysis performed in C57BL/6 revealed that the number of transitions between chambers in the light-dark box decreases significantly when comparing mice from 2-3 months with mice of 4-5 (Shoji *et al.*, 2016). Although this finding corroborates with our data, in which all mice from 8 weeks experiment exhibited lower number of transitions between chambers when compared with the 4 week experimental animals (Figure 14 C and D), it does not entirely explain our results, once in this same large-scale study no differences were found when comparing theses animals with the time spent in the light zone (Shoji *et al.*, 2016). Therefore, beyond aging, other environmental variants could have influenced this different behavior seen in all groups, and a replication of the experiment is mandatory to confirm our results.

In the end of the 6th week, mice were tested for memory performance with the NOR test in two time points: 2h and 24h. We found no differences between groups in either of them. However, it is important to state that 7 animals were excluded from the analysis, and maybe with all animals a significant difference could have been found. Nevertheless, interestingly, HSB-i group seemed to exhibit a poor memory performance than the other groups after 2 hours (Figure 15C) indicating that this disturbance in eating patterns could affect short memory. However, after 24 hours both groups with HSB diet access seemed to exhibit poor performance (Figure 15D), suggesting that maybe the long-term memory is impaired by both chronic HSB consumption and compulsivity. Chawla and collaborators (2017), when evaluating memory performance in rats, found out that only binge prone rats exhibited lower memory performance in a 2 days recall test when compared to the other groups, including the group with daily access to the high caloric food. However, in Chawla and colleagues work (2017), daily group had 1h daily access to high caloric food, while in our, HSB-d group had 24h access to the high caloric diet, which generates more severe impacts in animal's physiology and phenotype. In any case, a repetition of NOR experiment with a bigger sample is crucial to confirm (or not) these

suggestions and separate the effects of the high fat diet consumption and the compulsivity behavior in memory performance.

The impaired control of food consumption is partly driven by deregulation of PFC, an important region regulating executive function and self-control (Volkow, Wise and Baler, 2017). Since DAergic and GABAergic projections overlap in the PFC (Miller and Cummings, 2017) and have major influences in guiding behavior in accordance to motivation (Miller and Cohen, 2001; Pistillo *et al.*, 2015; Ayano, 2016; Baldo, 2016) we sought to evaluate the transcriptional profile of DA and GABA receptors in the PFC. In the 4 weeks experiment, no differences were found in the transcriptional profile of *Drd1* and *Drd2*, but in the 8 weeks experiment, while this pattern remained for *Drd2*, *Drd1* was significantly downregulated in HSB-i group when compared with the other groups (Figure 16 A, B, C and D). Indeed, BED has been associated with diminished activity in the impulse-control of cortical areas (Balodis et. al, 2013; Kessler *et al.*, 2016). Moreover, Ferenczi and colleagues' (2015) opotogenetic work showed that an elevation in excitability in medial PFC suppress striatal response to dopamine and the behavioral drive to seek for rewarding stimuli, being responsible, at least in part, for the anhedonia (loss of enjoyment) seen in patients with depression. This suggests that the hypoactivity in PFC seen in individuals in BED could be a cause or consequence of striatal response to dopamine, which ultimately leads to the reward seek (food) and loss of control over food consumption.

In an attempt to confirm DRD1 activity, we sought to investigate the availability of p-DARPP-32^{Thr34} in the PFC of mice. However, given the amount of proteins extracted from PFC, we could not produce reliable bands in western blot to compare groups, although, compared with the single control band, both HSB-i and HSB-d group seemed downregulated, corroborating with the transcriptional findings (Figure 19). Nevertheless, a repetition of this protocol with higher inputs of proteins is mandatory to confirm if the transcritptional downregulation of *Drd1* does also reflects hypoactivity of dopamine response in a molecular level in PFC.

Regarding the possible dopaminergic hypoactivity seen by the downregulation of *Drd1*, Chawla and colleagues work (2017), in a 4 week study, showed that binge prone rats exhibited a *Drd1* downregulation in Orbitalfrontal Cortex and an upregulation in Medial Prefrontal Cortex, suggesting that specific regions of the PFC may contribute differently with the phenotype. Corwin and colleagues (2016), however, found dopaminergic transcriptional alterations in the VTA of rats before the

binge episode, which was reinstated after binge episode, and a general reduced state of gene transcription in PFC, independently of the moment assessed. This suggests that dopamine response in VTA is important for the anticipation and seeking for the food, and that the hypoactivity in PFC could be driving this state. In our experiment, gene analysis was performed in the entire PFC, which suggests that the profile seen could be an overall neuroadpatation of PFC in mice after 8 weeks but not in 4 weeks.

It's important to recall that animals exhibited two different behaviours in 4 and 8 weeks. While in 4 weeks mice started eating more in the accesses days and established an ovearting habit, it was only in 8 weeks that they increased the severity in binge episodes, eating significantly more than all groups in accesses days, and significantly less than even the basal consumption of control group in the complementary days (Figure 8). In addition, it has been proposed that binge eating could be conceptualized in 3 elements: (i) habitual overeating, (ii) ovearting to relieve negative emotional states and (iii) overeating despite aversive consequences (Moore *et al.*, 2018). Despite the fact that our experimental protocol did not test animals' food response against aversive consequences, it is possible that in the first 4 weeks HSB played a role in the reinforcement learning and habit formation, and after an overstimulation, a desintetization occurred, contributing with the evolution of motivational deficits and a negative emotional state when food was not available. This would enhance food intake, as seen in our data. Indeed, Hildebrandt and colleagues (2018), in a pilot study, investigated the effect of binge behavior chronicity in reward, and found that, over time, binge rats exhibited a lower responsivity in NAc when compared with the early stages binge rats. This supports the idea that firstly, high fat diet acts as a positive reinforcement and after overstimulation a desintetization occurs. In addition, Corwin and colleagues (2016) work, binge rats presented a downregulation of *Drd1* in VTA. Considering that MC projects dopamine neurons from VTA to PFC, this result could also be a consequence of a dopamine lower activity in other reward regions due to desintetization. Nevertheless, the evaluation of other regions of the system should be taken into account in order to judge these assumptions. Moreover, the apparent lower *Drd1* in HSB-d group should not be ignored (Figure 15B). It is possible that the lower dopaminergic profile in PFC could be a response to HSB and not necessarily due to compulsivity itself, once a lower sample was used for transcriptional data in the 8 weeks experiment (n=5). Indeed, pharmacological modulation of D1 receptor in PFC did not altered eating consumption in rats (Corwin et. al., 2016). Taken

together, these data suggest that it is possible that the lower *Drd1* transcriptional profile in our experiment is a consequence of tolerance and reduced sensitivity in other reward brain areas, which would increase consumption over time, or also that the loss of control evidenced in our HSB-i group was not driven by dopaminergic alterations in the PFC but rather via other neurotransmitters system.

In addition, binge eating in these animals did not seem to be established via impairment in the neuronal inhibitory organization, or at least, via disruption of $GABA_B$ receptors, although chronic consumption of HSB led to downregulation of it (Figure 16E, F, G and H). This downregulation, seen in 4 and 8 weeks of chronic exposure to HSB diet remains in 12 weeks of chronic consumption of it (Carvalho *et al.*, 2018). This suggests that chronic consumption of this high fat diet impairs $GABA_B$ in PFC of animals regardless the time, which can be, at least partly, responsible for PFC alterations seen in obese mice. Indeed, the downregulation of *Bdnf* in HSB-d group after 8 weeks supports impairments in PFC.

Brain-derived neurotrophic factor (*Bdnf*) is the most widely expressed neutrophin in the brain, and also a marker of well function of the system, and has already been associated with different disturbances, including obesity, AN and BN (Rosas-vargas, Martínez-ezquerro and Bienvenu, 2011; Zai *et al.*, 2012; Wu *et al.*, 2016). However, in our study, binge mice exhibit normal amounts of *Bdnf* in both experiments indicating that both the establishment and the worsening of the phenotype are not marked by alterations in this neurotrophin transcription. However, *Bdnf* alterations have been reported in PFC of binge rats (Chawla et. al, 2017). This suggests that different mechanisms could lie in each specie and strain. Indeed, most animal BED studies through intermittent access are performed in rats (Corwin, 2004; Lardeux, Kim and Nicola, 2015;Corwin *et al.*, 2016; Chawla et. al., 2017; Hildebrandt *et al.*, 2018; Spierling *et al.*, 2018), but as C57BL/6 is an important complex neurobehavioral trait inbred strain and commonly used for energy homeostasis studies (Ellacott *et al.*, 2010; Babbs *et al.*, 2018), the present work contributes to extend the understanding of binge eating-like behavior in this strain and also to the comprehension of PFC role in this context.

We finally evaluated the availability of activated PKA in the PFC of mice. Although ANOVA indicated statistical differences between groups, post-hoc test did not, which could be due to the small sample per group (n=3) (Figure 18). Protein kinase A is one of the most effectors for cAMP and mediates numerous physiological responses elicited by G-protein-coupled receptors, including

dopamine's (Brady *et al.*, 2012; Søberg *et al.*, 2017). In our experiment, HSB-i group seemed to have higher amounts of phosphorilated PKA, indicating an increase in the concentration of cAMP in PFC of this group. Because cAMP can be produced via different types of G-protein-coupled receptors, this result gives us a general concept of regulation in PFC neurons of this group. However, considering *Drd1* results, this regulation may not be driven by dopaminergic signalling, but could be a result of other neurotransmitters receptors activation. Thus, although HSB-i did not alter *Bdnf* transcriptional expression, an important marker of well neuronal function, apparently it presented higher levels of PKA, an important regulatory kinase for different functions in the cell.

Taken together our results contribute to extend the understanding of binge eating behavior in a mouse animal model. It was clear that, although few weeks were sufficient to induce a pattern of eating behavior, over the course of the time this phenotype got more severe and a possible tolerance effect was evidenced. In addition, after the first 4 weeks, binge mice exhibited a high motivational drive to obtained food, although no dopaminergic transcriptional differences were seen then. After 8 weeks, binge animals presented a downregulation of *Drd1* gene in PFC, which could possibly be related to reduced dopamine sensitivity in VTA, once animals seemed to exhibit a tolerance effect in this period. Finally, binge animals also seemed to present higher amounts of phosphorilated PKA in PFC, indicating an impaired regulation of this region, which could possibly contribute to the impaired impulsivity control and cognitive functions. Nevertheless, it is in our perspective to replicate the model in order to obtain more samples and answer some of the questions that remained unanswered, as the impact of HSB diet and binge eating in memory, the impact of 8 weeks of experiment in motivation to obtain food and anxiety, as well as the evaluation of PKA and p-DARPP-32^{Thr34} in PFC and the evaluation of other reward brain regions.

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APPENDIX A

CHOW-i group does not differ from HSB-d group

Studies that investigate binge eating through intermittent access vary in time and present adaptations, but they share the source that generates eating compulsion: hypercaloric and high palatable foods (Corwin, 2004; King *et al.,* 2016; Lardeux, Kim and Nicola, 2015; Sirohi, van Cleef and Davis, 2017). To test if, indeed, the hypercaloric and high palatable food is the source that promotes binge eating and not the intermittent protocol itself, we tested a group that had continuously access to the high palatable food (HSB) and three times a week had access to the standard maintenance and not palatable mouse diet (CHOW), named CHOW-i. During the 8 weeks of experiment, this group presented a similar pattern of ingestion with HSB-d group, indicating that, even though they had periodically access to CHOW, they chronically consumed HSB diet (Figures 20 and 21). This supports the assumption that high palatable and caloric diets promotes loss of control over food, and not the access to an alternative diet per se.

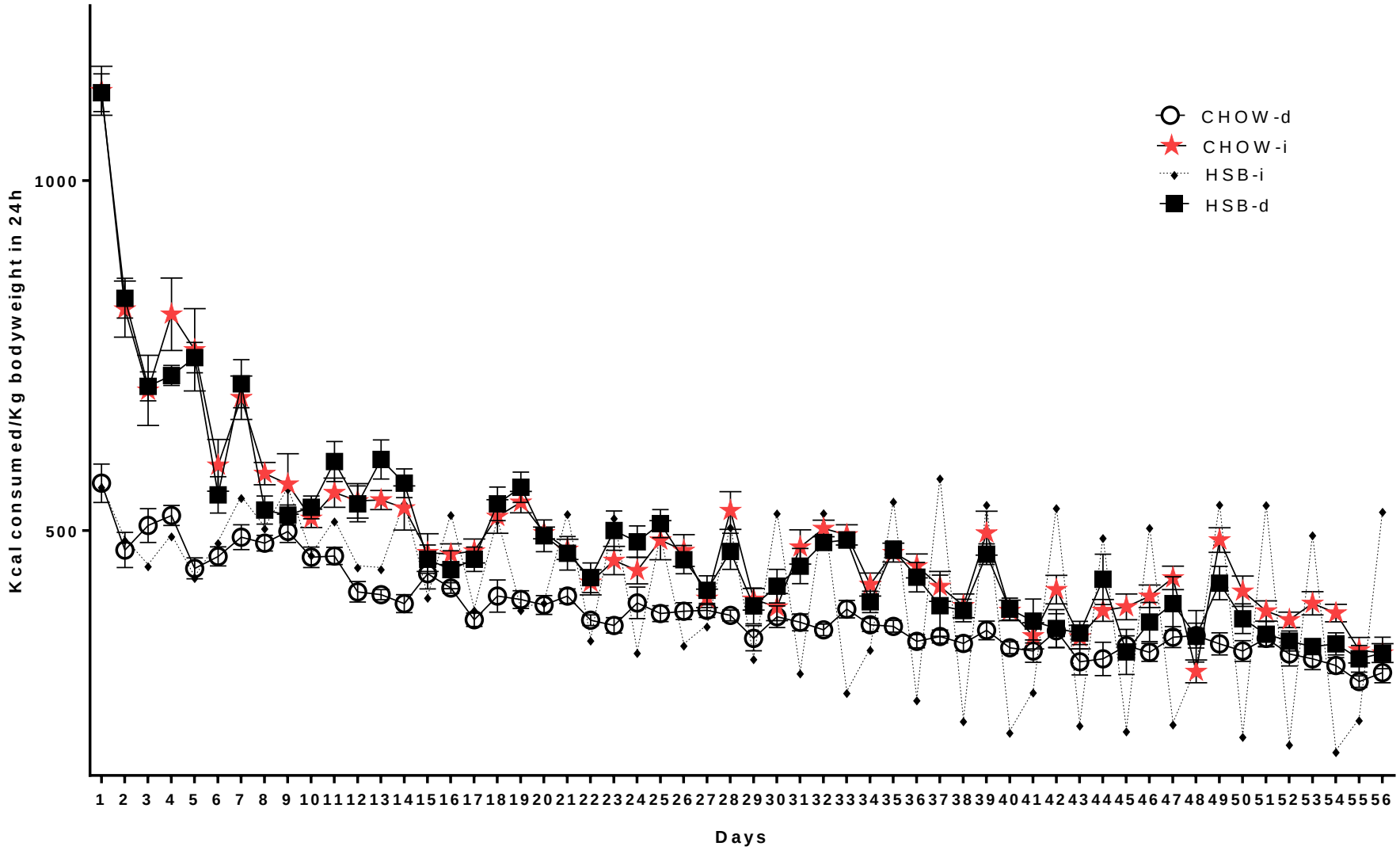

Figure 20. Kilocalories consumed by CHOW-i group does not differ from HSB-d in 24h schedule.

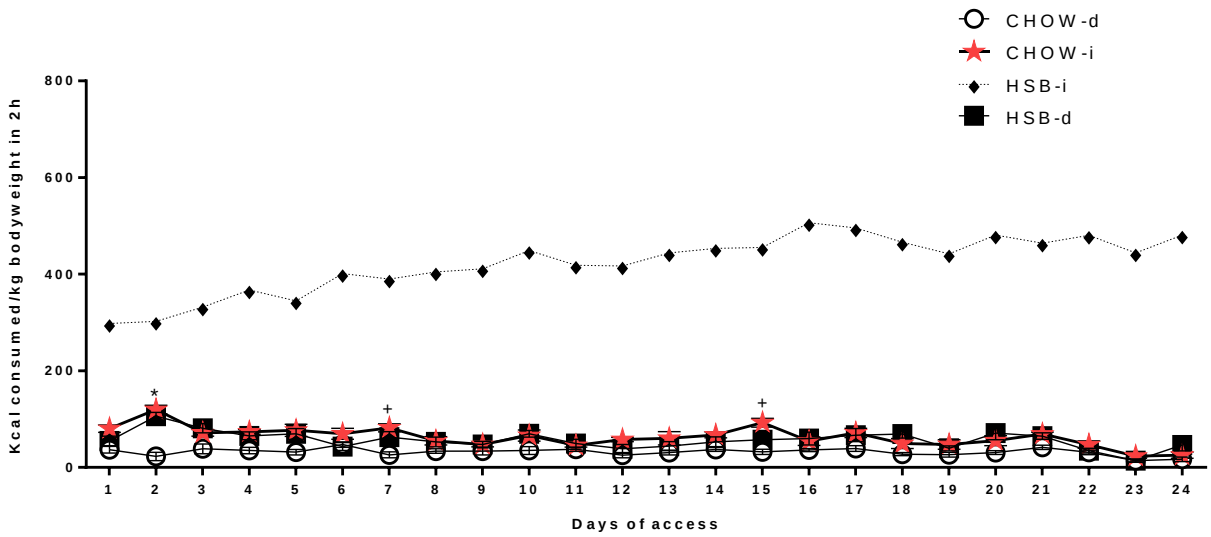

Figure 21. Kilocalories consumed by CHOW-i group does not differ from HSB-d in 2h access period. +, $p<0.05$ for CHOW-i vs CHOW-d.