UNIVERSITAT DE BARCELONA

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PROGRAMA DE DOCTORAT EN BIOMEDICINA

UNDERSTANDING THE MECHANISMS OF FOOD INTAKE AND OBESITY IN DOWN SYNDROME IS SUPPORTED BY BEHAVIORAL AND NEUROCHEMICAL ABNORMALITIES

Memòria presentada per Marta Fructuoso Castellar

per optar al títol de doctor per la Universitat de Barcelona

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Marta Fructuoso Castellar 2017

First of all, I would like to thank my Thesis supervisor, Professor Mara Dierssen for having given me the opportunity to work in her laboratory at the Centre for Genomic Regulation. By working in her group, I learned that being an exceptional scientist means being patient, strong, curious and a hard worker!

I would also like to thank my Thesis tutor, the Professor Jordi Alberch, and the members of my Thesis Committee at the CRG: Matthieu Louis, Cedric Notredame, Jordi García, who, along the Thesis gave me their support and scientific advice. Thanks to the members of the jury to accept to be a part of this important moment in a one's life. Thanks also to Imma Falero for your help during these years.

During my Thesis, I have the great honor to work with the Professor Nathalie Janel, who, beyond to be a passionate for science, is an excellent person who was always there for listening and helping me. MERCI. I also have some words to thank all the people I met during these years and I worked with. Thanks to Dr Hervé Le Sttuff and Dr Latif Rachdi, it has been a pleasure collaborating with you and learning from you. Thanks to Dr Grettje Vande, Dr Neus Martínez, and Rubén González who also contribute to this work. I also acknowledge Nadim Kassis and Axelle LeMarec for their help and their contagious happiness.

Gracias a la *familia científica*, constituida por los miembros de "Mara Dierssen lab". Pasamos sueño y estuvimos en "el pou", pero también lo pasamos bien. Gracias a la doctora María Martínez de Lagrán siempre ahí para consultas y favores (no imagino estos años aquí sin tí cerca). Gracias Júlia por tu disponibilidad y buen rollo; gracias a tu risa ensordecedora Mire, que me hace sonreír siempre desde el otro lado; gracias José por tu ayuda y amistad clave desde el día 0. Gracias a Linus e Ilario, "el dark side", que me han hecho replantearme el concepto de orden y me han hecho darme cuenta de la importancia de la etiología y el arte. Gracias Marcos por estar ahí para escuchar y ánimo, que se acaba, verás como todo tu esfuerzo vale la pena. Gracias Laura por tu paciencia con mis preguntas administrativas. Silvina, Ruggero, Marchin, Siân,…también Eduardo e Ibrahim, gracias a todos porque todos habéis sido importantes, como cada miembro de una familia lo es.

Quiero dedicar unas líneas a tres científicas con las que tuve el placer de coincidir espacio-temporalmente en el laboratorio de Mara Dierssen. Mónica Santos, de la que resalto su seriedad y rigor. Susanna Molas, mi "maestra de comportamiento", la personificación del trabajo y la fuerza. Y Belén de Sancristóbal, una gran amiga que irradia calidad científica y una bondad poco común. Gracias a las tres, probablemente sin saberlo, os habéis convertido en referentes para mí.

Un gran gracias a *La Familia*. Gracias por respetar y apoyarme en mi decisión de instalarme e iniciarme como científica en Barcelona en 2011. Gracias por vuestras visitas que tanto bien me han hecho durante estos años. En especial, gracias a mis chicas: mamá gracias por tus llamadas y tu amor, sé que para ti ha sido difícil no tenerme cerca, pero has sabido estarlo para mí cada día desde la distancia. Tía gracias por endulzarme (¡a mí y a todo el laboratorio!) los lunes después de un fin de semana por Valencia. Ana, mi hermana favorita, encarnas el éxito personal y profesional de quien lucha por conseguir su sueño, independientemente del tamaño de la barrera (corrijo, si es más alto, mayor será el triunfo). De ti aprendo cada día. Te quiero y espero que podamos trabajar juntas en un fututo doctora, sería un honor. Pero también gracias a mis chicos: papá gracias por venir a visitarme y estar ahí, tío por alegrarme cada vez que vuelvo por casa con tus risas y tus bailes y Hugo, porque tu felicidad y serenidad son un soplo de aire fresco. Y porque os quiero. Gracias también a mis *gachos* de Valencia.

Des remerciements aussi pour ma *belle-famille*, qui m'a tant chérie dès le début. Grâce à sa gentillesse et son hospitalité j'ai joui de moments de détente et de joie sur « le-paradis-sur-terre ». Catherine, je te remercie d'avoir nourri de livres mes étagères avec des milliers d'aventures et ma tête de pensées positives avec tes photos qui me souhaitaient une « bonne semaine ». Merci Marc pour ton aide précieuse avec mon *cv* et de me fournir tes meilleurs fromages. Merci « Const et Ol » car grâce à vous j'ai pu me loger et passer de bons moments en votre compagnie lors de mes stages à « la capitale ». Merci Nicole et Claude pour vous emails qui ont réchauffé mon cœur.

Marceau, que dire pour être à la hauteur de ce que tu mérites ! Ton sens du perfectionnisme et tes milliers de corrections et conseils - dans toutes les langues et domaines - m'ont énormément appris à apprendre de mes erreurs. Ta sensibilité et ta compréhension m'ont encouragé pour surmonter des moments difficiles. Merci d'avoir cru en moi-même quand je ne le faisais plus. Ma boussole, mon soutien le plus précieux. Merci de m'avoir attendue.

J'arrive, enfin.

Marta Fructuoso Castellar has been funded by a personal fellowship from Fundació Privada Centre de Regulació Genòmica (CRG) during the period 2013-2017.

The work has received financial help from Fondation Jérôme Lejeune, MINECO (SAF2013-49129-C2-1-R and SAF2016-79956-R), DURSI of Generalitat de Catalunya (SGR 2014/1125) and EU- Era NET Neuron (PCIN-2013-060).

ABSTRACT/RESUMEN

ABSTRACT

Obesity prevalence is higher in Down syndrome (DS) than in the general population. Beyond metabolic alterations, individuals with DS present increased impulsivity, a trait observed in obese people and in compulsive eaters that may affect their control of food intake. In this Thesis, we used a trisomic DS mouse model (Ts65Dn) to understand the behavioral component of obesity in DS and explore some possible underlying mechanisms.

Our meal pattern analysis revealed longer and slowly meals in Ts65Dn mice, leading to reduced eating rate, which may be associated to the mandible hypoplasia described in both human and mice. When exposed to obesogenic environments, Ts65Dn mice showed higher preference for energy-dense foods, gained more weight in specific conditions and scored higher in compulsivity and inflexibility tests than WT mice, as measured by binge eating during limited access and persistence of consumption of quine adulteration of energy-dense food. High performance liquid chromatography revealed reduced levels of dopamine in prefrontal cortex in Ts65Dn mice. This could lead to higher reward sensitivity that in turn would facilitate overeating as a compensatory response to restore optimal dopamine levels. Feeding behavior is also regulated by hormones and other circulating signals. We detected higher plasma leptin and glucose levels along with reduced insulin levels in Ts65Dn mice. Upon a glucose challenge, Ts65Dn mice showed reduced glucose-stimulated insulin response both *in vivo* and *in vitro*, suggesting a deficient insulin secretion or the reduced pancreatic mass. Indeed, we detected that Ts65Dn mice had altered plasma profile for some markers of inflammation and oxidative damage, in agreement with the high prevalence of autoimmune diseases and diabetes in DS people.

We also explored the involvement of the serine/threonine kinase DYRK1A, a candidate DS gene, in obesity and feeding behavior. *Dyrk1A* overexpression was sufficient to recapitulate some behavioral aspects associated to overeating in DS, but with a distinct profile. We conclude that increased obesity prevalence in DS is explained by both metabolic and behavioral alterations, in part driven by a hypodopaminergic status, and that *Dyrk1A* overexpression is only involved in specific DS obesity phenotypes.

RESUMEN

La prevalencia de obesidad es más alta en el síndrome de Down (SD) que en la población general. Más allá de las alteraciones metabólicas, los individuos con SD tienen mayor impulsividad, rasgo común en personas obesas y en comedores compulsivos, que pueden afectar el control de la ingesta de alimentos. En esta Tesis, se ha utilizado un modelo de ratón trisómico (Ts65Dn) para comprender el componente de comportamiento en el desarrollo de la obesidad en SD y explorar posibles mecanismos subyacentes.

Nuestro análisis del patrón de ingesta mostró que los ratones Ts65Dn comen más lento que los euploides, lo que podría estar asociado con la hipoplasia mandibular descrita en ratones y humanos con SD. Cuando los ratones Ts65Dn son expuestos a ambientes obesogénicos, comen mayores cantidades de dietas hipercalóricas, engordan más en determinadas condiciones y puntúan más alto en pruebas de compulsividad e inflexibilidad que los ratones euploides. La cuantificación de los niveles de monoaminas mediante cromatografía líquida puso en evidencia que los ratones Ts65Dn presentan niveles más bajos de dopamina en corteza prefrontal. Dado que las dietas hipercalóricas promueven la liberación del neurotransmisor en el circuito de recompensa, el sobre consumo de las mismas podría indicar un intento de restaurar los niveles óptimos de dopamina. La regulación de la ingesta también depende de hormonas y otras señales circulantes. Detectamos que los ratones Ts65Dn tienen mayores niveles de leptina y glucosa en plasma y niveles más bajos de insulina que los ratones euploides. La administración exógena de glucosa produjo una menor respuesta secretoria de insulina en los ratones Ts65Dn *in vivo* e *in vitro*, lo que sugiere una secreción deficiente o una masa pancreática reducida. Además, diversos marcadores de inflamación y estrés oxidativo son más elevados en los ratones Ts65Dn que en euploides, en consonancia con la mayor incidencia de enfermedades autoinmunes y diabetes en personas con SD.

En esta Tesis también se ha explorado la contribución de la proteína serina / treonina quinasa DYRK1A, un gen candidato para SD en la obesidad e ingesta. La sobreexpresión de *Dyrk1A* es suficiente para recapitular algunos comportamientos asociados a la ingesta compulsiva, pero con un perfil distinto al observado en el modelo trisómico. Concluimos que la prevalencia de la obesidad en SD se explica por alteraciones tanto metabólicas como conductuales, en parte como consecuencia de un estado de hipodopaminergia, y que la sobreexpresión de *Dyrk1A* está implicada en fenotipos específicos de la obesidad en SD.

PRESENTATION

PRESENTATION

Individuals with Down syndrome (DS; trisomy HSA21), a neurodevelopmental disorder causing intellectual disability, are more likely to become obese. In this Thesis, we aimed at understanding the behavioral and metabolic mechanisms underlying increased prevalence of obesity in DS using a validated trisomic mouse model (Ts65Dn).

In the first part of this work, we characterized the time-course and dynamics of obesity-related behavioral changes induced by the availability of energy-dense foods in Ts65Dn mice. We first studied how this "obesogenic" situation may affect the animal's behavior, and specifically its meal pattern. The fine behavioral characterization of feeding behavior was performed in the Animal Facility of the Barcelona Biomedical Research Park (PRBB), using an analysis pipeline specially conceived for longitudinal data. In non-obesogenic conditions Ts65Dn mice showed a distinct eating pattern, characterized by increased energy intake with reduced eating rate and longer meals as compared to wild-type (WT) mice. Upon free choice exposure to highly palatable and high-caloric diets, that play a key role in the development of overweight, Ts65Dn mice showed higher preference for energy-dense diets than WT and scored higher in compulsivity and inflexibility tests (limited access to energy-dense food and food adulteration with quinine hydrochloride).

To understand the mechanisms underlying the overeating phenotype, we studied the neurochemical correlates in basal conditions and upon long-term access to energy-dense diets. I performed this neurochemical characterization under the supervision of Professor Nathalie Janel, from the University of Paris Diderot during a short-term EMBO fellowship I benefited. High performance liquid chromatography revealed a selective reduction of the dopamine (DA) levels in the prefrontal cortex of Ts65Dn mice compared to WT, suggesting that trisomy may produce a reward-deficient phenotype. We also observed altered plasma levels of some anorexigenic and orexigenic signals such as leptin and ghrelin in Ts65Dn mice that might affect feeding behavior and energy balance inducing a pro-obesogenic state. Our work gave rise to a manuscript that is in preparation and will be submitted to *Biological Psychiatry*.

A second mechanism that could be involved in the overweight phenotype is inflammation, which is common in obese people. DS is associated with increased prevalence of autoimmune disease and impaired inflammatory mechanisms. However, the link of these alterations with adiposity has not been explored. To do so, during my Thesis, I studied the obesity-related

inflammatory status in mice maintained in non-obesogenic (standard rodent chow) and obesogenic (mice receiving high-fat) conditions since mice receiving chocolate-mixture gain less weight with no genotype-dependent differences. We detected that Ts65Dn mice had altered plasma levels of some markers of inflammation and oxidative damage. The work entitled "Increased levels of inflammatory plasma markers and obesity risk in a mouse model of Down syndrome" is in second revision in *Free Radical Biology and Medicine*.

Regarding the metabolic components of obesity in DS, thanks to an international collaboration with Professor Hervé Le Stunff from the University of Paris Diderot and Professor Latif Rachdi from the Institute Cochin (Paris), we explored how trisomy 21 affects the risk of diabetes mellitus. My work revealed morphological and functional deficiencies in exocrine pancreas of Ts65Dn mice that supports the higher prevalence of diabetes in DS people and we explored different mechanisms. These results will be submitted to *Endocrinology*.

The second main aim of the Thesis was to narrow down the genetic dependence of some of the DS-obesity phenotypes. We focused on DYRK1A, a serine protein kinase relevant for DS brain and cognitive defects. To this aim, we used a monogenic transgenic mouse model of DS (mBACTgDyrk1A, kindly provided by Professor Jean-Maurice Delabar, Institut du Cerveau et de la Moelle Épinière) with 1.5-fold overexpression of *Dyrk1A* in an otherwise disomic context. Meal pattern analysis showed no differences in feeding behaviors in TgDyrk1A mice, but they had specific food preferences and were protected against diet-induced obesity. The behavioral characterization was conducted in the CRG and the neurochemical characterization will be done at the University Paris Diderot in collaboration with Professor Nathalie Janel. The results of this work will be submitted to *Neurobiology of Disease*.

In summary, my work has shown central and peripheral obesity phenotypes in DS with feeding behavioral traits and region-specific neurochemical defects explaining overeating and overweight in DS. It has also narrowed down the genetic dependence of these phenotypes, showing the importance of *Dyrk1A* overexpression to drive reward-related food preferences.

During my PhD experience, I had the opportunity to present my work in several national (16 congress of the "Sociedad Española de Neurociencias, Granada and "VIII Cajal Winter Conference", Salamanca) and international meetings (1st T21RS International Conference, Paris, France). I have also participated in a number of science dissemination activities and in

the Gender Balance Committee of the CRG. I had the specific support of a predoctoral fellowship of the Center for Genomic Regulation, and my project was funded by grants of the Jerome Lejeune Foundation, and the Spanish Ministry of Economy and Competitiveness.

List of scientific publications

1. **Fructuoso M**, Rachdi L, Philippe E, Denis R, Magnan C, Le Stunff H, Janel N, Diessen M. **Increased levels of inflammatory plasma markers and obesity risk in a mouse model of Down syndrome.** Free Radical Biology & Medicine (*submitted*).

2. **Fructuoso M,** Espinosa-Carrasco J, di Toma I, Janel N, Dairou J, Dierssen M. **Overeating in a mouse model of Down syndrome is associated to dopaminergic deficit in the prefrontal cortex.** Biological Psychiatry (*in preparation*).

3. **Fructuoso M**, Denom J, Magnan C, Le Stunff H, Janel N, Diessen M, Scharfmann R, Rachdi L. **Glucose homeostasis and pancreatic dysfunction in a mouse model of Down syndrome.** Endocrinology (*in preparation*).

4. **Fructuoso M,** Espinosa-Carrasco J, Martín-García E, González Colom R, Martínez-Abadías N, Vande Velde G, Janel N, Dierssen M. **Increased chocolate craving in a mouse model of DYRK1A overexpression : implications for compulsive overeating** (*in preparation*).

5. **Fructuoso M,** González Colom R, Martínez-Abadías N, Vande Velde G, Dierssen M. ***Dyrk1A* overexpression in mice is sufficient to recapitulate the brachycephalic phenotype associated to Down syndrome** (*in preparation*).

The already submitted paper and the results that are not included in the main text of the Thesis are fully available as Supplementary Results in Annex II.

Besides my Thesis project, I have been involved in collaborations which gave rise to two papers. In both cases, I did the behavioral analysis of the work and revised the paper.

1. De Toma I, Grabowicz IE, **Fructuoso M**, Trujillano D, Wilczynski B, Dierssen M. **Brain region specific transcriptional responses to diet are driven by highly coordinated changes confined to regulatory domains.** Genome Research (*in preparation*). My contribution in Figure 1 and Supplementary Figure 1.

2. Espinosa-Carrasco J, Burokas A, **Fructuoso M**, Erb I Martin-Garcia E, Gutiérrez-Martos M, Notredame C, Maldonado R, Dierssen M. **Time-course and dynamics of obesity-related behavioural changes induced by different energy-dense foods in mice.** Addiction Biology (*in first revision*). My contribution in Figure 2 and Supplementary Figure 4.

TABLE OF CONTENTS

INTRODUCTION

1. INTRODUCTION

1.1 Obesity in Down syndrome

Down syndrome (DS) is a neurodevelopmental disorder caused by the partial or total trisomy of chromosome 21, leading to intellectual disability (Antonarakis et al. 2004). As a consequence of the extra genetic contribution, DS gives rise to a plethora of different alterations affecting both peripheral and central nervous system. Among those, the most common features having an impact in daily life of DS patients are the intellectual impairments, heart abnormalities and obesity (Roizen and Patterson 2003; Karmiloff-Smith et al. 2016).

The prevalence of obesity is high in DS population. Bell and collaborators found that 70.58% of males and 95.83% of females with DS were categorized as overweight and obese compared with 40% of males and 32% females in that category from general population (Bell 1992). Other studies have shown that the rate of obesity or overweight in DS was two to four times higher than in non-disabled youths matched by age and gender (Rimmer et al. 2010; Must et al. 2014).

The identification of the causes leading to increased obesity risk in DS in human studies is complicated since existing data are for different countries with different dietary habits, making it difficult to compare populations. Traditionally, the increased obesity risk in DS individuals has been mainly attributed to their endocrine disturbances and reduced physical activity but feeding practices may also play an important role in the development of obesity in this population (Melville et al. 2005). In fact, there is an association between body mass index (BMI) and overeating (Mallan et al. 2017) and several reports suggest unhealthy dietary habits as an important factor for obesity risk in DS population (Grammatikopoulou et al. 2008). The management of the excessive weight gain has been usually focused on food restriction, and increased exercise that have proven moderate success in preventing or treating DS obesity (Spanos et al. 2013).

1.2 Metabolic and lifestyle factors contributing to obesity development in Down syndrome

DS individuals have higher BMI and percentage of body fat (% BF) than age- and sex-matched persons without DS (González-Agüero et al. 2011; Loveday et al. 2012). Within the DS population there is a sexual dimorphism with a higher value for BMI, % of BF and overweight prevalence in females than in males (Bell 1992; Slevin et al. 2014). The mechanisms of this increased obesity risk have not been studied systematically in the DS population but genome wide association studies have found that the chromosome 21 (HSA21) shows overlap with quantitative traits loci (QTL) for body weight (BW276_H) and seven obesity related loci (Dasouki et al. 2011; Kunej et al. 2013).

Energy expenditure and glucose homeostasis in Down syndrome

Energy expenditure depends of the circulating messengers controlling energy balance (López et al. 2013). Among those circulating signals, leptin is secreted by adipocytes, acting in the hypothalamus to suppress appetite and regulate body weight (Considine et al. 1996). Obesity in the general population is associated with increased body fat accumulation. Leptin levels are positively correlated with the percentage of body fat so that it has been proposed that obese individuals have some degree of leptin resistance (Considine et al. 1996). Obesity in DS has been attributed partially to leptin resistance since blood levels are higher in this population compared to non-obese DS individuals (Proto et al. 2007; Magge et al. 2008).

Another endocrine condition in DS that may affect body weight increase is hypothyroidism that is the most common endocrine alteration in the DS population, particularly in the form of subclinical hypofunction (Lavigne et al. 2017). This endocrine abnormality might also directly affect fat accumulation since thyroid hormones are involved in the activation of gene pathways controlling thermogenesis, glucose homeostasis and fat oxidation (Mullur et al. 2014).

Diabetes has also a higher prevalence in DS than in the general population. Diabetes is defined by reduced insulin secretion and insulin sensitivity, resulting in abnormally high blood glucose levels in plasma. Type 1 diabetes (T1D) is commonly associated to immunological problems leading to deficient insulin production whereas type 2 diabetes (T2D) is associated with insulin deficiency and insulin resistance (American Diabetes

Association). While obesity is mainly related to T2D, the clinical manifestation of DS diabetic patients usually is closer to a TD1, with an earlier onset peak compared to non-DS children (Anwar et al. 1998; Rohrer et al. 2010) and with increased level concentrations of autoantibodies, which is a marker of β pancreatic cells damage (Steck et al. 2015). It has been shown that in general population the major genetic determinants of T1D are polymorphisms of class II HLA genes located in the human chromosome 6 (Dean et al. 2004). One study conducted in DS patients detected an increase in the frequency of the classic risk allele compared to the neutral or protective ones when analyzing the usual major type 1 diabetes risk locus located in the HLA region (Bergholdt et al. 2006). Authors conclude that, besides HLA region of chromosome 6, other regions such as chromosome 21 (HSA21), might be contributing to type 1 diabetes risk (Anwar et al. 1998). The increased risk of diabetes in DS could also be related to proteins encoded in the chromosome 21 that are expressed in pancreas (Esterhazy et al. 2011; Peiris et al. 2016; Rachdi et al. 2014a). It has been recently shown that basal insulin secretion by DS fetal pancreatic cells was drastically decreased compared to non-DS cells (Helguera et al. 2013) the secretory defects being attributed to RCAN1 overexpression (Peiris et al. 2016) that is located in the HSA21.

Physical activity in Down syndrome individuals

Physical activity is the main factor determining energy expenditure. Sedentarism has been positively associated to obesity in the general population (Jebb and Moore 1999). Many young people and adults with ID have a sedentary lifestyle (Dairo et al. 2016) and undertake low levels of exercise (Temple and Stanish 2008) compared to general population. Specifically, most of the studies show that DS population did not comply with the recommended physical activity (Izquierdo-Gómez et al. 2014; Phillips and Holland 2011). Independently of the age, people with DS spent more time in sedentary activities compared to people with intellectual disabilities without DS (Phillips and Holland 2011). Improving physical activity may require the involvement of family and carers, as there is a positive association with the implication of the parents and the healthy habits of the DS children (Spanos et al. 2013).

Mechanical problems affecting feeding behavior in Down syndrome

Finally, it is also important to account for the mechanical aspects of feeding that may have an impact of the final feeding behavior phenotypes. Very often, DS children and adults present oral-motor difficulties (Faulks et al. 2008a-b) that produce feeding behavior disturbances. Although some are corrected during development, some persist to adulthood (Smith et al. 2014). The orofacial dysfunction has direct repercussions on mastication and swallowing and can potentially lead to texture selectivity but also lead to life-threatening conditions such as malnutrition and dehydration in the most severe conditions (Lazenby 2008). DS subjects present hypotonia of tongue and lips, underdeveloped facial mid-third and lax temporomandibular joints (Faulks et al. 2008a-b). As a consequence, diverse problems such as delayed initiation and poor coordination and sequencing of oromotor movements and difficulty for chewing are common in DS individuals (Hennequin et al. 1999; Lazenby 2008). This might be a factor influencing the choice of softer food, such as high-fat food.

1.3 Brain mechanisms of control of feeding behavior and their alterations in Down syndrome

Energy intake depends on multiple and redundant signaling pathways modulated by interoceptive and exteroceptive cues (Figure 1). The nutritional information about food is mediated by gastric distension, release of hormones, neuropeptides whereas the hedonic value of the food requires sensorial information that is provided by our sensory organs (Morrison et al. 2012). Appetite and food preferences and choices depend on two distinct -though interconnected- pathways at the central nervous system (CNS) level: one related to the homeostatic control of energy balance and the other controlling the non-homeostatic or hedonic food intake.

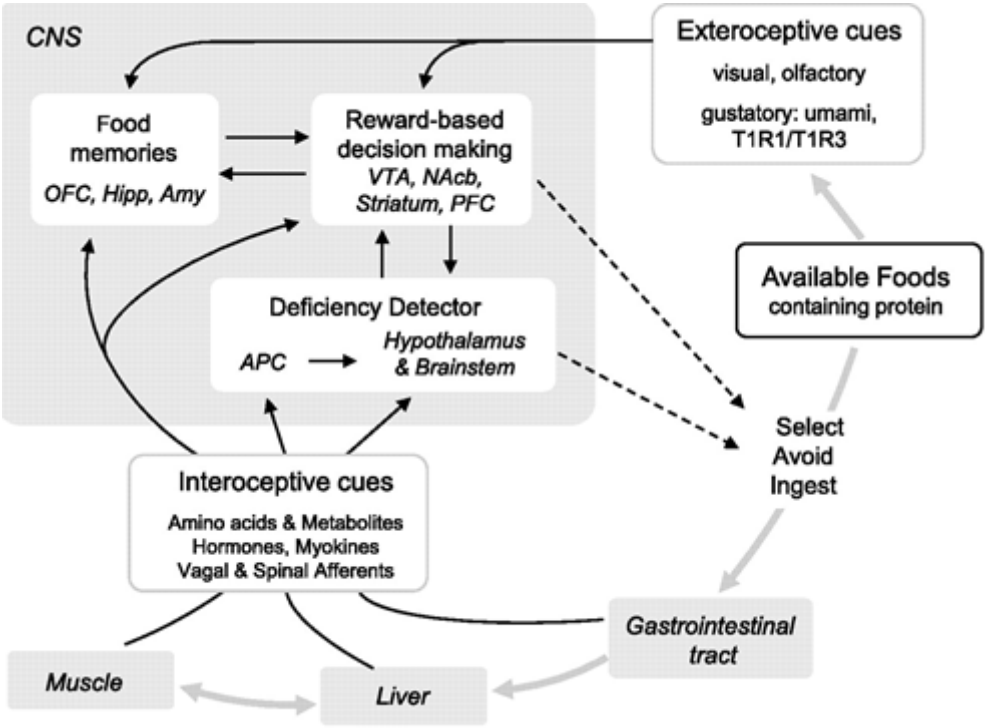


Figure 1. Food selection is controlled by both energy requirements and reward-related mechanisms. In mammals, feeding is controlled by external factors such as food availability and internal factors such as the hormones produced in starvation and upon feeding by internal organs. Eating is decided by a homeostatic- based decision in which the hypothalamus acts as a "deficiency detector". Moreover, there is a "reward-based decision making" controlled by prefrontal cortex and striatum that mediates feeding beyond energy demand. Our choices also depend on the sensorial experience and our memories and areas such as the hippocampus are also involved in feeding behavior. In the figure: orbitofrontal cortex (OFC), hippocampus (Hipp), amygdala (Amy), ventral tegmental area (VTA), nucleus accumbens (NAcb), prefrontal cortex (PFC), anterior piriform cortex (APC), taste receptors (T1R1/T1R3) (Morrison et al. 2012).

Homeostatic control of feeding behavior

The hypothalamus is the main region involved in homeostatic control of feeding behavior. It is critical in the relaying of afferent signals from the gut and brainstem as well as processing efferent signals that modulate food intake and energy expenditure. The hypothalamus is subdivided into interconnecting nuclei, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial nucleus (VMN), dorsomedial nucleus (DMN) and lateral hypothalamic area (LHA). Neuronal pathways between these nuclei are organised into a complex network in which orexigenic (promoting food intake) and anorexigenic (inhibiting food intake) circuits influence food intake and energy expenditure. The ARC is in contact with the circulating blood being key for the regulation of appetite, so that its lesion results in obesity and hyperphagia in mice (Olney 1969). It integrates circulating hormonal and chemical signals that act on neuronal populations that 1/ co-express neuropeptide Y (NPY)

/agouti-related protein (AgRP) and which activation increase food intake, or 2/ co-express proopiomelanocortin (POMC) /cocaine- and amphetamine-regulated transcript (CART) that inhibit food intake (Figure 2) (reviewed in Waterson and Horvath 2015). Regarding the other nuclei, LHA receives neuronal projections from the ARC and contains the orexigenic neuropeptides melanin concentrating hormone (MCH) and orexins. It has been shown that microinjection of almost all known orexigenic peptides into the PVN stimulate feeding whereas the destruction of DMN and VMN results in hyperphagia and obesity (reviewed in Waterson and Horvath 2015). All these data suggest that the concerted action of central and peripheral orexigenic and anorexigenic signals is integrated at the hypothalamic level.

The anatomical and functional integrity of the hypothalamic circuits have not been studied in depth in DS brains. However, one morphometric study has shown diminished number of neurons in the arcuate nuclei of the hypothalamus of DS patients (Wisniewski and Bobinski 1991). Deficits of other hypothalamic dependent functions such as sleep regulation and growth hormone secretion have been reported in DS, (Castells et al. 1992; Castells et al. 1996; Anneren et al. 1999) suggesting that the alterations of brain regions involved in homeostatic control of energy intake could be contributing to obesity in DS.

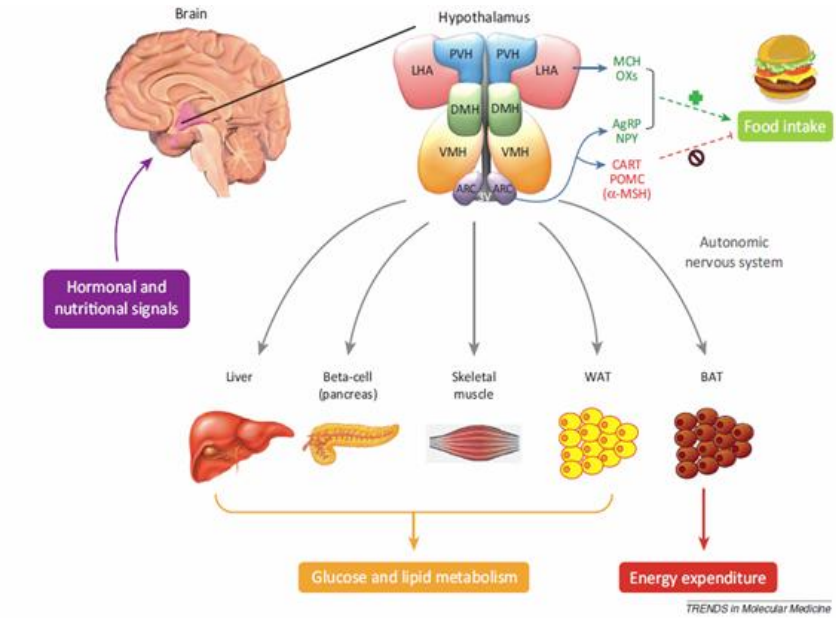


Figure 2. Hypothalamic regulation of feeding and energy balance. Specific nuclei in the hypothalamus respond to alterations in food availability, energy stores, and nutritional requirements. In the arcuate nucleus (ARC), AgRP/NPY and POMC/CART neurons act as a hunger and satiety centers and elicit functional changes in a range of tissues including the liver, pancreas, muscle, adipose white adipose tissue (WAT) and brown adipose tissue (BAT) (López et al. 2013).

Hedonic control of feeding behavior

The regulation of food intake involves a close interrelationship between homeostatic and nonhomeostatic factors. The former are related to nutritional needs and monitor available energy within the blood and fat stores, whereas the latter are considered unrelated to nutritional or energy requirements. Most nonhomeostatic mechanisms are related to the brain reward system (Kelley et al. 2005). Contrary to the hypothalamus which main role is the energy detection, regions such as the striatum and the prefrontal cortex are mostly implicated in the hedonic feeding and the behavioral control to eat. The mesocorticolimbic pathway is a central component of this system. It arises from dopaminergic neurons located in the ventral tegmental area of the midbrain that send projections to target areas in the limbic forebrain, particularly the nucleus accumbens, as well as the prefrontal cortex and the striatum. The prefrontal cortex, in turn, provides descending projections to the nucleus accumbens and the ventral tegmental area. Several reports have observed that obese patients had reduced orbitofrontal volume (Maayan et al. 2011; Cohen et al. 2011) suggesting its involvement in disinhibited eating and obesity. Also, lower baseline prefrontal cortex metabolism has been correlated to higher BMI (Kg/m^2) in both PET (Wang et al. 2011) and SPECT (Willeumier et al. 2011). At the behavioral level, obese patients have worse performance in prefrontal cortex-dependent tasks requiring decision-making, response inhibition and cognitive flexibility, as compared to healthy controls (Lena et al. 2004; Fagundo et al. 2012). Inappropriate sensitivity to punishment and difficulties to focus on long-term goals has also been observed in compulsive eaters (Danner et al. 2012; Wolz et al. 2016; Navas et al. 2016). This mesocorticolimbic circuit, then, is a key player in the final common pathway that processes reward signals and regulates motivated behavior in rats and, according to imaging data, in humans.

Other regions are also involved in the reward-related food consumption, including regions implicated in sensory reception (insular and gustatory cortex), attribution of the salience (orbitofrontal cortex) and emotion processing (amygdala and hippocampus) (Lee et al. 2012) (Figure 3). Obesity promotes changes in this circuit sustaining overeating (Volkow et al. 2013). Neuroimaging studies have shown that compared to lean controls, the striatum of obese adults show higher brain activation in response to food cues and lower brain activation upon feeding, respectively. This may suggest that

both increased reactivity to reward expectation but decreased sensitivity to food reward might underlie overeating (Volkow et al. 2011; Michaelides et al. 2012).

A higher consumption of energy-dense foods has been reported in DS individuals (Grammatikopoulou et al. 2008; Nordstrøm et al. 2015) that could reflect alterations in the reward response to diet. In adulthood, the dietary intake is unbalanced in DS individuals with higher protein and fat intake and low carbohydrate intake than recommended (Sharav and Bowman 1992; O'Neill et al. 2005; Marin and Graupera 2011). This leads to excessive intake of inappropriate nutrients, which is common in children with DS and may result in overfatness (Wong et al. 2014). These unhealthy food choices and the consequent body weight gain normally occur during adolescence or in adulthood, which coincides with the time when DS individuals leave their parental house and lose the dietary supervision (Sharav and Bowman 1992), which suggests that their autoregulatory mechanisms are impaired. In fact, lower food variety is also common suggesting a biased in food choices based on personal food preferences. As a consequence, dietary intake in DS adults used to be unbalanced with higher protein and fat intake (Marin and Graupera 2011).

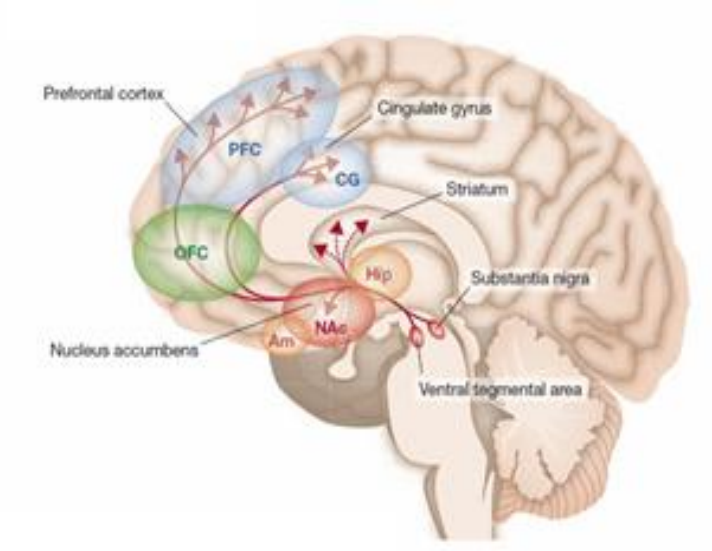


Figure 3. Schematic representation of brain circuits implicated in hedonic feeding. The nucleus accumbens (NAc) assess reward and saliency. The orbitofrontal cortex (OFC) is involved in sensory integration for decision-making and determining the expected rewards and punishments of an action. The amygdala (Am) and hippocampus (Hip) are involved in forming memories of the stimulus/reward relationship, whilst the prefrontal cortex (PFC) and the anterior cingulate gyrus (CG) are responsible of the inhibitory control and emotional regulation. The arrows in red depict the mesolimbic reward pathway. Energy-dense foods cause neurons from the ventral tegmental area to release dopamine in the nucleus accumbens. These regions regulate activity in the frontal cortical regions. Adapted from Lee et al. 2012.

In DS reduced frontal cortex volume (Whittle et al. 2007) and a decreased functional connectivity (Pujol et al. 2015) have been reported. This may compromise their executive functions and lead to impulsivity and motor abnormalities that require the integrity of the fronto-striatal network functioning (Brunamonti et al. 2011). In fact, difficulties in sustained attention, planning and problem-solving, working memory, inhibition and perseveration are common (Jernigan et al. 1993; de la Torre et al. 2016; Lanfranchi et al. 2010; Pritchard et al, 2015). However, no work has specifically address the striatal activation upon food stimuli in DS individuals and thus, the implications of an altered frontal-striatal in feeding control are unknown.

1.4 Monoamines as regulators of homeostatic and reward aspects of feeding

Among the multiple messengers that mediate and control feeding, monoaminergic neurotransmitters are especially relevant since they act both at the "energy-sensing" and at the "reward-based decision making" level. We will briefly review the involvement of each of the monoaminergic systems in feeding behavior.

One of the main monoaminergic systems is the serotonin or 5-hydroxytryptamine (5-HT). 5-HT is involved in both the modulation of feeding behavior and the regulation of glucose levels. It is synthesized from tryptophan by the enzyme Tryptophan Hydroxylase (TPH) and primarily degraded by the mitochondrial bound protein Monoamine Oxidase A (MAOA), leading to the generation of the metabolite, 5-hydroxyindoleacetic acid (5-HIAA) (Lu 2017). Serotonergic neurons are located in the raphe nuclei send projections to the peripheral nervous system (Fuxe et al. 1988). The dorsal raphe (B7), in particular, is a midbrain nucleus that contains a substantial portion of the total brain 5-HT and has distinct projections to hypothalamic nuclei and feeding-related forebrain areas such as the frontal cortex (Celada et al. 2013), supporting its dual role in energy-sensing and food-derived reward. This multifaceted control is reflected even at the molecular level with peculiar contribution of the serotonin receptor 5-HTR2C in the hypothalamus (homeostatic control) and serotonin receptor 5-HTR4 in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) (non-homeostatic control) (Compan 2013). In mice, constitutive activity of NAc-5-HTR4 triggers anorexia, while its inhibition leads to overeating (Compan 2013). In the hypothalamus 5-HT acts as a satiety signal. Concretely, it has been shown that serotonin released from projections of the raphe innervating the hypothalamus serves for meal termination

(Leibowitz and Alexander 1998). Carbohydrate intake increases the levels of 5HT in hypothalamus probably by direct increase of 5-HT synthesis since tryptophan is a metabolite of carbohydrates but also a precursor of serotonin. The serotoninergic system is also implicated in the non-homeostatic feeding control. This might be related to their role in mediating behavioral flexibility (Puig and Gulledge 2011) since both increased (Dalley et al. 2002) and decreased (Harrison et al. 1997; Clarke et al. 2004) serotonin levels in prefrontal cortex are associated with increased impulsivity, impaired reversal learning and increased perseveration. Regarding obesity, it has been shown that humans with a higher BMI had increased density of 5-HTR4 in the NAc and orbitofrontal cortex, that has been explained as an adaptive mechanism in which upregulation of the 5-HTR4 may result from the frequent lower levels of serotonin in obese individuals (Haahr et al. 2012).

Dopamine (DA) has also a key role in functions related to feeding behavior. It mediates flexible behaviors (Winstanley et al. 2005) and participates in the control of impulsive choices and perseveration (Brozoski et al. 1979; Laskowski et al. 2016). DA is synthesized in the dopaminergic neurons located in the midbrain, specifically in the substantia nigra and the ventral tegmental area (VTA), and is mainly released in the striatum (Stott and Ang 2013). In the brain, there are three main DA pathways: the nigrostriatal, related to motor function, the tuberoinfundibular and mesocorticolimbic (Stott and Ang 2013). In the mesocorticolimbic pathway, the VTA projects to the cingulate gyrus and orbital and medial prefrontal cortex. This pathway is related to the cognition and motivation and has a main role in reward mechanisms controlling food intake (reviewed by Palmiter 2008). Striatal DA is necessary for food seeking and ingestive behavior as shown by previous reports in which DA depletion (genetically or pharmacologically by inhibiting their limiting synthesis enzyme) causes death by starvation in mice (Szczypka et al. 1999; Cannon et al. 2004; Palmiter 2008). In humans, ingestion of fatty or sweet foods has been shown to release dopamine (DA) in the dorsal striatum proportional to the self-reported level of pleasure derived from eating the food (Small et al. 2003). Probably because of that, humans overconsume pleasurable food in spite of the negative health consequences (Duffey et al. 2007; Schulze et al. 2004). Upon first exposure, a food reward activates the dopaminergic neurons in the ventral tegmental area (VTA), which leads to an increase of DA tone and release to the striatum and its ventral extension, the nucleus accumbens (NAc) (Volkow et al. 2013). Indeed, altered dopaminergic tone has been proposed as a vulnerability

factor to overconsumption (Mizoguchi et al. 2010). In obesity-prone rats there is a global presynaptic deficit in DA in prefrontal cortex, likely due to differences in the expression of genes that regulate dopamine exocytosis (Geiger et al. 2008). Conversely, DA injection in the hypothalamus inhibits eating in rats suggesting that it locally acts as a satiety signal (Leibowitz and Rossakis 1979).

The brain noradrenaline (NA) system plays an important role in the central nervous control of energy balance and is thus implicated in the pathogenesis of obesity. The NA producing cells are mainly located in the locus coeruleus (LC) and send noradrenergic fibers that diffusely innervate areas such as cortex, thalamus, hypothalamus, hippocampus (Samuels and Szabadi 2008). Evidence for a direct involvement of NA in mediating food intake derives mainly from pharmacological and lesioning studies performed in animals. Noradrenergic fibers arriving to hypothalamus can either elicit hypophagia to hyperphagia, depending upon the subset of adrenoceptors they active (Wellman 2000). It has been shown in rats that NA acting in the hypothalamic arcuate, ventromedial and paraventricular nuclei stimulates feeding (Leibowitz 1970; Date et al. 2006), whereas it inhibits feeding in the lateral hypothalamic area through α- and β-receptors (Leibowitz 1970). Furthermore, ablating hindbrain noradrenergic neurons, which project to the hypothalamic ARC and PVN eliminates orexigenic and potentiates anorexigenic actions of feeding hormones in rats, respectively. The appetite-suppressing drugs amphetamine, methamphetamine and phentermine all possess a monoaminergic mechanism of action; in particular they potently cause release of NA from synaptic vesicles as well inhibiting/reversing extracellular NA reuptake by the NA transporter (NAT) at the synaptic cleft (Adan et al. 2008).

Natural stimuli induced an increase in NA release in the mPFC of mice that is proportional to their salience (Ventura et al. 2007). In addition, the NA prefrontal transmission is a necessary condition for motivational processing of reward related stimuli probably through modulation of dopamine release within the NAc (Puglisi-Allegra et al. 2012), including feeding stimuli. In fact, it has been suggested that excessive NA tone could be the starting point to aberrant motivation and leading to excessive palatable food intake (Latagliata et al. 2010). Critically, the NA system is a key substrate that mediates stress and emotional eating, so that food seeking despite harmful consequences is preventable by selective NA inactivation of the mPFC (Dallman 2010). Hence, prefrontal NA plays a critical role in maladaptive eating

behavior whislt prefrontal cortex and striatal NA transmission are required for food-related motivated behavior.

Monoaminergic profile in Down syndrome

The monoaminergic systems have been explored in DS patients mainly in relation with age-related cognitive impairment and Alzheimer's disease. Initial postmortem studies in older DS patients demonstrated reduced brain concentrations of serotonin (5-HT), and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA) and norepinephrine (NE) (Nyberg, et al. 1982; Yates et al. 1983; Reynolds and Godridge 1985; Godridge et al. 1985; Seidl et al. 1999). This possibly results from the reduced cell numbers in the locus coeruleus and dorsal tegmental nucleus of the raphe complex (Mann et al. 1984; Mann et al. 1985) while the source of the dopaminergic deficits is unclear. More recent works have also shown monoamine reductions in entire brain or in different regions of DS younger adults (Mann et al. 1987; Risser et al. 1997). Significant reductions in tissue concentrations of serotonin, noradrenaline, and dopamine in the frontal cortex of fetal DS cases at a gestational age of approximately 20 weeks have been also found compared to age-matched control fetuses (Whittle et al. 2007).

In biosamples, only few studies have explored monoaminergic biomarkers in DS. Plasma concentrations of homovanillic acid (HVA) and 5-HIAA have been reported to accurately indicate alterations in regard to DA and 5-HT metabolism in the brain. Moreover, the (nor) adrenergic metabolite 3-methoxy- 4-hydroxyphenylglycol (MHPG) diffuses freely over the blood-brain barrier, thereby reflecting central NA activity. Shapiro and collaborators reported that cerebrospinal concentrations of 5-HIAA, HVA, MHPG, and norepinephrine were significantly higher in young DS adults as compared to controls, while in serum concentrations were unchanged, suggesting increased turnover of monoamines in adults with DS (Schapiro et al. 1987; Kay et al. 1987).

Several studies have implicated trait differences in DA signaling on executive functioning based on genetic polymorphisms (COMTVal158Met, VNTR-DAT1) in the catechol-O-methyltransferase (COMT) and in the gene encoding for the dopamine transporter (DAT, SLC6A3). Recently, a study of genetic polymorphisms of the enzymes related to the synthesis and disposal of dopamine (DA) in a cohort of DS adults (Del Hoyo et al. 2016) revealed that those individuals having better performance

in executive function tasks were those bearing the genotypes conferring higher DA availability, such as Met carriers and VNTR-DAT1 10-repeat homozygotes (del Hoyo et al. 2016). This study supports that problems in flexibility in DS patients could be partially due to dopaminergic alterations. Dekker and collaborators showed recently that serum concentrations of 3-methoxy-4-hydroxyphenylglycol (MHPG) the principal metabolite of NA, are significantly lower in demented and dementia converted DS subjects compared to non-demented DS individuals and healthy controls (Dekker et al. 2017). The DS individuals with MHPG levels below median had a tenfold increased risk of developing dementia, suggesting that NA deafferentation is linked to dementia onset in DS (Dekker et al. 2017). However, no previous studies have linked the monoaminergic phenotypes with obesity.

1.5 Narrowing the genetic dependence of Down syndrome obesity phenotypes: DYRK1A

DS phenotypes are the result of a trisomy, and thus present overdosage of a large number of genes. Among the triplicated genes, accumulating evidences suggest that the gene encoding for the Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1A (DYRK1A), could be implicated in both homeostatic and non-homeostatic aspects of feeding behavior.

DYRK1A is the homologue of the Drosophila minibrain (mnb), which was named from the description of the brain phenotype observed in hypomorphic mutant flies (Tejedor et al. 1995). The DYRK family includes four additional mammalian subtypes including DYRK1B, DYRK2, DYRK3, and DYRK4. DYRK proteins show little sequence homology with other kinases outside of their catalytic domains but are highly conserved across species (Becker et al. 1998). DYRK1A can catalyze its own activation through auto-phosphorylation of a single tyrosine residue in its activation loop (Lochhead et al. 2005; Soundararajan et al. 2013; Soppa and Becker 2015).

DYRK1A is highly expressed during embryonic development both in brain and outside the CNS in vertebrates (http://www.proteinatlas.org/ENSG00000157540-DYRK1A/tissue). In the adult, its expression is reduced and restricted to certain regions such as cortex, hippocampus, cerebellum, deep motor nuclei, hypothalamic nuclei and olfactory bulb (Marti et al. 2003), part of which pertain to the brain circuits involved in both homeostatic and hedonic control of food intake. Outside the brain, DYRK1A is

highly expressed in tissues such as the gallbladder, the salivary gland, the intestine and the thyroid gland (Figure 4).

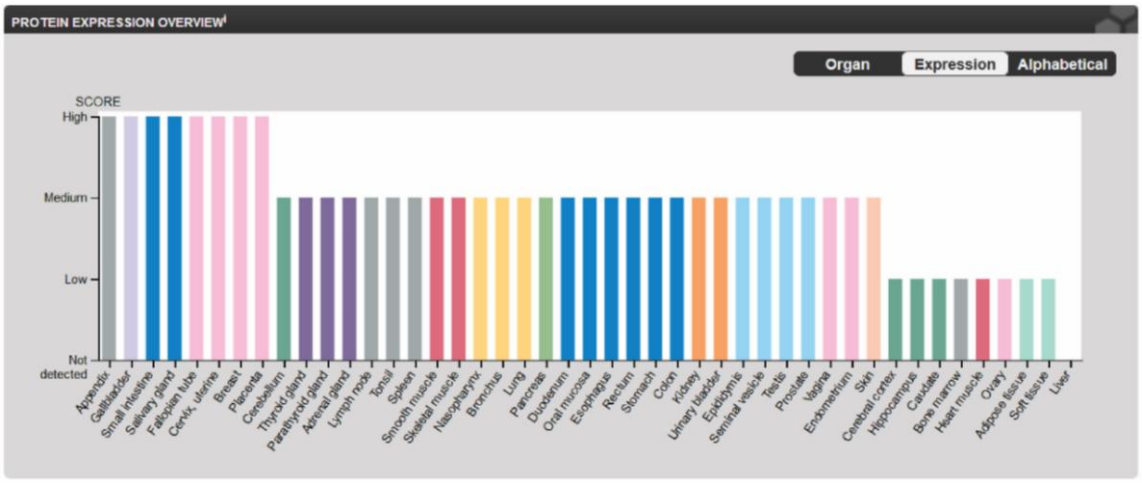


Figure 4. DYRK1A is expressed in different organs across the body. The figure, adapted from human protein atlas (http://www.proteinatlas.org) depicts the DYRK1A expression across the human body ranked from higher (right) to lower (left) protein levels.

Involvement of DYRK1A *in hedonic feeding control*

Feeding control requires optimal executive capabilities, such as behavioral inhibition and flexibility to avoid hedonic overeating. The involvement of DYRK1A in reward related mechanisms implicated in the control of food intake has not been addressed before, but several studies suggest a relationship between DYRK1A and dopamine circuits. First, *Dyrk1A* dosage is relevant for the developing ventral mesencephalon since DYRK1A regulates the activity of the caspase 9 and thus controls the apoptotic wave of dopaminergic neurons during development (Barallobre et al. 2014). Second, *Dyrk1A* is highly expressed in the striatum (Marti et al. 2003) that receives numerous dopamine projections and is implicated in motor control, but also in compulsive behavior (O'Connor and Kenny 2016). Some of the DS-related corticostriatal-dependent phenotypes of TgDyrk1A mice, such as hyperactive behavior, motor-coordination defects and sensorimotor gating could be reversed or improved upon striatal injection of an inhibitory RNA against *Dyrk1A* (AAVsh*Dyrk1A*) (Ortiz-Abalia et al. 2008). Interestingly, *Dyrk1A*$^{(+/-)}$ mice hypoactivity occurred along with decreased striatal dopamine levels, reduced number of dopaminergic neurons in the substantia nigra pars compacta, as well as altered behavioral responses to dopaminergic agents (Martinez de Lagrán et al. 2007). Whilst these data indicate that DYRK1A

is essential for a proper function of dopaminergic neurons in nigrostriatal circuit (Martinez de Lagrán et al. 2007), the role of DYRK1A in mesolimbic circuits remains unknown.

On the other hand, DYRK1A is also strongly expressed in prefrontal cortex (Marti et al. 2003) where its correct levels of expression are necessary for several cortical dependent functions. *Dyrk1A* overexpression in mice leads to defective cortical pyramidal cell morphology (Martinez de Lagrán et al. 2012), synaptic plasticity deficits and altered excitation/inhibition balance in vitro (Thomazeau et al. 2014; Souchet et al. 2014). Recently, its relevance at the local cortical network dynamics has been demonstrated in TgDyrk1A and Ts65Dn mice (Ruiz-Megías et al. 2016). These mice have decreased prefrontal cortex excitability with decreased firing rate and gamma frequency power along with reduced vesicular GABA transporter (VGAT) punctae on parvalbumin-expressing interneurons that suggest impaired modulation of inhibition (Ruiz-Megías et al. 2016). Along with these network alterations, TgDyrk1A mice show reduced cognitive flexibility (Altafaj et al. 2001) which could also be involved in the persistence of inadequate inhibition of feeding choices. Interestingly, some of those abnormalities are rescued using a DYRK1A kinase inhibitor (epigallocatechin-3-gallate) (De la Torre et al. 2014; Thomazeau et al. 2014).

Involvement of DYRK1A *in energy homeostasis*

Regarding the homeostatic brain circuits, *Dyrk1A* is expressed in hypothalamic nuclei controlling energy balance (Spiegelman and Flier 2001). Recent data showed that in the hypothalamus, DYRK1A activates a positive feedback loop where Sir2-dFOXO induces NPY gene expression through the PKA-CREB up-stream pathway (Hong et al. 2012). This might be relevant for feeding behavior, since NPY, as explained above, acts as an orexigenic signal promoting food intake. Accordingly, *Dyrk1A* transgenic mice exhibited decreased FOXO acetylation and increased NPY expression in the hypothalamus, as well as increased food intake compared to wild-type mice (Hong et al. 2012). Since *Dyrk1A* is overexpressed in DS, one of the explanations of the propensity for excessive food intake could result from the reinforced DYRK1A-dependent positive NPY feedback mediated by the kinase leading to an orexogenic phenotype.

Dyrk1A is also expressed in peripheral organs such as pancreas and adipose tissue, but the existing data suggest that it might not be involved in the metabolic phenotypes

associated to obesity in DS humans. In the adipose tissue *Dyrk1A* expression is not as relevant as in other organs (Figure 4), but some evidence suggests its role in adiposity through glycogen synthase kinase 3β (GSK3β) phosphorylation. GSK3β overexpression in mice induces adiposity and obesity (Pearce et al. 2004) and DYRK1A specifically inhibits GSK3β activity through phosphorylation of Thr (P)(356) (Song et al. 2015). Accordingly, mice overexpressing *Dyrk1A* show reduced fat mass along with increased Thr(P)(356)-GSK3β in the white adipose tissue and downregulation of adipogenic proteins, including peroxisome proliferator-activated receptor γ as compared to wild-type mice (Song et al. 2015). Taking together, these results may suggest an obesity-resistant phenotype in conditions of DYRK1A overexpression.

Dyrk1A is highly expressed in pancreas where it induces the expression and nuclear accumulation of p27Kip1 (Rachdi et al. 2014a), which is a negative regulator of proliferation via the inhibition of cyclin-CDK activity (Lloyd et al. 1999). This could explain the increased β-cell mass and improved glucose homeostasis observed in mouse overexpressing *Dyrk1A* (Rachdi et al. 2014b), and that *Dyrk1A*-haploinsufficient mice show the opposite phenotype: they have severe glucose intolerance, reduced β-cell mass and decreased β-cell proliferation (Rachdi et al. 2014a). Surprisingly, recent *in vitro* studies in both mice and human cells have reported that the pharmacological inhibition of DYRK1A induces β-cell proliferation in vitro, likely by causing NFAT activation in beta cells along with other DYRK1A-independent effects, for example harmine activation of Myc signaling (Wang et al. 2015a; Wang et al. 2015b; Belgardt and Lammert 2016). This led to the proposal that targeted modulation of the DYRK1A activity in pancreas could be a successful treatment for diabetic patients (Belgardt and Lammert 2016). In fact, this *in vitro* result would be in line with the finding that overexpression of human DYRK1A suppresses cell proliferation in multiple cell lines.

Recent data have revealed the role of DYRK1A in thyroid development. *Dyrk1A* overexpression in mice is associated to the up-regulation of important transcription factors for early thyroid morphogenesis (Nkx2–1, Foxe1, and Pax8). As a consequence, young adult transgenic *Dyrk1A* mice show significant heavier, but dysgenetic thyroid glands, with large disorganized regions, consist of small follicles lacking colloid and are therefore non-T4-producing. Moreover, they are less functional since they have lower thyroxine (T4) hormone excretion than wild-type mice and increased level of plasma

Thyroid-Stimulating Hormone (TSH), although this did not reach statistical significance (Kariyawasam et al. 2015).

1.6 Using mouse models to study feeding behavior abnormalities in Down syndrome

In this Thesis, we were interested in analyzing the contribution of behavioral factors in DS obesity risk, and their possible mechanisms. To do so, we used a trisomic mouse model, the Ts(17^{16})65Dn mice (abbreviated Ts65Dn mice), one of the most commonly used mouse model of DS. The model carries a small chromosome derived primarily from mouse chromosome 16, causing dosage imbalance for approximately half of human chromosome 21 orthologues (Reeves et al. 1995). However, it also bears in triplicate a region that is not present in DS. Some interesting new models are being generated with better construct validity than the Ts65Dn mice (Birling et al. 2017), but in the context of the Thesis we had not access to those.

A recent study has shown that Ts65Dn mice are hyperglycemic (Peiris et al. 2016) a phenotype that has been attributed the overexpression of the *RCAN1* that is involved in the insulin secretion pathway and mitochondrial function (Peiris et al. 2016). Although other mechanisms controlling both homeostasis and no-homeostatic feeding have not been addressed before in this mouse model, behavioral and neurochemical alterations such as impulsivity, perseveration (Escorihuela et al. 1998; Driscoll et al. 2004; Heller et al. 2014) and cognitive inflexibility (Coussons-Read and Crnic 1996; Faizi et al. 2011) may contribute to obesity development and overeating (Meule et al. 2014; Blanco-Gomez et al. 2015; Mallorqui-Bague et al. 2016).

In this Thesis, we have also used a transgenic mouse model of *Dyrk1A* overexpression to narrowing-down the contribution of the kinase to overeating in DS. Several transgenic mice are available for scientific purposes (Altafaj et al. 2001; Yabut et al. 2010; Guedj et al. 2012; Song et al. 2015). We chose mBACTgDyrk1A mice (Guedj et al. 2012) because this model uses the endogenous promoter of the gene and recapitulates several DS phenotypes at the behavioral and molecular level (Figure 5). The gene construct contains the *Dyrk1A* gene with their endogenous regulatory sequences that allow the interaction of the transcription factors and thus, mimics the best the endogenous *Dyrk1A* expression (Guedj et al. 2012). Also, several metabolic

contributors to obesity phenotypes have been already described (Rachdi et al. 2014b) so we can here concentrate in the behavioral dimension of feeding behavior.

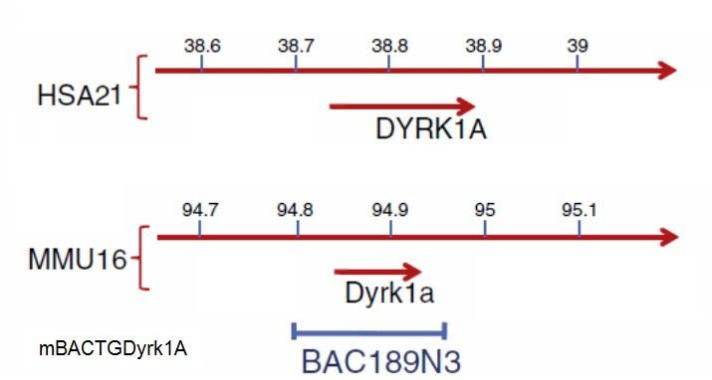


Figure 5. Schematic representation of the Murine BAC model of *Dyrk1A* overexpression. Comparison of the map and clone size of mBACTgDyrk1A transgenic mice with the coordinates on HSA21 and MMU16 (adapted from Guedj et al. 2012).

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Individuals with Down syndrome (DS; trisomy HSA21), a neurodevelopmental disorder leading to intellectual disability, are more likely to be obese. This higher obesity risk has been mostly related to lifestyle with deficient exercising and endocrine disturbances, such as leptin resistance or thyroid gland dysfunction. However, differences in feeding practices may also contribute to obesity, since there is an association between the body mass index and overeating and DS individuals consume more energy-dense foods, like snacks and sweets beverages than the general population. The working hypothesis of my work has been that, beyond the proposed homeostatic and lifestyle alterations leading to increased obesity risk, feeding behavior disturbances in DS might account for overeating contributing to obesity and could depend on reward sensitivity alterations.

Among the hundreds of triplicated genes, the dual-specificity tyrosine-(Y)-phosphorylated regulated kinase 1A (DYRK1A) is relevant for appetitive behavior regulation, insulin sensitivity and for the proper function of nigrostriatal dopaminergic neurons. Our secondary hypothesis was that *Dyrk1A* is a main player in this phenotype, and thus its overexpression could recapitulate some of the feeding behaviors associated to obesity in DS.

2.2 Objectives

The main objective of this work is to study the contribution of behavioral abnormalities to DS obesity risk using the Ts65Dn mice, a validated trisomic mouse model. To understand the underlying mechanisms we also studied possible metabolic and neurochemical players and pancreatic function in this model. Finally, we dissected the impact of in vivo *Dyrk1A* overexpression in DS-related obesity phenotypes using a mouse model overexpressing *Dyrk1A* in an otherwise disomic context.

My specific objectives have been:

1. To characterize the feeding behavior phenotypes associated to the trisomy in Ts65Dn mice.
2. To study the behavioral adaptation of Ts65Dn mice to energy-dense foods and the appearance of compulsive overeating.

3. To determine the changes in key neurochemical players with a role in non-homeostatic control of feeding, such as the monoaminergic systems in Ts65Dn mice.
4. To determine the changes in key metabolic players with a role in homeostatic control of feeding such as leptin and ghrelin.
5. To understand pancreatic function and peripheral control of glucose homeostasis in Ts65Dn mice.
6. To elucidate the impact of DYRK1A on feeding phenotypes using a mouse model overexpressing *Dyrk1A* (mBACTgDyrk1A).

MATERIAL AND METHODS

3. MATERIAL AND METHODS

The main objective in this Thesis has been to investigate the contribution of abnormal feeding behaviors, such as compulsive overeating of energy-dense foods, in obesity development in Down syndrome (DS). To this aim we have used a well-validated mouse model of DS bearing a partial trisomy of chromosome 16, the Ts65Dn. We first characterized feeding behavior and obesity-related phenotypes in non-obesogenic conditions. Then, we induced obesity and compulsive overeating using a protocol previously validated in our group (Espinosa-Carrasco al. submitted) based on long-term free access to energy-dense diets. Mice exposed to those conditions were sacrificed for the neurochemical and histological analysis. The second objective of this Thesis was to study the implication of the tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A), an interesting candidate for DS obesity. We analyzed feeding behaviors in transgenic mice (mBACTgDyrk1A) only overexpressing *Dyrk1A* to determine if we could recapitulate trisomic obesity-related phenotypes. We used the same experimental design used for studies in Ts65Dn mice.

3.1 Animals

In this Thesis, we used two mouse models: a partial trisomic mouse model of DS (Ts65Dn) to define obesity-related DS phenotypes and a transgenic mouse model (mBACTgDyrk1A) overexpressing only *Dyrk1A* to confirm the implication of this DS candidate gene on the trisomic obesity phenotypes.

Ts(17^{16})65Dn (Ts65Dn) and wild-type (WT) littermates were obtained crossing B6EiC3Sn a/A-Ts (17^{16})65Dn (Ts65Dn) females to B6C3F1/J males purchased from The Jackson Laboratory (refs. 001924 and 001875, the Jackson Laboratory Bar Harbor, Bar Harbor, ME, USA). In B6EiC3Sn a/A-Ts(17^{16})65Dn (Ts65Dn) female the chromosomal rearrangement was generated by radiation that induced a spontaneous and unplanned reciprocal translocation of the telomere proximal region of *Mmu16* to the centromere and pericentromeric region of *Mmu17* (Davisson et al. 1993).

mBACTgDyrk1A mice were obtained crossings transgenic males overexpressing *Dyrk1A* (mBACTgDyrk1A) with C57BL6/SJL females. mBACTgDyrk1A (Tg189N3) mice were generated in the laboratory of Jean-Maurice Delabar (University Paris

Diderot, Sorbonne Paris Cité) as previously described (Guedj et al. 2012). Briefly, a bacterial artificial chromosome 189N3 (BAC-189N3) strain carrying the entire murine *Dyrk1A* gene with endogenous promoter and regulatory regions was introduced by electroporation in the HM-1 embryonic stem (ES). ES clone was selected for overexpression of *Dyrk1A* close to 1.5 times, and injected into blastocysts to generate the *Dyrk1A* overexpressing mouse. Of note, the WT controls of Ts65Dn and mBACTgDyrk1A are of different genetic backgrounds, and thus we specifically compared the wild-type mice of the two strains to control for strain-dependent effects.

Genotyping

Genotyping was performed by amplifying genomic DNA obtained from the tail. For Ts65Dn model, quantitative polymerase chain reaction (qPCR) was done following (Liu et al. 2003). The overexpression of *Dyrk1A* in mBACTgDyrk1A mice was confirmed as previously described (Guedj et al. 2012).

Housing and husbandry

The mouse colonies of Ts65Dn and mBACTgDyrk1A mice were bred in the Animal Facility of the Barcelona Biomedical Research Park (PRBB, Barcelona, Spain). Mice were housed individually in conventional (325 weight × 145 depth × 132 height mm Plexiglas cages; Figure 6A), in controlled laboratory conditions, with temperature maintained at 22 ºC ± 1ºC and humidity at 55 ± 10% on a 12 hours light/dark cycle (lights off 20:00 hours). Prior to the study all mice were fed *ad libitum* on a standard rodent chow (SC, see composition below) with free access to clean drinking water.

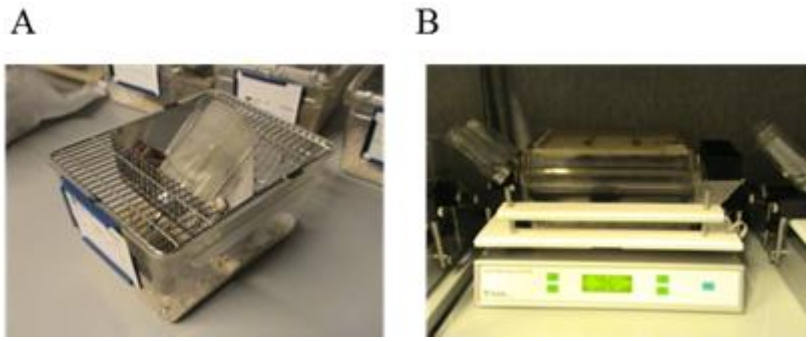


Figure 6. Representative images for housing cages used during the study. (A) Standard individualized cage for mouse housing and (B) a PheCOMP cage (Panlab-Harvard Instruments, Spain), the equipment used for automatically record the food/water intake and activity of individualized mice.

The characterization of the time-course and dynamics of obesity-related behavioral changes required isolation. For this reason, we selected male mice, since females show greater responses to stress than males (Senst et al. 2016) that may alter metabolism and lead to body weight loss being a potential confounder. Also, since some reports suggest that Ts65Dn mice have delayed growth (Davisson and Costa 1999), experiments were conducted in 5 months-old male mice once body weight had stabilized. Thus, we also used 5 months-old male mBACTgDyrk1A mice allowing comparison between the two models. Experiments and analysis were performed blind to genotype and experimental condition.

Ethical statement

All experimental procedures were approved by the local ethical committee (Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB; procedure number MDS-12-1464P3), and met the guidelines of the local (law 32/2007) and European regulations (EU directive n° 86/609, EU decree 2001-486) and the Standards for Use of Laboratory Animals n° A5388-01 (NIH). CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14). Methods and results have been written according to the ARRIVE Guidelines (Kilkenny 2010).

3.2 Feeding behavior analysis

We characterized behavioral, neurochemical and metabolic phenotypes in Ts65Dn mice to define DS-obesity phenotypes and unravel possible mechanisms. Two different models of diet induced obesity were used based in free access to standard chow (SC) and (i) a chocolate-mixture (CM) diet or (ii) or high-fat (HF) diet.

We then investigated if the single overexpression of *Dyrk1A* could be sufficient to replicate the feeding behavior phenotypes detected in trisomic mice. For both mouse models, the experiments were performed in at least 4 batches of animals to get the sufficient number of mice and of technical replicates, and control for possible litter and mothering effects. The sample size of the groups was calculated based on similar experiments previously performed in our laboratory.

For the study of feeding behavior and the influence of energy-dense diets, Ts65Dn mice were placed in the experimental cages (PheCOMP, Panlab-Harvard Instruments,

Barcelona, Spain) by randomization using Excel (RAND() function) and were assigned to each experimental group balanced by body weight. PheCOMP cages (Panlab-Harvard Instruments, Barcelona, Spain) allowed the precise detection of food and water intake.

We used a total of 19 wild-type (WT) and 16 Ts65Dn (TS) mice distributed in three groups: mice receiving only standard chow (SC, n = 8 per genotype), mice having free choice between standard chow (SC) and a chocolate-mixture (CM) diet, (SC+CM, n = 10 per genotype) or mice having free choice between standard chow (SC) and high-fat (HF) diet, (SC+HF, n = 9 per genotype). However, along the experiment some of them registered "abnormal signals" (e.g. some cages registered continuous intake signals or showed values that were higher than the expected ones for a mouse's consumption). For this technical reason, some mice were excluded of the meal pattern analysis and behavioral tests. The final number of mice analyzed was: SC group: WT n = 8, TS n = 7; SC + CM group: WT n = 9, TS n = 10; SC + HF group WT n = 8, TS n = 7.

For the characterization of the role of overexpression of *Dyrk1A* on obesity development and compulsive eating due to the mBACTgDyrk1A (TG) we isolated the mice in their home cages and performed the stand-alone tests of compulsivity 8 weeks after the continuous exposure to energy-dense food. TG mice and their WT littermates were also distributed in 3 experimental groups: SC (n = 8 per genotype), free choice SC+CM (n = 8 per genotype) or free choice SC+HF (n = 8 per genotype).

In a separate group, we also characterized the meal pattern using PheCOMP cages, in n = 4 WT, n = 5 TG mice. Mice were given only SC for 2 weeks and then were switched to a free choice SC+CM diet. For this experiment, due to a breeding problem in our colony, the sample size of SC+CM group is small and we could not include a SC+HF group. However, we decided to include the results in the Thesis, since, although preliminary they shed light to *Dyrk1A*-dependent phenotypes.

Body weight and feeding behavior analysis

Short-term laboratory-based measures of eating in weight stable obese individuals may not be functionally relevant to understand long-term changes in body weight gain. To solve this, here we used longitudinal meal pattern analysis for the study of obesity development. Body weight was registered twice weekly for whole experimental period, always at the same time of the day. Body weight gain was determined by subtracting the

body weight on the 1st day of CM or HF exposure from the body weight on subsequent days.

Apparatus

For meal pattern studies, we used PheCOMP high-resolution monitoring system (Panlab-Harvard Instruments, Spain). The equipment contains experimental cages (Allentown Caging Equipment, ACE, 197 weight × 306 depth × 212 height mm, see Bura et al. 2010) provided with a grid floor and a filter top. The platform supporting each cage contains 2 infrared frames (16 × 16 beams, 16 mm spaced) allowing simultaneously recordings of horizontal activity and rearing. The system is equipped with 2 water and 2 food dispensers that continuously register food consumption with 20 mg precision by means of weight transducers mounted into the platform supporting each cage. The quantity of water drunk from a nipple, and any possible dripping, are also accounted using the same system of transducers with 20µl precision. The modular structure allows multiple dispenser combinations made them suitable for our free-choice experiments. In our experiments of free choice, one dispenser always contained standard chow (SC) and the other a chocolate-mixture (CM) or a high-fat (HF) diet. The side was counterbalanced between cages and genotypes. Infrared beams allowed recording of locomotor activity and rearing. Animals were recorded uninterruptedly during periods of 3 or 4 days. Between these periods the system was paused during approximately one hour for refilling the feeders and the cleaning of the cages.

The continuous signals from each weight transducer and the infrared frame are amplified, digitized and sent to the computer software for data acquisition and storage. Along the acquisition process, meal pattern analysis is performed using the COMPULSE software (Panlab-Harvard Instruments, Spain). Meal pattern analysis included recording duration and recurrence intervals of eating or drinking acts, to calculate circadian and ultradian periodicities, and event-related frequency distributions. Feeding/drinking acts were considered as a meal or a drinking bout when separated by more than 120 seconds from the next feeding/drinking act. Hence, feeding/drinking acts were merged into single food/water bouts when the intermeal interval was shorter than 120 seconds (see Heyne et al. 2009).

The feeding parameters computed by the COMPULSE software and their biological significance are listed in Table 1.

Table 1. Parameters defining meal pattern and its behavioral significance

Parameters of feeding behavior	Significance of the behavior
Meal duration (min)	Shorter duration of meals is related to the repetitive access to these source. "Snacking" is defined by shorter and recurrent meals
Number of meals	A high number of meals could indicate a high preference for a particular food
Eating Rate (mg/sec)	A high eating rate means the food is rapidly consumed. Could be used as an indicator of the desire of feeding a particular food
Intermeal interval (min)	The smaller the time between consecutive meals the higher the frequency of refeefingm indicating altered satiety mechanisms
Satiety ratio (min/KJ)	In homeostatic conditions, the satiety signals modulate the end of a meal when the energy demand is covered. However, the increased motivation of consuming preferred foods (such as highly palatable or energy-dense foods), fail this regulation. This can be reflected in shorter inter-meal intervals or bigger meal size reducing satiety ratio in both cases
Energy intake (grams * KJ)	When energy demand is covered, a sustained increased energy intake (overeating) is an indicator of compulsive feeding

Besides we also calculated the preference (P) for energy-dense foods against standard chow is a measure of the rewarding and reinforcing effect of the energy-dense diets, and was calculated as:

(i)

$$P = \frac{(CM\ or\ HF)\ intake\ (g) - (CM\ or\ HF) estimated\ intake\ (g)}{Total\ (SC + CM) or\ (SC + HF) intake\ (g)}$$

(ii)

$$P = \frac{(CM\ or\ HF)\ intake\ (KJ/BW) - (CM\ or\ HF) estimated\ intake\ (KJ/BW)}{Total\ (SC + CM) or\ (SC + HF) intake\ (KJ/BW)}$$

Where estimated CM/HF is calculated as:

$$Estimated\ CM/HF = \frac{Total\ (SC + CM) or\ (SC + HF) intake}{2}$$

In (i) in grams and in (ii) in KJ corrected by the body weight (BW) of the animal in grams.

Therefore $P > 0$ means preference for CM/HF whereas $P \leq 0$ means no preference or preference for SC.

For the visualization of the longitudinal data, and specifically of food consumption and temporal distribution of feeding events of each mouse, we used a customized open-

source software developed in our laboratory (Pergola; http://cbcrg.github.io/pergola) designed for the manipulation, modeling, visualization and integration of (high-throughput) longitudinal behavioral data. For quantification of locomotor activity, we used the ActiTrack software (Panlab-Harvard Instruments, Spain).

Diets

The diets used in this Thesis (Figure 7) were:

(i) Standard chow (SC). This group had access only to standard chow. Standard chow (SDS, UK) contained 10.76 MJ kg^{-1} digestible energy (17.5 % coming from protein, 75 % from carbohydrate and 7.4% from fat; Table 2). Typically, rodent chow carbohydrate is contributed to by 45% starch and approximately 4% simple sugars (monosaccharides plus disaccharides) as a proportion of total carbohydrates by weight.

(ii) Pellets of a mixture of commercial chocolates (CM) made from equal amounts of Bounty®, Snickers®, Mars® and Milka ® chocolates. CM pellets contained 20.6 MJ kg^{-1} digestible energy (17 % coming from protein, 52 % from carbohydrate and 24% from fat; carbohydrate is contributed to by 8% starch and approximately 44% simple sugars (monosaccharides plus disaccharides, Table 2).

(iii) High-Fat diet (HF) consisted of commercial pellets of purified diet w/60% energy from fat. HF pellets (58G9; Test Diet ®, USA) contained 22 MJ kg^{-1} digestible energy (24 % coming from protein, 30 % from carbohydrate and 35 % from fat; carbohydrate is contributed to by 5.4% starch and approximately 6.4 % simple sugars (monosaccharides plus disaccharides, Table 2).

Food was renewed each of 3 - 4 days to ensure the maintenance of the organoleptic properties.

Figure 7. Standard chow and energy-dense diets. Representative photographs showing the different diets used during this Thesis. (A) Control mice are fed with standard chow (SC). Free choice mice have always SC and (B) chocolate-mixture (CM), or (C) high-fat (HF) diet pellets.

Table 2. Nutritional composition and energy content of the experimental diets. The composition of the SC and HF diets was provided by the manufacturers. CM diet that was prepared in the lab and its composition was analyzed by Alquimisa laboratories S.L (Salamanca, Spain).

Nutrients	Standard chow (SC)	Chocolate-mixture (CM)*	High-fat diet (HF)
Protein (%)	17.49	17	23.7
Fat (%)	7.42	24	34.7
Carbohydrates (%)	75.09	52	29.6
Sugar (%)	4.05	44.4	6.36
Starch – fiber (%)	44.97	7.6	5.4
Energy (Kcal/g)	2.85	4.92	5.25
Energy (KJ/g)	10.76	20.6	22

Experimental design

For the analysis of feeding behavior, at 5 months of age, animals were individually housed during 15 weeks (2 weeks for habituation, 8 weeks for obesity development and 5 weeks of behavioral testing phase, Figure 8) in multi-take metabolism and activity cages (PheCOMP, Panlab-Harvard Instruments, Barcelona, Spain, Figure 6B).

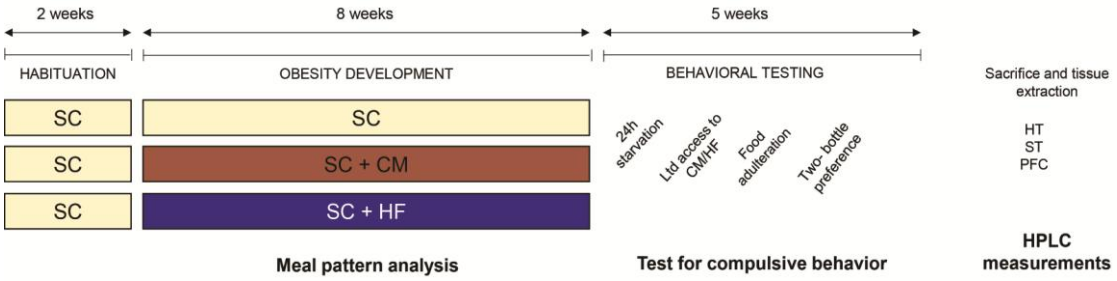


Figure 8. Experimental design. Mice were individually housed in PheCOMP cages to register longitudinal activity and meal pattern. During the habituation period, all mice received standard chow (SC). Then they were allocated to receive free access of chocolate-mixture (CM) or high-fat diet (HF) for the study of the obesity development. After animals gained stable overweight (8 weeks after introduction of energy-dense diets), a battery of behavioral tests was used to characterize the appearance of compulsive and inflexible behavior during 5 weeks. At the end of this experimental phase animals were sacrificed, and the prefrontal cortex (PFC), hypothalamus (HT) and striatum (ST) were dissected and collected for HPLC neurotransmitter measurements (Neurochemical studies).

Habituation phase. All mice were individually housed in PheCOMP cages and provided with standard chow (SC) and water *ad libitum* during two weeks to allow habituation to the housing conditions and to define the baseline meal pattern.

Obesity development phase. After habituation, mice were assigned to each diet-condition, i.e. free access to either chocolate-mixture (CM) or high-fat chow (HF) and

standard chow (SC). Control group continued receiving sole access to standard chow. The development of overweight in the experimental groups was followed during 8 weeks.

Testing phase. At the end of the obesity development phase, we evaluated the appearance of diet-induced non-adaptive behaviors, such as compulsive behavior or behavioral inflexibility. Several behavioral tests were performed, usually one test per week to give the animals enough time to re-establish their regular food intake during the days between tests: starvation, limited access to energy-dense food, food adulteration and preference for sweet solutions.

Starvation

Mice of all groups (SC, SC+CM and SC+HF) were food deprived during 24 hours (water still provided *ad libitum*) and we calculated the increase in energy intake (KJ) after refeeding compared to their behavior in free feeding conditions. Energy intake after starvation provides information on the influence of hunger on food seeking and food taking.

Limited access to energy-dense food

Access to chocolate-mixture or high-fat diet was restricted to 1 hour during the light (resting) phase of the light-dark cycle (from 14:00 to 15:00h) for 3 consecutive days in overweight mice (SC+CM and SC+HF). Standard chow and water were provided unrestrictedly *ad libitum*. The test evaluates (i) inflexible behavior and (ii) compulsive intake binge-like eating episodes characterized by rapid consumption of high quantities of CM or HF during refeeding (Espinosa-Carrasco et al. submitted).

(i) Behavioral flexibility is studied by measuring if the animals compensate for the restriction of access to the high caloric food by increasing the intake of standard chow. An inflexible response would be revealed by the neglect of standard chow. To this aim we compared the standard chow (SC) energy intake (KJ/grams of body weight) during periods where the CM/HF was not available with the SC energy intake in free feeding conditions.

(ii) Binge-like eating episodes upon limited access are revealed by measuring the energy intake (KJ/grams of body weight) and the eating rate (mg/s) of CM and HF consumed

during the periods of CM/HF access (1h for 3 consecutive days). We also analyzed the evolution of binges, to analyze the effect of repetitive restriction to the preferred food. The measurements of each day of limited access where compared to the feeding behaviors at the same hour of the day (14:00 to 15:00h) in non-restricted conditions. The CM/HF energy increase between the *ad libitum* conditions and during the third day of the limited access was also calculated.

Food adulteration with quinine hydrochloride

Compulsive and inflexible components are also reflected by maintenance of consumption despite an adverse bitter taste. The protocol was adapted from Heyne and collaborators (Heyne et al. 2009). Briefly, for inducing bitter taste we used quinine hydrochloride (Sigma-Aldrich, UK). To adulterate the chocolate-mixture (CM), we added 3g of quinine hydrochloride per Kg of CM during the mash preparation. The high-fat (HF) pellets were triturated, we added 3g of quinine hydrochloride per Kg of HF to the mash, and then the pellets were reconstituted. To adulterate the standard chow, the quinine hydrochloride was diluted in water and then pellets were impregnated with the quinine solution. For two consecutive days, SC+CM mice received unpleasantly bitter-tasting CM and SC+HF mice received bitter-tasting HF. SC mice were given bitter-tasting SC as a control of taste detection. For all conditions, unadulterated SC and water were always available. We compared the average food and energy intake of bitter SC/CM/HF of the two days to the daily SC/CM/HF energy intake in non-adulterated conditions (in SC mice, SC+CM mice and SC+HF mice, respectively).

Preference for sweets solutions

The two-bottle test was used for assessing reward and taste perception. For 2 consecutive days, all mice had the choice between water and a sweet solution: either 0.05 % saccharin or 2 % sucrose solution (each sweet solution spacing of one week). Preference scores were calculated as the following ratio:

$$P = \frac{sweet\ solution\ intake\ (ml/BW)}{Total\ (sweet\ solution + water)\ intake\ (ml/BW)}\ x\ 100$$

To prevent possible bias of side preference in drinking behavior, the position of the bottles was counterbalanced. No food or water deprivation was applied before the test.

3.3 Neurochemical analysis in Ts65Dn mice

At the end of the behavioral testing phase of the study, the monoamine content (NA (noradrenaline), AD (adrenaline), DA (dopamine), DOPAC (3-4dihydroxyphenylacetic acid), 5-HIAA (5-hydroxy-3-indolacetic acid), 5-HT (serotonin) and HVA (homovanillic acid) was measured in hypothalamus, prefrontal cortex and striatum of SC mice (WT n = 6, TS n = 6) to define genotype-dependent differences. We then measured changes induced by long-term free access to energy-dense food in SC+CM mice (WT n = 6, TS n = 6), and SC+HF mice (WT n = 5, TS n = 5).

Tissue dissection and preparation

Mice were sacrificed using carbon dioxide. The hypothalamus, prefrontal cortex and striatum were removed and stored at -80ºC until analysis. For monoamines measurements, the frozen tissues were weighed and suspended in 400 µl of ice-cold 0.1M perchloric acid solution with 0.4% EDTA. Tissues were homogenized with 1.5 mm diameter beads during 2 min using a tissue lyser II (Qiagen). The homogenates were centrifuged at 3000 rpm during 30 min at 4°C and the supernatants were collected and centrifuged at 16400 rpm during 2 min.

High-performance Liquid Chromatography with Electrochemical Detection (HPLC-ED)

The HPLC–ED system was composed of Shimadzu apparatus equipped with a LC20AD pump, a SiL20AC auto-sampler coupled with an electrochemical detector (Waters 2465). Separation was obtained using a 150 x 4.6 mm C18 5µM Beckman Ultrasphere column equipped with two Phenomenex C18 filters in a security guard system. The mobile phase which contains 150 mM ammonium acetate, 8.2 mM octane-sulfonic-acid, 15% methanol (v/v) adjusted to a pH of 3.8 with glacial acetic acid, was filtered through a 0.2µM membrane filter, degassed before use and pumped at a flow rate of 0.8ml/min. Eluates were detected at an oxidation potential of 700 mV versus a reference electrode. The column and the detection cell were housed within the Faraday cage of the

electrochemical detector that was set at 25.5°C. Samples with an injection volume of 40 µl were placed in the auto-sampler and kept at 4°C.

The monoamines NA (noradrenaline), AD (adrenaline), DA (dopamine), DOPAC (3-4dihydroxyphenylacetic acid), 5-HIAA (5-hydroxy-3-indolacetic acid), 5-HT (serotonin) and HVA (homovanillic acid) were purchased from (Sigma-Aldrich, UK). Ultrapure water was obtained with a Milli-Q system (Millipore, USA). Standards solutions of each monoamine or metabolite were prepared at a concentration of 50ng/ml in ultrapure water for indoleamines and in 0.1 N HCl solution for catecholamines and were stored at -20°C. The retention time allowed by this system was 7 min, 10 min, 13.5min, 16.5 min, 21 min, 32 min and 42 min for NA, AD, DA, DOPAC, 5-HIAA, 5-HT and HVA respectively. The peak areas of the external standards allow the metabolites quantification in the tested samples. The content of each monoamine was expressed as ng per mg of wet weight. The outlier values defined as above/below the group mean ± 2 x standard deviation were excluded of the analysis, concretely two samples from prefrontal cortex of SC mice (1 WT and 1 TS) were discarded.

3.4 Metabolic studies in Ts65Dn mice

The metabolic analysis was carried out in three different batches of Ts65Dn mice. For this experiment, we characterized (i) body composition, (ii) glucose homeostasis and (iii) pancreatic phenotypes in non-obesogenic conditions (standard chow, SC): WT n = 10, TS n = 8.

Body composition analysis in Ts65Dn mice

Non-invasive analysis of body composition was estimated by nuclear magnetic resonance (NMR) technology via a scanner EchoMRI 900 (Echo Medical Systems, Houston, USA), that creates contrast between soft tissues taking advantage of the differences in relaxation times of the hydrogen spins and/or hydrogen density. Tissue contrast is high between fat, body free fluid, and muscle based on NMR signal amplitude and relaxation times, and can be further enhanced by application of certain radio frequency sequences (http://www.echomri.com/). Briefly, the mouse is placed in a specially sized, clear plastic holder without sedation or anesthesia. The holder is then inserted into a tubular space in the side of the EchoMRI™ system.

Lean mass is equivalent to muscle mass and organs. Body fat does not distinguish between different types of fat and fat depositions in every organ including the brain, muscles, other organs, bone marrow, and circulating blood lipids, and fatty acids. In our experiment, lean and fat body composition was directly obtained from EchoMRI™ measurements in grams. From this data, body composition was calculated for each mouse and corrected for body weight.

Glucose homeostasis and insulin levels

5-month old male mice fed with standard chow weeks were used for characterization of the glucose homeostasis using a glucose tolerance test.

First for the study of the basal glucose and insulin blood levels, mice were food deprived for 5 hours. Blood samples were collected from the tail vein and blood glucose levels were measured using the OneTouch Vita blood glucose meter (LifeScan). Plasma levels of insulin were determined by using Ultra Sensitive Mouse Insulin ELISA Kit (France).

Glucose tolerance test (GTT) was then performed to study the glycemic and insulinemic profiles after administration of a glucose load. Mice were food deprived for 5 hours prior to an oral administration of 2g/Kg of 30% glucose (Lavoisier, France). Blood was sampled from the tail vein at 0, 10, 30, 60, 90 and 120 min in order to assay glucose concentration using OneTouch Vita blood glucose meter (LifeScan). At 0, 15, 60 and 120 min blood was taken to measure plasma insulin concentration with Ultra Sensitive Mouse Insulin ELISA Kit (France).

Morphological and functional studies of islets

For immunohistochemistry, pancreases were immersed in 10% formalin and embedded in paraffin sections (4-µm-thick) as previously described (Rachdi et al. 2014a). Mouse anti-insulin (1:2000; Sigma) was used for quantification of insulin staining. We analyzed the pancreases of mice fed with standard chow (WT n = 4, TS n = 4). 5 sections for each pancreas were used for the analysis.

For in vitro insulin secretion study, islets from pancreases of WT and Ts65Dn 5-months-old mice were isolated from by collagenase digestion followed by purification through a Histopaque gradient (Sigma-Aldrich, France). Insulin secretion was assessed by static incubation of isolated islets in Millicell insert (Millipore). Following overnight

culture in RPMI containing 5.6 mmol/l glucose, islets were pre-incubated for 1 hour in Krebs-Ringer medium containing 2.8 mmol/l glucose. Groups of 50 islets in triplicate were then incubated in Krebs-Ringer medium containing 2.8 mmol/l glucose or 20 mmol/l glucose for 1 hour. Secreted insulin and insulin content were measured using an ultrasensitive mouse insulin ELISA (Mercodia AB, Sweden).

3.5 Statistical analysis

Different statistical methods were applied depending of the characteristics of each experiment. Mann-Whitney-U-test analysis was used for the comparison of feeding behavior and obesity phenotypes between genotypes (Ts65Dn/mBACTgDyrk1A vs. their wild-type littermates) in SC mice. Paired sample Mann-Whitney-U-test test was used to compare feeding behaviors between CM/HF vs. SC in free choice mice, since they are two measures for the same subject. The time course evolution of body weight was analyzed by a mixed analysis of variance (ANOVA) repeated measures.

For the analysis of meal pattern data, we used a linear mixed model in which we included the diet (SC from SC group, SC and CM separately from SC+CM group; SC and HF separately from SC+HF group) as a fixed effect. The variable 'Mouse ID' was included as a random effect since we disposed of repeated measures in the case of the free choice mice. Differences were considered significant at $P< 0.05$ upon subsequent Sidak post-hoc analyses. To quantify the circadian feeding rhythmicity loss, we used three-way repeated measures ANOVA (genotype, diet and phase). For analysis of energy compensation during the limited access to energy-dense food test and for the two-bottle tests with sweets solutions, we used two-way ANOVA with genotype (Ts65Dn/mBACTgDyrk1A vs. their wild-type littermates) and group (SC, SC+CM, SC+HF) as factors between subjects. For each test, significance was considered when $P< 0.05$. For ANOVA, subsequent Bonferroni post-hoc analyses were performed when required and differences were considered significant at $P< 0.05$. All results are expressed in mean ± SEM. The statistical analysis was performed using the Statistical Package for Social Science program SPSS® 12.0 (SPSS Inc., Chicago, USA).

For the principal component analysis (PCA) we used physical, behavioral, and neurochemical data. Physical data included body weight (BW, Kg; BWfinal, BWincrease, % of increase). Behavioral data included: locomotor activity during the light phase (beam cuts), locomotor activity during the dark phase (beam cuts); number

of meals (n_meals_total); food intake (Food_intake_(g)_total); energy intake (KJ/BW; Total_Intake_week8); meal duration (Meal_Duration_(min)_av); intermeal interval (Intermeal_Interval(min)_av); eating rate (Eating_Rate(mg/sec)_av); satiety (Satiety_(min/g)_av; increase of energy intake upon starvation (Energy after starvation_TOTAL); SC intake (KJ/BW) during the quinine hydrochloride adulteration test (Energy(SC)_adulteration_test); SC/CM/HF intake (KJ/BW) during the quinine hydrochloride adulteration test (Energy (bitter SC/CM/HF)_adulteration_test); preference for saccharine (Pref_saccharine); preference for sucrose (Pref_sucrose). As neurochemical variables, we include the HPLC measurements (ng/g) in hypothalamus (HT), striatum (ST) and prefrontal cortex (PFC) of: NA (noradrenaline), AD/DOPAC (adrenaline/ 3-4dihydroxyphenylacetic acid); DA (dopamine), 5-HIAA (5-hydroxy-3-indolacetic acid), 5-HT (serotonin) and HVA (homovanillic acid); 5-HIAA/5-HT levels.

Before performing the PCA, we apply a log-transformation in case the transformation was improving the "normality" of the distribution (using the Shapiro test for testing the 0 hypothesis of normality). PCA was performed using the R function prcomp, after shifting all variables to be zero centered and scaling them to have unit variance. We then looked at the correlation between our variables. Spearman Rho's were converted in P-values using the Fishers R-Z transformation (Myers and Sirois 2006).

RESULTS

4. RESULTS

4.1 Behavioral contributors to obesity in Ts65Dn mice

Obesity in people with DS has been traditionally attributed to endocrine issues such as leptin resistance and thyroid gland dysfunctions (Anwar et al. 1998; Bergholdt et al. 2006) or to deficient exercising (Heller et al. 2003). However, differences in feeding practices may also contribute to obesity, since there is an association between body mass index and overeating (Mallan et al. 2017) and DS individuals consume more energy-dense foods like snacks and sweets beverages than the general population (Grammatikopoulou et al. 2008; Nordstrøm et al. 2015). In the first part of this Thesis we characterized feeding behavior and obesity-related phenotypes in a well validated mouse model of DS bearing a partial trisomy of chromosome 16, the Ts65Dn (Davisson et al. 1993).

4.1.1 Somatometric characteristics and feeding behavior in Ts65Dn mice

Since feeding behavior had not been characterized in Ts65Dn mice, we first had to establish the face validity of this model. The body weight and mass index of 5 months-old Ts65Dn mice fed with standard chow was similar to WT littermates (Figure 9A and 9B, respectively). Ts65Dn mice presented a significant reduction of body length (Mann-Whitney-U-test, z = -3.12; P<0.01; Figure 9C).

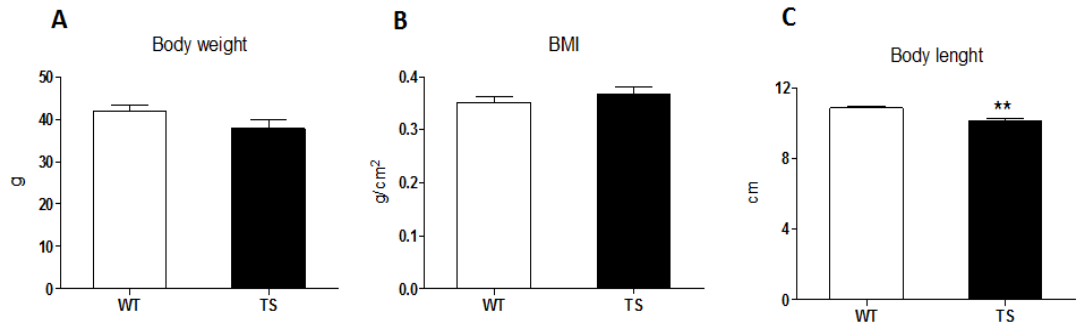


Figure 9. Somatometric characteristics of Ts65Dn mice. Bar blots represent (A) Body weight (grams), (B) body mass index (BMI; grams/cm^2) and (C) body length (cm) in mice fed with standard chow (SC) of Ts65Dn (TS, n = 11) and wild-type mice (WT, n = 13). Data expressed as mean ± SEM. Mann-Whitney-U-test for independent samples ** P<0.01.

Using Nuclear Magnetic Resonance (NMR) we detected reduced lean body mass (Mann-Whitney-U-test, z = - 2.58; P<0.05; Figure 10A) and a higher percentage of

body fat per body weight in Ts65Dn as compared to their WT controls (Mann-Whitney-U-test, z = -2.40; P<0.01; Figure 10B) suggesting increased risk for fat accumulation.

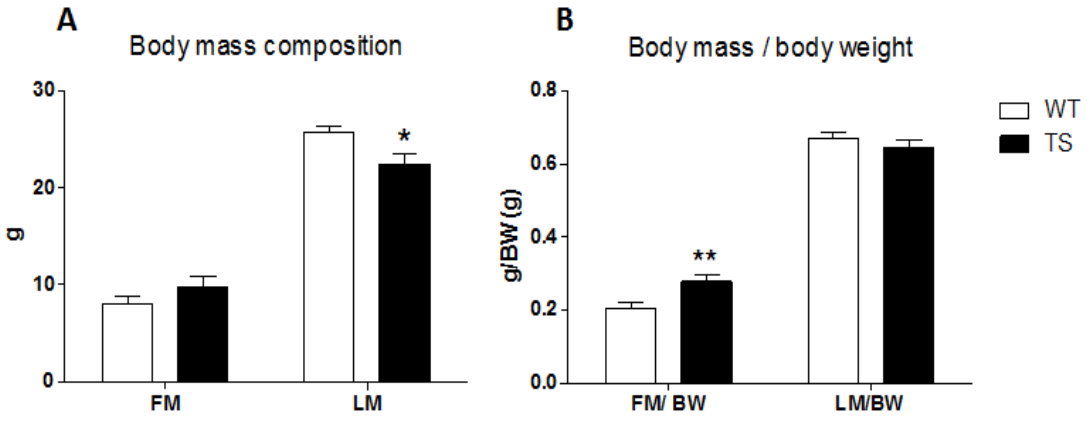


Figure 10. NMR non-invasive analysis of body mass composition in wild-type (WT) and Ts65Dn (TS) mice. Bar blots show (A) body composition in lean mass (LM) and fat mass (FM) represented in grams, and (B) body composition corrected for body weight (BW in TS (n = 8) and WT (n = 10) mice. Data expressed as mean ± SEM. Mann-Whitney-U-test for independent samples * P< 0.05, ** P<0.01.

Meal pattern analysis

Meal pattern analysis revealed that Ts65Dn mice fed with SC have longer meals (Mann-Whitney-U-test, z = P<0.01; Figure 11A) and reduced eating rate (milligrams of food eaten per second) compared to WT (Mann-Whitney-U-test, z = P<0.001; Figure 11B). Ts65Dn also showed reduced intermeal intervals (Mann-Whitney-U-test, z = - 2.47; P<0.05, Figure 11C) indicating higher frequency of refeeding. However, we detected only slight, non-significant differences in the total number of meals (Figure 11D).

We also characterized drinking behavior. The total amount of fluid consumed was similar in both genotypes (Figure 11E), but Ts65Dn mice showed significantly longer (Mann-Whitney-U-test, z = -4.33; P<0.01, Figure 11F) and slowlier drinking events (drinking rate, Mann-Whitney-U-test, z = -5.72; P<0.001, Figure 11G) than WT. These results could reflect oral-motor dysfunction in trisomic mice, similar to what has been described in humans that may lead to problems initiating and maintaining a smooth sequence of feeding actions (Hennequin et al. 2005).

Finally, continuous recording of food intake and locomotor activity tracked by the actimeter in the PheCOMP cages showed similar circadian patterns in both genotypes (Figure 11H). Ts65Dn and WT littermate controls, showed normal photoentrainment

under a 12:12 light-dark cycle, with feeding events concentrated during the active period (the nocturnal phase, Figure 11H).

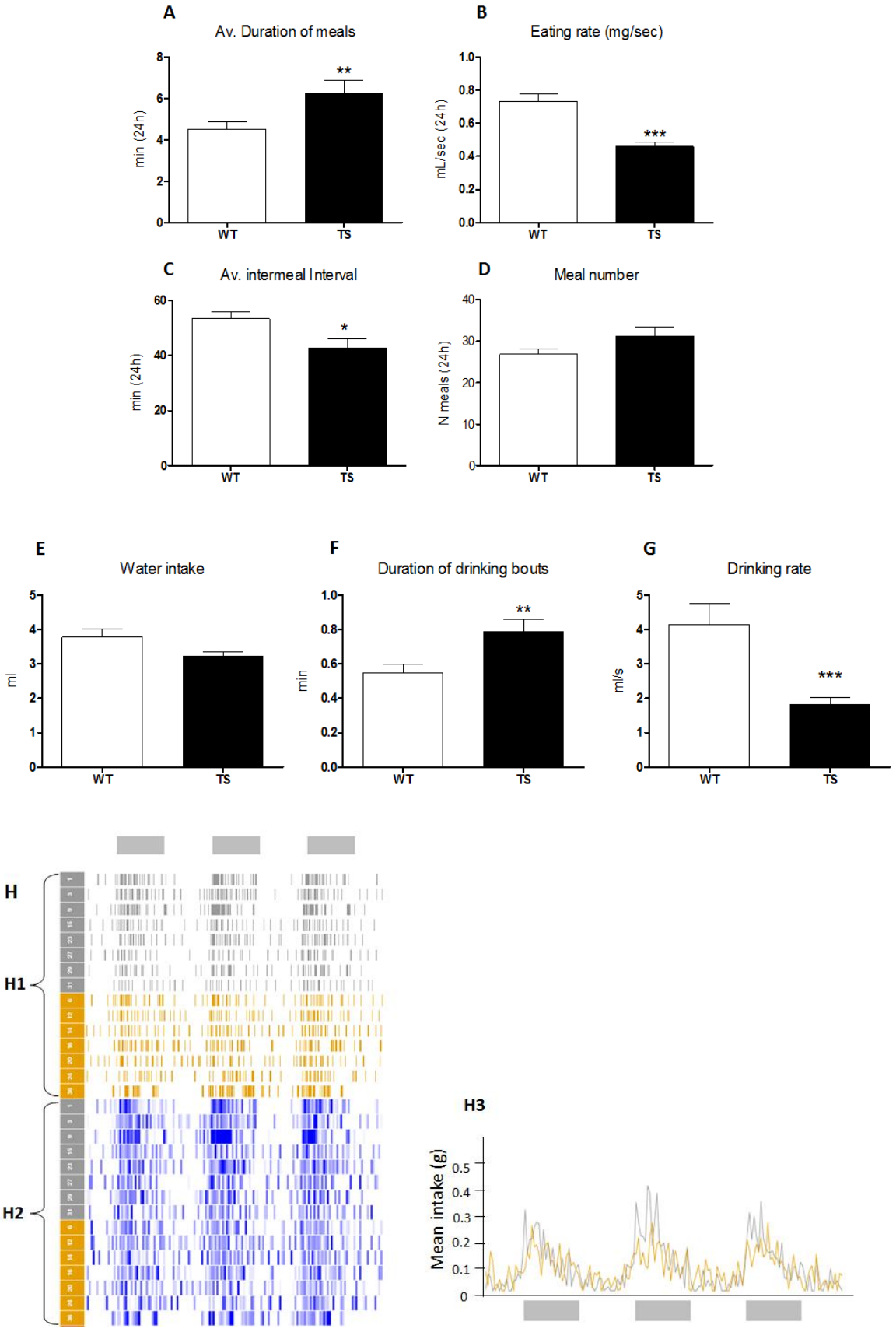


Figure 11. Meal pattern analysis and circadian rhythmicity in wild-type (WT) and Ts65Dn (TS) mice fed with standard chow (SC). Bar plot shows (A) the average duration (min), (B) the eating rate (mg/s), (C) the average intermeal interval (min) and (D) the number of meals in 24 hours at the end of the habituation period. Bar plots for (E) the amount of water consumed (ml), (F) the average duration of the drinking events (min) and (G) the drinking rate (ml/s) in 24 hours at the end of the habituation phase. (H) Longitudinal behavioral recordings. The figure depicts the feeding bouts along 3 consecutive days of 8 WT (gray) and 7 Ts65Dn (orange) mice receiving standard chow (H1). Each line represents feeding bouts of individual mice. Each line depicts a meal along time as an indicator of meal frequency. The width of each bar represents the meal duration. Individual intakes corresponding to WT mice are depicted in the upper part of the graph and those corresponding to Ts65Dn (TS) mice in the bottom part. (H2) Each blue bar represents the cumulative value of food consumed in 30 minutes. Color intensity indicates the accumulated value of the intake (white = 0 grams, blue ≥ 0.5 grams). (H3) Time course of the average SC intake in 30 minutes of WT (grey) and TS (orange). Metadata in the figure include: (i) identifiers (left bar in H1 and H2) of each WT (gray) and Ts65Dn (orange) mice and (ii) dark (active) periods (light-dark phase track) represented as grey rectangle on the top of H1-H2 and on the bottom of H3. Data from A-G are expressed as mean ± SEM. WT mice (n = 19), TS mice (n = 16). Mann-Whitney-U-test for independent samples * P<0.05; ** P<0.01; *** P<0.001.

4.1.2 Changes in feeding behavior upon exposure to free access to energy-dense food in Ts65Dn mice

To determine if voluntary consumption of energy-dense foods, reported to be increased in DS patients, may lead to obesity, overeating and compulsive-like behaviors, we used an experimental diet-induced obesity model based on free choice of standard chow (SC), and chocolate-mixture (CM) or high-fat diet (HF). We continuously recorded feeding behavior during eight weeks and the changes of feeding behavior upon exposure to the test diets. After long-term access to CM or HF we evaluated the appearance of compulsive and inflexible feeding responses in stand-alone laboratory tests (starvation, limited access to energy-dense food, quinine adulteration and preference for sweet solutions).

Body weight gain

No changes in body weight of mice fed with only SC along 8 weeks were observed, but upon free access to CM or HF, all mice showed an increase of body weight (Repeated measures ANOVA, $F(2,47) = 5.34$; $P<0.05$), and the effects were more marked in SC+HF group (genotype effect: Repeated measures ANOVA $F(2,47) = 8.31$; $P<0.01$; Figure 12A). In SC+HF group weight gain significantly differ between both genotypes being the percentage of body weight increase after 8-weeks exposure to HF significantly higher in Ts65Dn mice than in WT (diet x genotype effect: two way ANOVA $F(1,52) = 4.74$, $P<0.05$; Bonferroni as post-hoc: $P< 0.05$; Figure 12B).

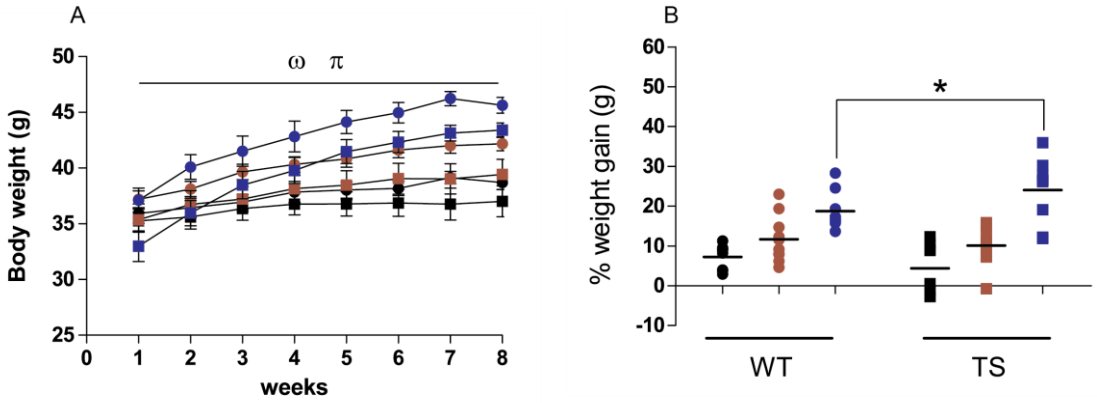


Figure 12. Body weight increase upon 8 weeks of free access energy-dense diets in wild-type (WT) and Ts65Dn (TS) mice. (A) Time course of body weight increase along the 8 weeks of exposure to energy-dense foods. (B) The plot represents the percentage of body weight gain at the end of the experiment. Genotypes are represented as dots (WT) and squares (TS), diet conditions are shown as colors (SC = black; SC+CM = brown and SC+HF = blue). (A) Depicts the mean of the group whereas in (B) each dot/square represents one individual. SC group: WT n = 9, TS n = 9; SC + CM group: WT n = 10, TS n = 10; SC + HF group WT n = 9, TS n = 9. (A) ANOVA Repeated measures diet effect ω $P<0.01$, genotype effect π<0.05. (B) Two-way ANOVA, Bonferroni as a post-hoc, * $P<0.05$.

Feeding behavior and locomotor activity changes

At the end of the obesity development phase we observed that both WT and Ts65Dn mice receiving SC+CM and SC+HF increased their energy intake compared to SC mice (diet effect ANOVA $F(1,48) = 13.67$, $P<0.001$; Figure 13A). SC+HF mice consumed more HF than SC (in WT, HF vs. SC, Paired Mann-Whitney-U-test, $z = -2.67$, $P<0.01$, in Ts65Dn, HF vs. SC, Paired Mann-Whitney-U-test, $z = -2.52$, $P<0.001$), being the grams of HF consumed significantly higher in Ts65Dn mice (Ts65Dn vs. WT, HF intake (g) Mann-Whitney-U-test, $z = -2.32$, $P<0.05$; Figure 13B), suggesting increased preference for HF. However, the intake in grams of CM was similar to SC indicating less preference for this food when given in a free choice access (Figure 13B).

Energy consumed (KJ of food consumed/ grams of body weight) from CM and HF was significantly higher in Ts65Dn mice than in WT (Ts65Dn vs. WT, CM intake (KJ/BW) Mann-Whitney-U-test, $z = -3.03$, $P<0.01$; Ts65Dn vs. WT HF intake (KJ/BW), Mann-Whitney-U-test, $z = -2.46$, $P<0.01$, Figure 13C). Whereas in WT mice the energy intake from HF was significantly higher than CM (HF vs. CM intake, Mann-Whitney-U-test, $z = -2.53$; $P<0.01$, Figure 13), the energy intake of CM and HF was similar for Ts65Dn mice.

Finally, regarding energy expenditure, we observed hyperactivity in Ts65Dn independently of the diet compared to WT mice (genotype effect $F(1,50) = 33.87$, $P<0.001$, Figure 13D).

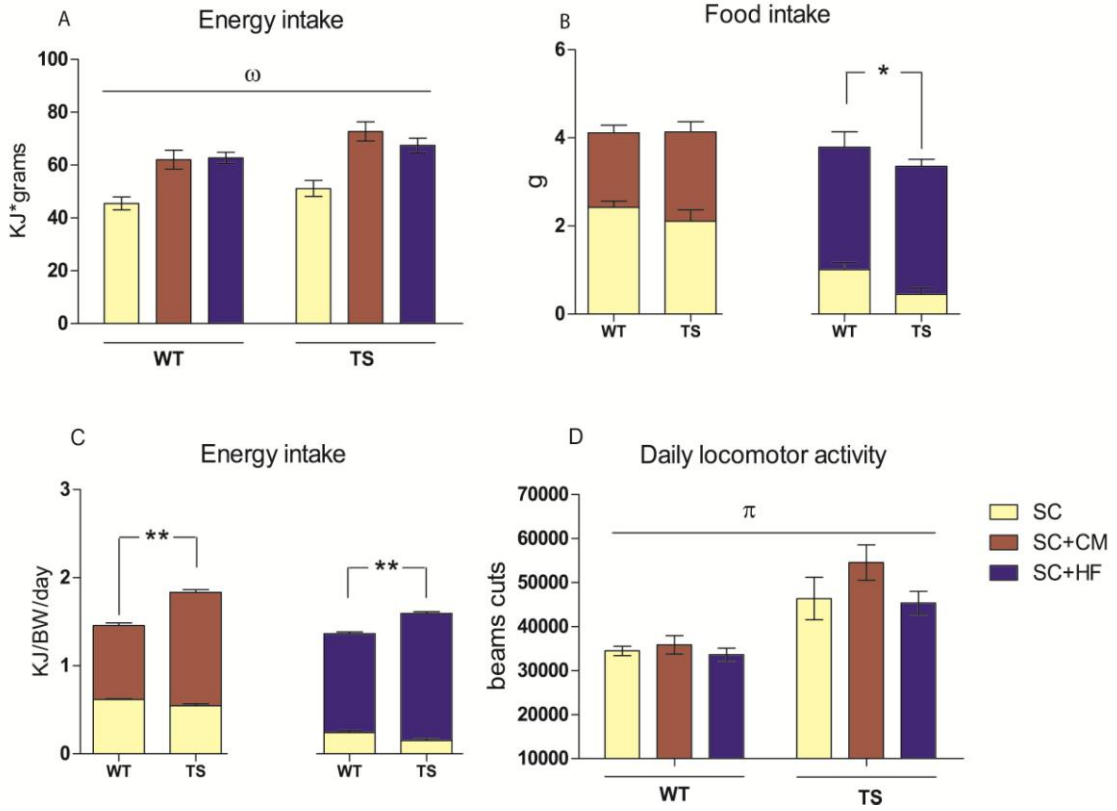


Figure 13. Energy intake, food consumption and locomotor activity in wild-type (WT) and Ts65Dn (TS) mice. (A) Bar plots shows the daily total energy intake (KJ consumed) by genotype of each group: SC (yellow), SC+CM (brown) and SC+HF (blue). Bar plots show separately (B) the daily intake (g) and (C) energy intake (KJ/g body weight (BW) of SC (yellow segment of the bar) and CM (brown segment of the bar) in SC+CM mice of either genotype (left panel) and of SC (yellow segment of the bar) and HF (blue segment of the bar) in SC+CF mice of either genotype. (D) Daily locomotor activity measured as beams cuts in PheCOMP cages. Data are expressed as mean ± SEM. SC group: WT n = 8, TS n = 7; SC + CM group: WT n = 9, TS n = 10; SC + HF group WT n = 8, TS n = 7. (A) ANOVA diet effect ω $P<0.001$. (B, C) Mann-Whitney-U-test for TS vs. WT comparisons, * $P<0.05$, * $P<0.01$. (C) Two-way ANOVA genotype effect π $P<0.05$.

In terms of food intake, neither WT nor Ts65Dn mice showed a clear preference for consuming CM (grams) along the experiment. Even so, Ts65Dn mice showed higher preference for CM than in WT that consumed less CM than SC showing a negative preference ($P<0$) value (see material and methods for calculation of preference, Figure 14A). We then calculated the preference corrected per body weight and observed that in Ts65Dn mice their main source of energy comes from CM along time while in WT the preference is close to zero, although the difference did not reach statistical significance

(Figure 14C). For SC+HF mice, the preferred food and the main energetic source was HF for both genotypes along the whole experiment (Figure 14B-D).

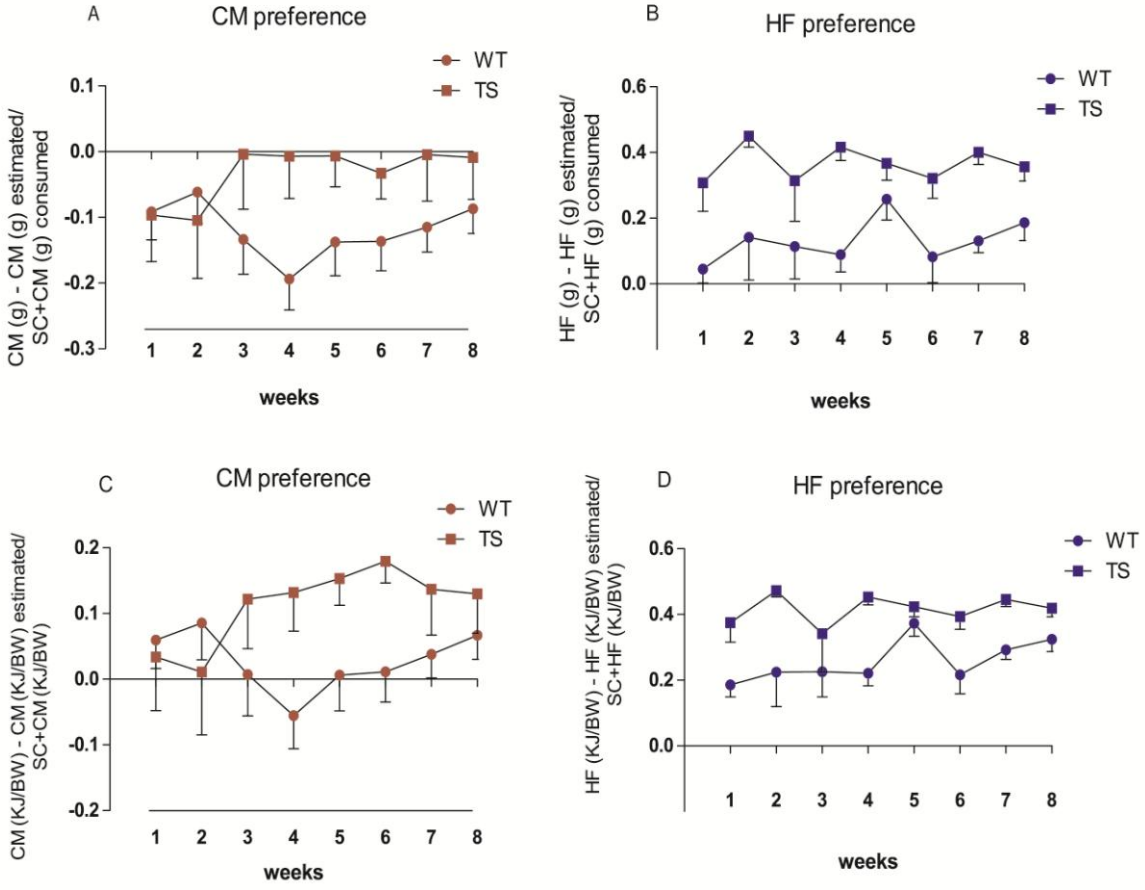


Figure 14. Preference for energy-dense foods in wild-type (WT) and Ts65Dn (TS) mice. (A) Time course of the CM and (B) HF preference in grams across the experimental time. (C) Time course of the preference for CM and (D) HF relative to the energy intake (KJ) per grams of body weight. Data are expressed as mean ± SEM. In (A-C) WT mice are depicted as brown dots and Ts65Dn (TS) mice as brown squares. In (B-D) WT mice are depicted as blue dots and Ts65Dn (TS) mice as blue squares. SC group: SC + CM group: WT n = 9, TS n = 10; SC + HF group WT n = 8, TS n = 7. ANOVA Repeated measures.

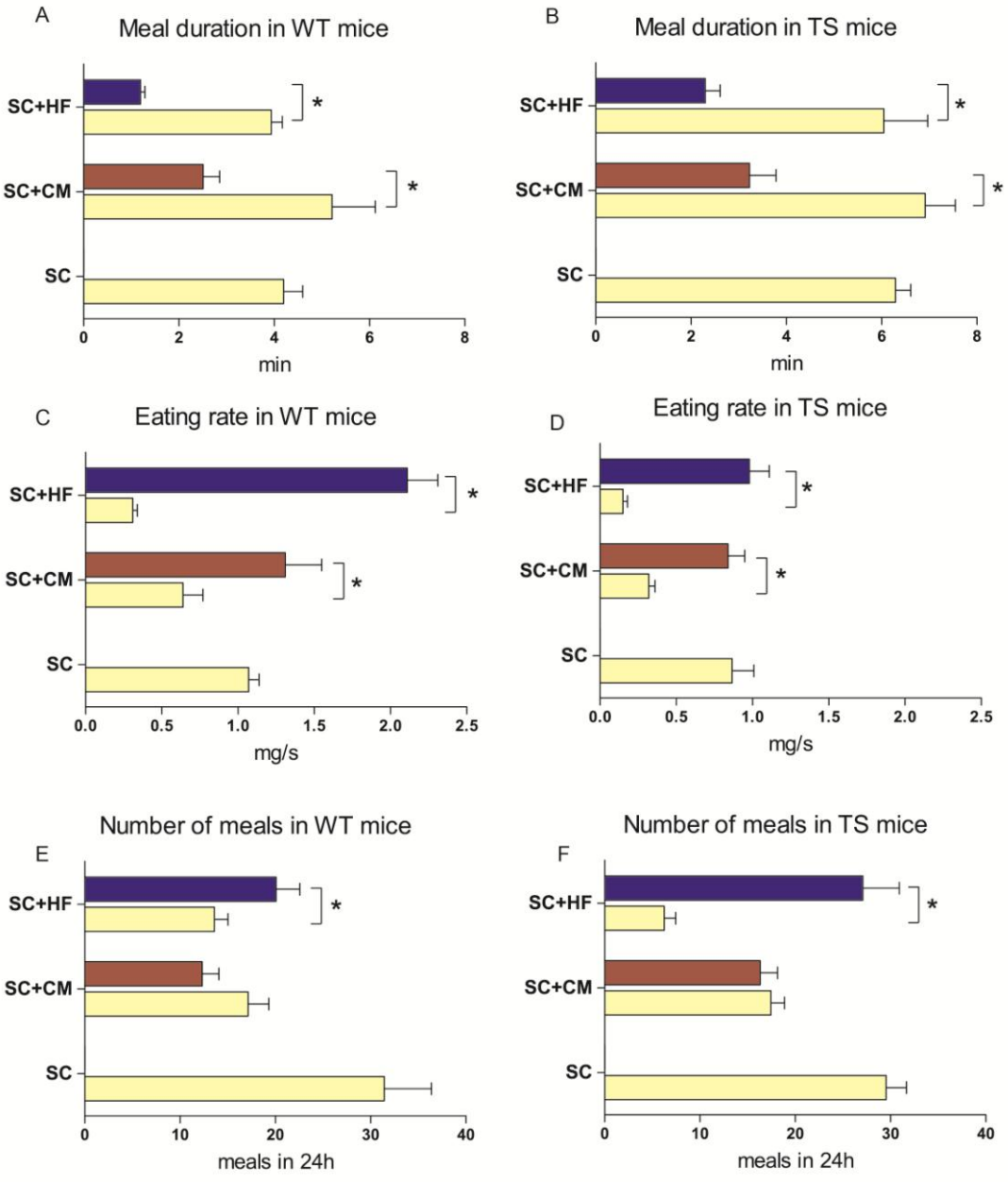
Meal pattern analysis

We detected similar diet-induced changes in meal pattern in both genotypes compared to baseline (SC) feeding behaviors (Figure 15). Diet has an effect on duration of meals (diet effect, $F(4,83) = 15.14$; $P < 0.001$, Figure 15A-B), being CM and HF meals shorter than SC meals for both genotypes (Sidak as a post-hoc; $P<0.05$). The eating rate was also influenced by diet (diet effect, $F(4,83) = 20$; $P<0.001$, Figure 15C-D), thus, for both genotypes, CM and HF meals were consumed faster than SC meals (Sidak as a post-hoc; $P<0.05$). Although energy-dense diets had reduced average meal duration and increased their eating rate as compared to SC meals, Ts65Dn still ate slowlier than WT

mice, (genotype effect for meal duration, $F(1,83) = 13.63$; <0.001; genotype effect for eating rate, $F(1,83) = 19.85$; <0.001).

Energy-dense diets had an effect on the number of meals (diet effect, $F(4,83) = 21.76$; <0.001, Figure 15E-F). The number of HF meals in SC+HF mice was significantly higher compared to their SC meals (Sidak as a post-hoc; $P <0.001$) and to CM meals of SC+CM group (Sidak as a post-hoc; $P<0.05$). Compared to the SC meals of SC mice both CM and HF meals were reduced (Sidak as a post-hoc; $P<0.05$).

Energy-dense diets had differential effect on satiety since we observed a diet effect on the time between meals (diet effect intermeal interval, $F(4,83) = 2.79$; $P <0.001$, Figure 15 G-H). In SC+CM mice the intermeal interval was higher for CM than for SC meals (Sidak as a post-hoc; $P<0.05$) but no changes were observed in SC+HF mice. Conversely, SC+HF mice had increased satiety for SC than for HF (diet effect $F(2,83) = 5.12$; $P<0.001$, Sidak as a post-hoc; $P<0.05$, Figure 15 I-J).


A
Meal duration in WT mice
SC+HF
SC+CM
SC
*
*
0
2
4
6
8
min
B
Meal duration in TS mice
SC+HF
SC+CM
SC
*
*
0
2
4
6
8
min
C
Eating rate in WT mice
SC+HF
SC+CM
SC
*
*
0.0
0.5
1.0
1.5
2.0
2.5
mg/s
D
Eating rate in TS mice
SC+HF
SC+CM
SC
*
*
0.0
0.5
1.0
1.5
2.0
2.5
mg/s
E
Number of meals in WT mice
SC+HF
SC+CM
SC
*
0
10
20
30
40
meals in 24h
F
Number of meals in TS mice
SC+HF
SC+CM
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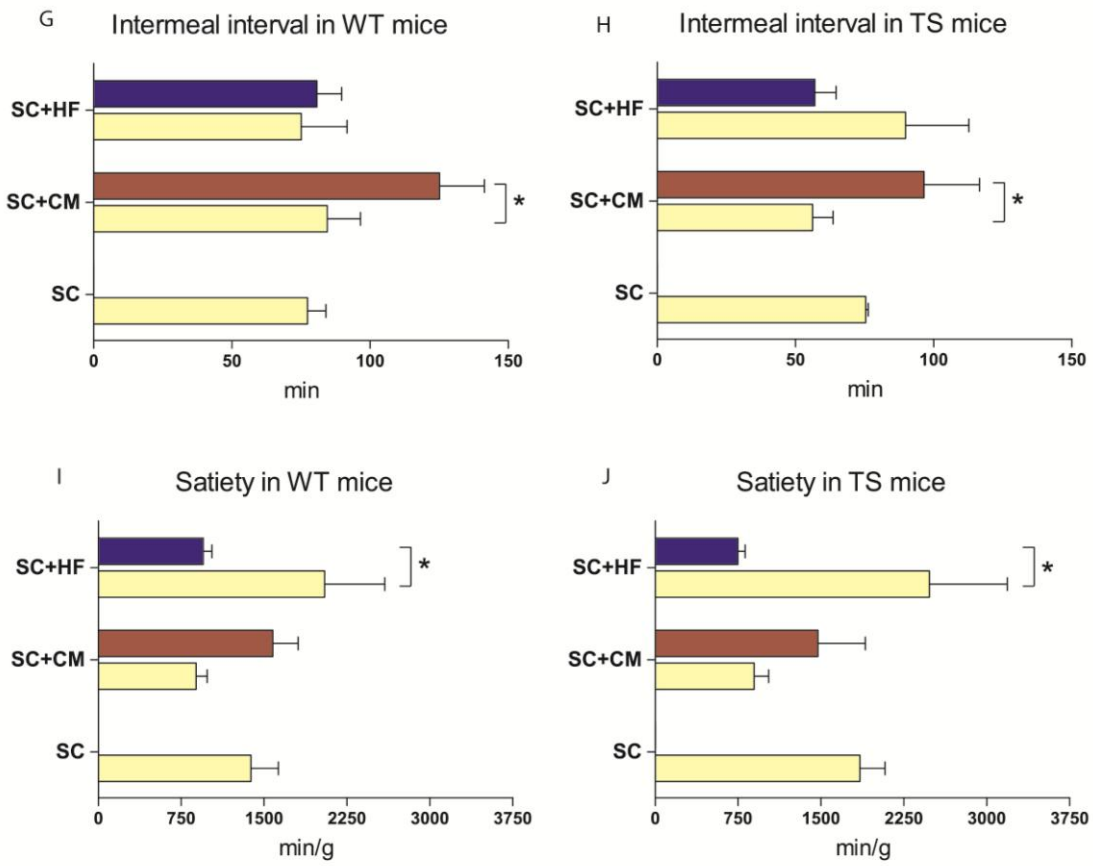

Figure 15. Meal pattern changes upon free access to energy-dense diets in wild-type (WT) and Ts65Dn (TS) mice. Bar plots represent the meal patterns for each type of food for each experimental group [yellow (SC), in brown (CM) and in blue (HF)] left panel shows WT mice values, and right panel results of TS. (A-B) Average meal duration (min) of SC, CM and HF meals in WT (A) and in TS mice (B). (C-D) Eating rate (min) of WT (C) and TS (D) mice. (E-F) Number of meals in WT (E) and TS (F) mice. (G-H) Average intermeal interval (min) in WT (G) and in TS (H) mice. (I-J) Satiety (min/g) in WT (I) and in TS (J) mice. Data are represented as mean ± SEM. SC group: WT n = 8, TS n = 7; SC + CM group: WT n = 9, TS n = 10; SC + HF group WT n = 8, TS n = 7. Linear mixed model analysis, Sidak as a post-hoc, * P< 0.05. In the figures only the significance of the intra-genotype CM vs. SC (in SC+CM mice) HF vs. SC (in SC+HF mice) is depicted.

Circadian feeding pattern

In mice, feeding is a patterned activity subject to well-controlled daily oscillations with feeding bouts accumulated over the dark (active) phase of the light/dark cycle (nocturnal meals) and almost absent during the light (resting) phase. However, when mice have access to energy-dense foods, these foods are also consumed during the resting phase, which causes early and stable circadian activity disruption, as it has been previously shown (Espinosa-Carrasco et al. submitted). To capture long-term fine-grained pattern of feeding changes promoted by CM and HF, we used Pergola (http://cbcrg.github.io/pergola) an open-source software developed in our group for the visualization and explorative analysis of (high-throughput) longitudinal behavioral data.

Pergola allows the interactive visualization of a complete experiment of each individual, which makes easier the detection of phenotypic quantitative and qualitative differences within subject. Figure 16A-C shows the amount of food intake and the temporal distribution of feeding events along 3 weeks. We selected 3 representative periods of the early (1st week), mid (4th week) and late time changes (8th week) of the circadian feeding activity.

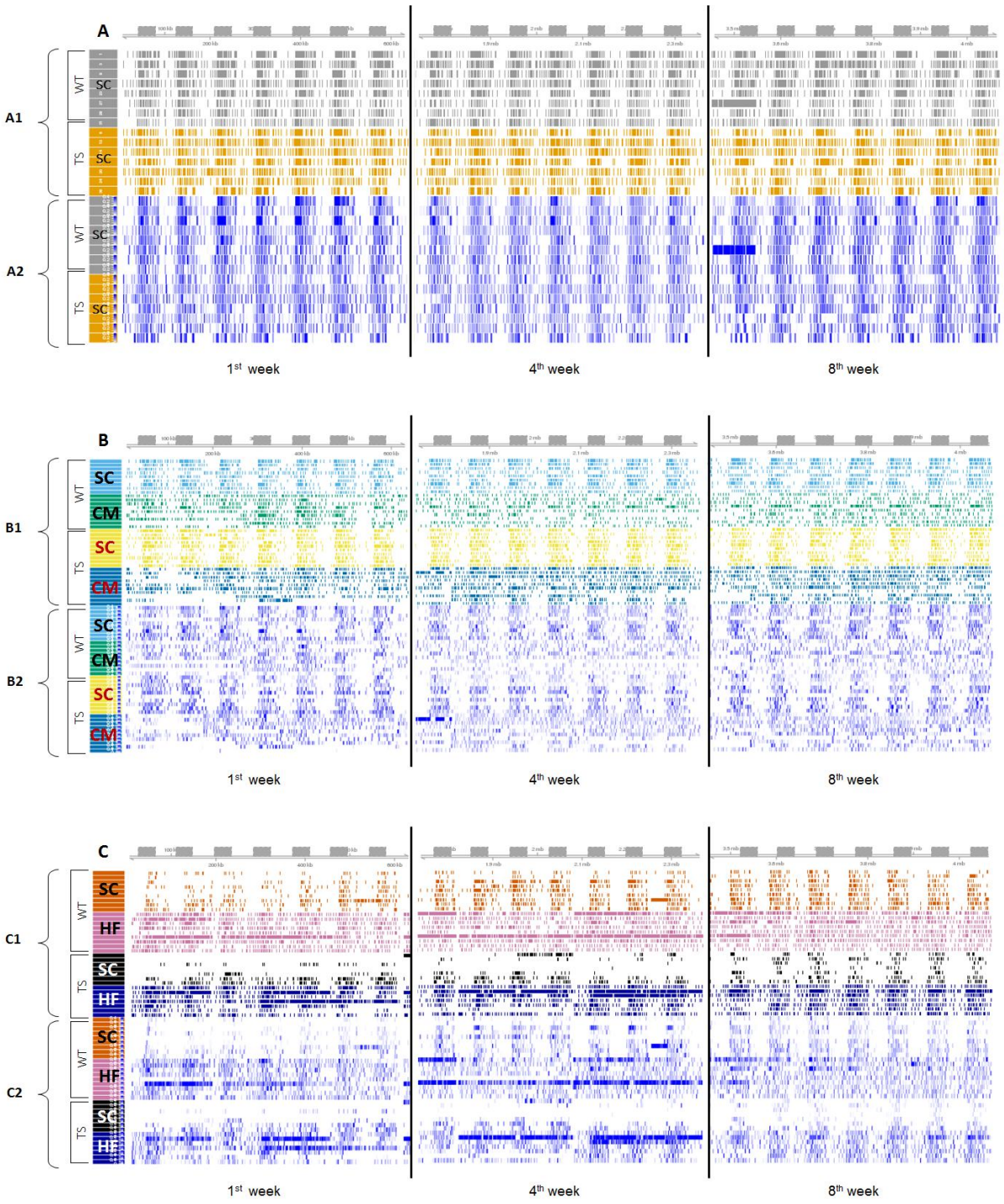

Figure 16. Longitudinal behavioral recordings from the 1st, 4th and 8th week of free access to energy-dense food in wild-type (WT) and Ts65Dn (TS) mice. Pergola visualization of feeding bouts along time for (A) SC mice (WT n = 8, TS n = 7); (B) SC+CM mice (WT n = 9, TS n = 10) and (C) SC+HF mice (WT n = 9, TS n = 7). Metadata included in the figure are: (i) identifiers for each mouse. In A we depict SC mice: WT (grey) and TS (orange); B represents SC + CM mice WT (light blue for SC and green for CM); TS (yellow for SC and dark blue for CM); C represents SC + HF mice: WT (orange for SC and pink for HF), TS (black for SC and blue for HF). (ii) Dark (active) periods (light-dark phase track) represented as grey rectangle on the top of each panel. (iii) The weeks of the longitudinal recordings are written on the bottom of panel. (A) SC meals of the SC group, (B) SC and CM meals of the SC+CM group and (C) SC and HF meals of the SC+HF group. Each row corresponds to one animal. For each group, in the upper part of the figure (A1, B1, and C1) each bar represents a feeding bout and its size corresponds to meal duration. WT mice are depicted in the upper panel and those corresponding to Ts65Dn mice in lower panel. For the same mice, in A2, B2 and C2 each line shows the accumulated intake (g) of the individual during a period of 30 minutes. The intensity of the blue indicates the value of the accumulated food intake (white = 0 grams, blue ≥ 0.5 grams).

The visualization of the longitudinal behavioral data showed, from the first week and along all the experiment, increased feeding events in the inactive period in mice given free-choice access to energy-dense food (Figure 16B-C).

We detected that in WT the SC mice had the highest intake, followed by SC+CM mice > SC+HF mice (Figure 17). In Ts65Dn, we observed that SC and SC+CM groups had similar SC intake whereas in SC+HF group the SC was almost neglected (Figure 17). Compared to SC group-genotype matched, WT mice consumed les grams of CM and HF whereas Ts65Dn mice showed equivalent intake.

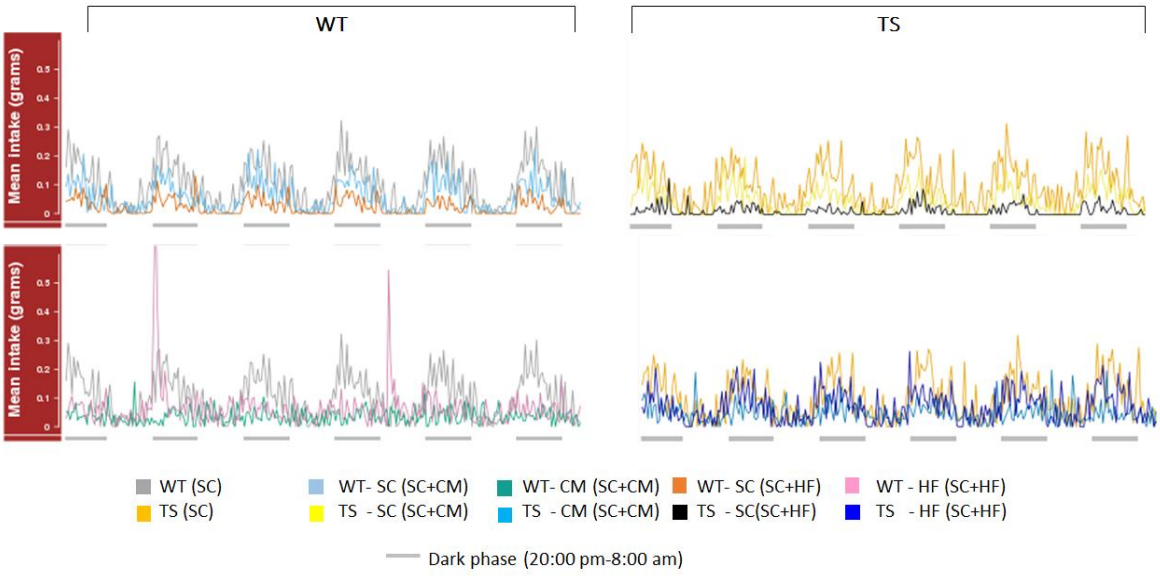

Figure 17. SC and energy-dense foods circadian intake in wild-type (WT) and Ts65Dn (TS) mice during the 8th week of the obesity development phase. Active (dark) phases are marked by a grey line in the bottom of the plot. The plot depicts, the group average of the WT (on the right) and Ts65Dn (TS, on the left), of the amount (in g) of SC (in the upper part) and CM or HF (in the lower part). In the bottom are the code colors for each diet of each group.

To quantify the circadian feeding rhythmicity loss, we calculated the number of accumulated meals within 30 minutes time windows distinguishing the light and dark phases during each week of the obesity development (Figure 18).

In non-obesogenic conditions, both WT and Ts65Dn mice concentrate their meals during the nights (SC number of meals light vs. dark phase, Bonferroni as a post-hoc, $P<0.001$ in WT, $P<0.001$ in Ts65Dn) without differences among genotypes (Figure 18). Statistical analysis revealed that diet had an effect on the meal frequency in both genotypes (Three-way repeated measures ANOVA (genotype, diet and phase), $F(9,75) = 10.48$; $P< 0.001$).

In SC+CM group the meals were also concentrated during the active (dark) phase. The number of SC meals was higher compared to CM meals in both genotypes (Bonferroni as a post-hoc, P<0.001 in WT, P<0.001 in Ts65Dn, Figure 18) but Ts65Dn mice showed significantly higher number of CM meals than WT mice (Bonferroni as a post-hoc, P<0.001). During the inactive phase, the number of CM meals was similar to the number of SC meals in both genotypes and the total number of meals was not different from the number of meals of SC group (Figure 18).

In the SC+HF group, during the inactive period, the number of HF meals was higher in both genotypes as compared to SC meals (Bonferroni as a post-hoc, P<0.001 for WT and for Ts65Dn). WT mice showed an increased number of HF meals as compared to SC group (Bonferroni as a post-hoc, P<0.001) during the dark (active) phase. However, the number of HF meals was similar in both active and inactive phases of the dark/light cycle (Figure 18), suggesting a loss of rhythmicity. This circadian rhythm disturbance was not detected in Ts65Dn mice that showed a significantly higher number of HF meals during the dark phase (Bonferroni as a post-hoc, P<0.001). In this genotype, we detected a significantly higher number of HF than SC meals (Bonferroni as a post-hoc, P<0.001, Figure 18).

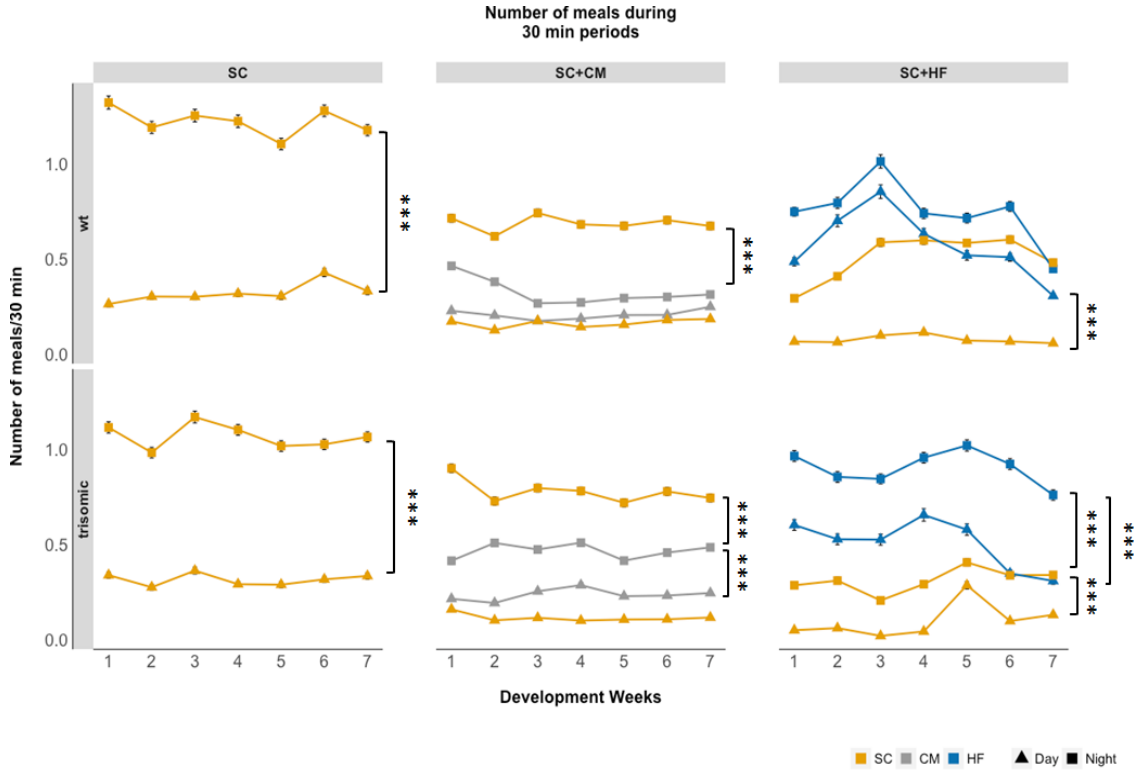

Figure 18. Quantitative measure of the circadian feeding activity along the experiment during the day and nights in wild-type (wt) and Ts65Dn (trisomic) mice. The figure depicts for each diet group (in columns) and genotype (upper part WT and Ts65Dn, trisomic, in the bottom) the number of meals consumed in a time window of 30 min across the whole experiment during the day (triangles) and during

the nights (squares). The color code for the foods is as follows: SC (orange), CM (grey) HF (blue). Three-way ANOVA was used for statistical analysis. Bonferroni was used as a post-hoc. *** P< 0.001. Only the significant comparison intra genotype and group are depicted in the figure.

Our analysis confirmed that WT mice have a more severe circadian feeding disruption than Ts65Dn when HF is provided *ad libitum* (Figure 17, 18). Interestingly, the disruptive behavior was food specific since circadian rhythmicity for SC meals was affected neither in SC+CM nor in SC+HF mice (Figure 16).

4.1.3 Testing phase

Consumption of energy-dense foods led to addictive-like behaviors such as inflexible food choices and compulsive (binge-like) eating as previously described (Heyne et al. 2009; Espinosa-Carrasco et al. submitted). The evaluation of these compulsive components of feeding was performed at the end of the obesity development phase, after 8 weeks of exposure to energy-dense foods. We limited the access and adulterated the flavor of the energy-dense foods to evaluate the appearance of diet-induced non-adaptive behaviors, such as compulsive intake or behavioral inflexibility.

Starvation

24 h starvation lead to increased energy intake (KJ) upon refeeding in all mice compared to their energy in *ad libitum* free feeding conditions (Two-way ANOVA, ($F_{(1,44)} = 67.51$, $P<0.001$). In the SC group, upon refeeding Ts65Dn compensated more their energy intake than WT mice (Ts65Dn vs. WT, Mann-Whitney-U-test, $z = -2.32$, $P<0.05$, Figure 19A). In SC+CM mice the increase of SC intake upon refeeding was similar between genotypes but WT mice tended to consume more CM than Ts65Dn, although the difference did not reach statistical significance (Figure 19B). In SC+HF mice, starvation preferentially increased HF intake in both genotypes but the increase was only significant in Ts65Dn mice (paired Mann-Whitney-U-test, SC vs. HF in Ts65Dn $z = -4.07$, $P<0.01$, Figure 19C).

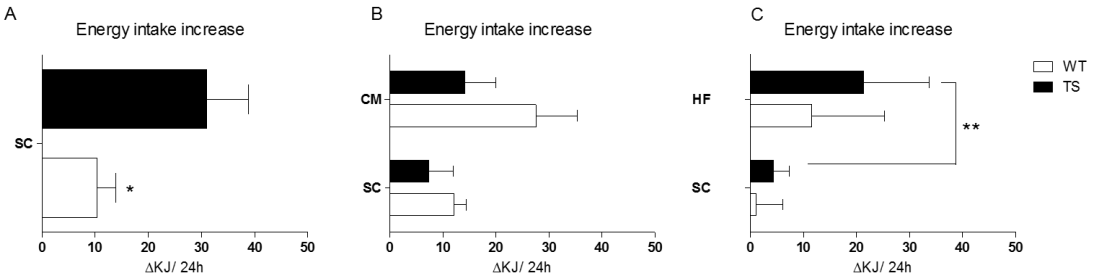

Figure 19. Energy intake upon 24 hours of starvation in wild-type (WT) and in Ts65Dn (TS) mice. Bar plots show the increase in energy intake calculated as the KJ consumed 24h after refeeding - KJ consumed in 24 hours in free feeding conditions. (A) Increase in daily energy upon refeeding in the SC group (B) the SC+CM group, and (C) the SC+HF mice. Data are expressed as means ± SEM. SC group: WT n = 8, TS n = 7; SC + CM group: WT n = 9, TS n = 10; SC + HF group WT n = 8, TS n = 7. Mann-Whitney-U test for genotype comparisons, Paired Mann-Whitney-U-test for within genotype comparisons (SC vs. CM and SC vs. HF), * P<0.05 ** P<0.01.

Limited access to energy-dense food

Access to HF or CM diet was restricted to 1 hour during the light (resting) phase of the light-dark cycle (from 14:00 to 15:00h) for 3 consecutive days in overweight mice (SC+CM or SC+HF; Figure 20A). SC and water were provided unrestrictedly *ad libitum.*

We observed that during the periods of CM/HF restriction, the SC energy intake (KJ/body weight) of free choice mice was increased as compared to *ad libitum* feeding condition (SC+CM, SC+HF; ($F(1,30) = 129.55$; $P<0.001$, Figure 20B). Two-way ANOVA analysis revealed that during the periods where the energy-dense foods were not available, the energy intake from SC was different between the free choice groups (diet effect, $F(1,33) = 10.015$; $P<0.01$) and between the genotypes (genotype effect, $F(1,33) = 5.345$; $P<0.05$). However, mice from any group were able to increase SC intake to compensate their basal energy levels (Figure 20C).

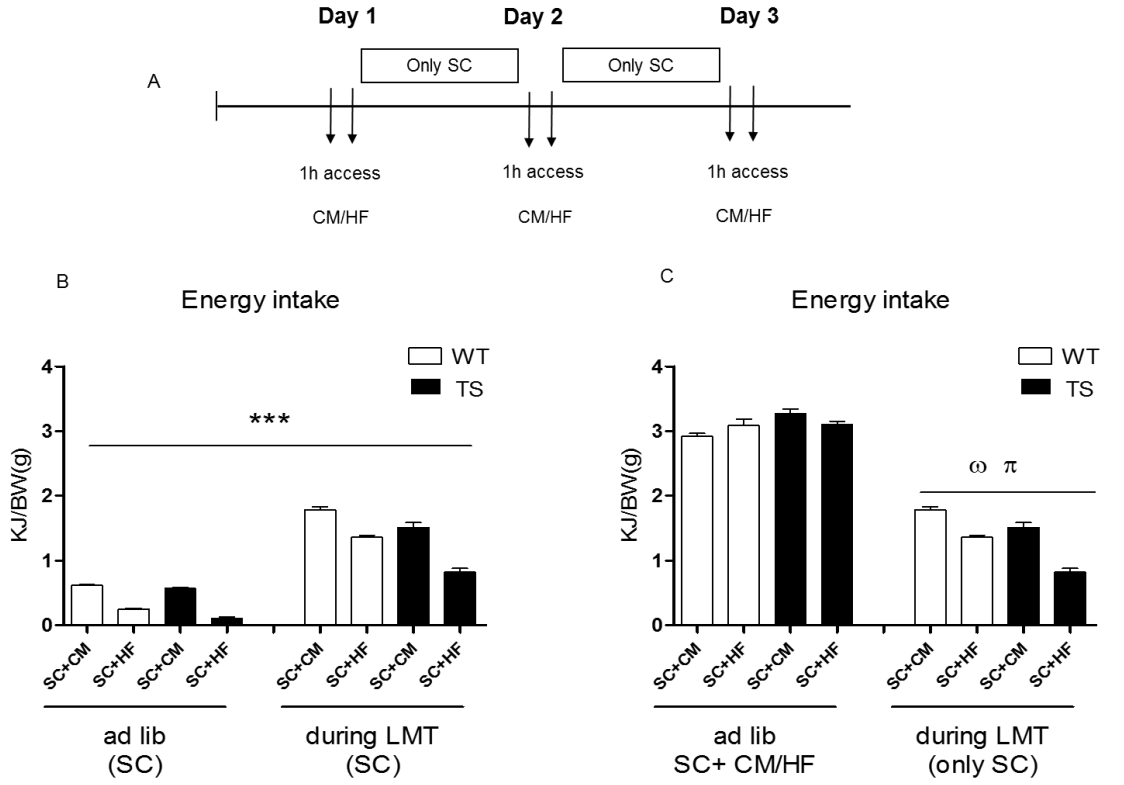

Figure 20. SC energy intake during the periods of absence of energy-dense foods wild-type (WT) and in Ts65Dn (TS) mice. (A) Schedule of the three days of limited access experiment. (B) The bars plots show the mean values of energy intake of SC in *ad libitum* feeding conditions (when CM and HF are freely available, "*ad lib*") and when energy-dense foods are not available (limited, "LMT"). (C) The bars plots show the mean values of total energy intake in *ad libitum* feeding conditions (SC+CM or SC+HF according to the experimental group) and the SC intake when energy-dense foods are not available (limited, "LMT"). Wild-type mice (WT) are depicted in white and Ts65Dn mice (TS) in black. Data are expressed as mean ± SEM. SC + CM group: WT n = 9, TS n = 10; SC + HF group: WT n = 8; TS n = 7. ANOVA Repeated measures, factor (test day) *** P< 0. 001; Two-way ANOVA, genotype effect π P<0.05; diet effect ω P<0.05.

Binge-like eating episodes upon limited access are revealed by measuring the energy intake and the eating rate (mg/s) of CM and HF consumed during the periods of CM/HF access. Since mice have continuous access to standard chow, the increased energy intake of energy-dense foods during the 1h access compared to *ad libitum* conditions, when they have both SC and CM/HF access are indicators of a compulsive like intake rather than a hunger effect.

When analyzing separately the consumption of energy-dense foods and SC along the three days of the experiment, we found a nonsignificant trend for increasing the consumption of CM and HF in the limited access periods along the test days in Ts65Dn and in WT mice (Figure 21A-B), suggesting that this schedule of access was not sufficient to induce strong binge-like episodes. Even so, the increase in energy intake between the *ad libitum* conditions and the third day of the limited access to energy-

dense food, was significantly increased and was higher in Ts65Dn compared to WT for both CM (Ts65Dn vs. WT, Mann-Whitney-U-test, z = -2.98; P<0.01) and HF (Ts65Dn vs. WT Mann-Whitney-U-test, z = -2.23; P< 0.01; Figure 21C).

We also calculated the eating rate as a measure of the avidity since more rapid intake would be an indicator of a compulsive-like feeding. The eating rate for CM intake showed a nonsignificant tendency to increase in WT mice (Repeated measures ANOVA, genotype effect F (1,17) = 3.95; P = 0.06, Figure 21D) but not in Ts65Dn mice. In SC+HF mice, WT mice showed a significant increased eating rate as compared to Ts65Dn mice the second and the third day of the test (Repeated measures ANOVA, genotype effect F(1,13) = 8.09; P<0.05, Figure 21E).

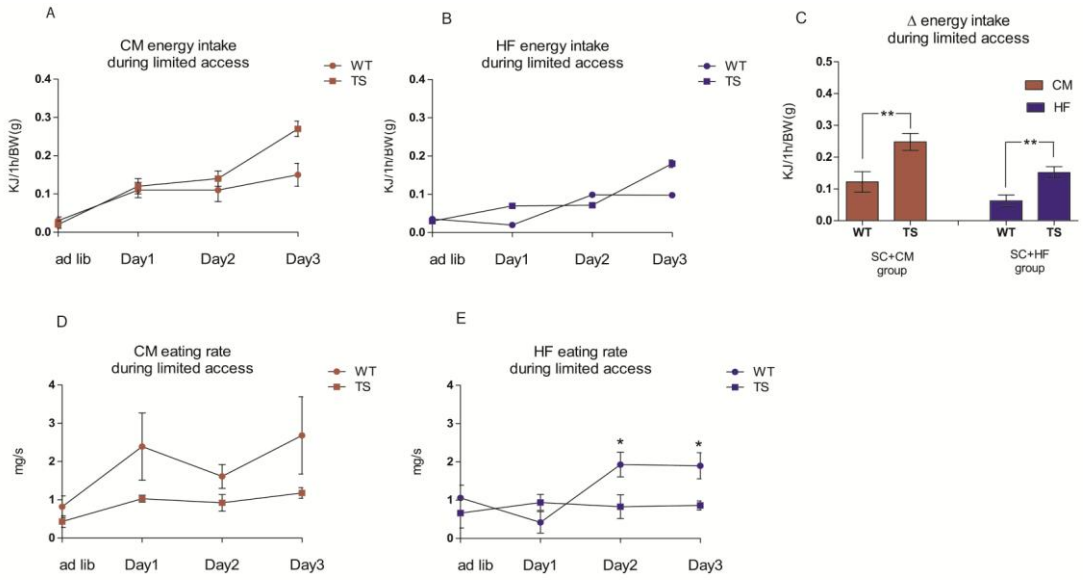

Figure 21. Energy intake and eating rate during limited access to energy-dense food in WT and Ts65Dn (TS) mice. Time course of the energy intake of (A) CM and (B) HF during the periods of access. (C) CM/HF energy increase between the *ad libitum* conditions and during the third day of the limited access. Time course of the eating rate of CM (D) and (E) HF intake during the periods of limited access. *ad lib= ad libitum*; Day 1, Day 2 and Day 3 are the three consecutive days of restricted access (1h) to CM/HF. Data are expressed as mean ± SEM. SC + CM group: WT =9, TS = 10; SC + HF group: WT = 7; TS = 7. * P<0.05 in (E) genotype effect, Repeated measures ANOVA, Bonferroni as a post-hoc. (C) Mann-Whitney-U-test ** P<0.01.

Food adulteration with quinine hydrochloride

The inability to neglect the preferred food after the modification of its hedonic properties (i.e. the taste) is a sign of inflexibility (Heyne et al. 2009). Here, food was manipulated with quinine hydrochloride to address if Ts65Dn mice still consumed energy-dense foods in spite of their aversive taste.

In the SC group, mice were given free choice between SC and SC adulterated with quinine hydrochloride and both genotypes showed reduced energy intake of adulterated SC as compared to non-adulterated chow (paired Mann-Whitney-U test, z = -2.02 in WT; P<0.05 and z = -2.36; P<0.05 in Ts65Dn, Figure 22A, 23A) suggesting no problems in bitter taste detection. WT mice slightly increased their intake of non-adulterated SC (Figure 22 A-B), but not significantly.

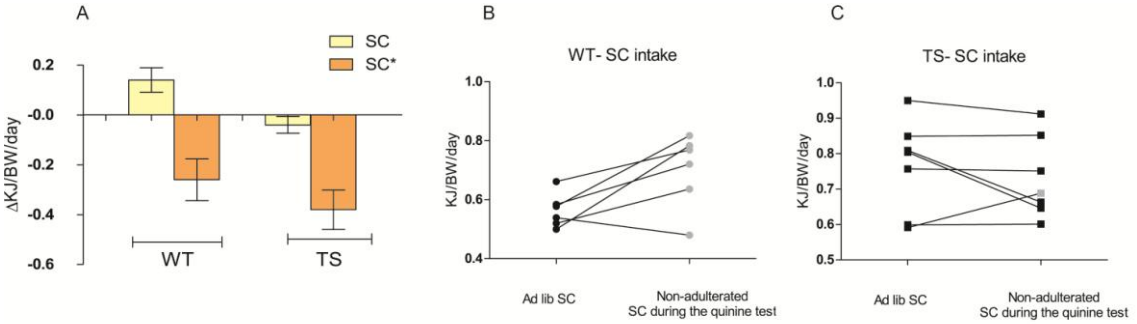

Figure 22. Energy compensation during quinine adulteration test in non-obesogenic conditions in wild-type (WT) and in Ts65Dn (TS) mice. (A) Percentage of reduction of adulterated and increased of non-adulterated SC consumption (percentage of KJ/g of body weight/day) in WT and TS mice. (B-C) Individual values of 24 hours intake of non-adulterated SC (KJ/g of body weight) in basal conditions (black symbols) and upon presentation of quinine adulterated SC (grey symbols) in WT (B) and TS (C) mice. Data are expressed as mean ± SEM. SC group, WT n = 8, TS n =7. Paired Mann-Whitney-U-test.

In the SC+CM group, 30% of mice ate adulterated CM despite the bitterness. The rest of mice reduced its consumption, but the reduction was not significant as compared to non-adulterated conditions although it was more important in WT than Ts65Dn (Figure 23B).

In the SC+HF group, quinine adulteration reduced HF intake in 80% of mice (8/9 WT and 6/7 Ts65Dn). However, whereas WT mice ate 50% less compared to non-adulterated conditions (paired Mann-Whitney-U-test, HF vs. bitter HF intake in WT, z = -2.253; P<0.01), the reduction of adulterated HF intake in Ts65Dn mice (30%) was not significant compared to non-adulterated conditions (Figure 23C).

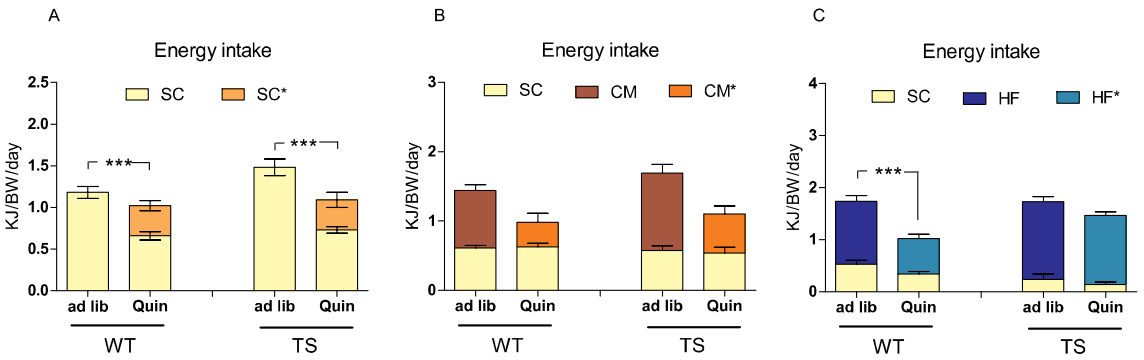

Figure 23. Energy intake of quinine-adulterated food in wild-type (WT) and Ts65Dn (TS) mice. Bar plots show food intake (KJ/g of body weight) in 24 hours in non-adulterated conditions (*ad libitum*, "*ad lib*") and the energy intake after quinine hydrochloride adulteration (Quin) of (A) SC , (B) CM (C) and HF. Adulterated food is marked by "*" . In (A) light orange SC* = bitter SC; in (B) dark orange CM* = bitter CM and in (C) light blue HF* = bitter HF. (A) SC adulteration in SC group. (B) CM adulteration in SC+CM group. (C) HF adulteration in SC+HF group. In each boxplot, the horizontal line corresponds to group median and individual dots are also plotted. SC group, WT = 8, TS = 7; SC + CM group: WT = 9, TS = 10; SC + HF group: WT = 8; TS = 8. Mann-Whitney-U-test ** P< 0.01; ** P< 0.001.

Preference for sweet solutions

The two-bottle test was used for assessing reward and taste perception. Ts65Dn mice showed similar preference for sweets solutions than WT in non-obesogenic conditions (Figure 24).

Compared to SC mice, mice on free choice access to energy-dense diets reduced the preference for saccharin different between the genotypes (genotype effect Two-way ANOVA, $F(1,52) = 5.67$; $P<0.05$) and diet condition (diet effect Two- way ANOVA $F(1,52) = 6.24$; $P<0.01$, Figure 24A). Also compared to SC mice, the preference for sucrose was reduced in free choice groups (diet effect Two- way ANOVA $F(1,52) = 13.13$; $P<0.001$; Figure 24B). In WT mice SC+HF group showed the lowest preference for sucrose whereas in Ts65Dn mice both SC+CM and SC+HF groups shower similar sucrose preferences but the diet x genotype interaction was not significant (genotype x diet interaction, Two- way ANOVA $F(2,52) = 2.75$; $P= 0.07$).

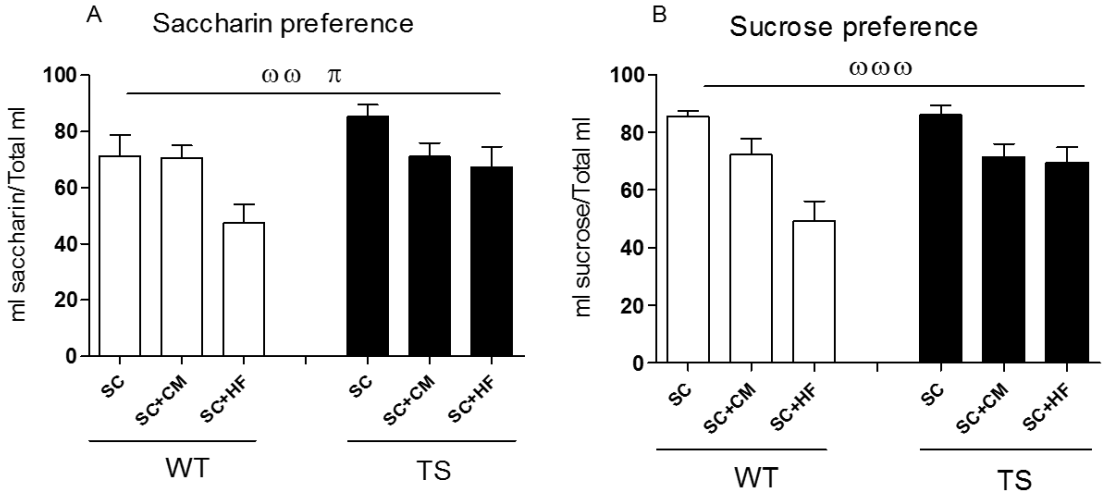

Figure 24. Preference for sweets solutions upon long-term access to energy-dense diets in wild-type (WT) and Ts65Dn (TS) mice. (A) Bar blots show the preference for (A) saccharin and (B) sucrose against water [ml sweet solution/ total liquid intake (water + sweet solution)] in SC, SC+CM and SC+HF mice. Data are expressed as mean ± SEM. SC group: WT = 8, TS = 8; SC + CM group: WT = 10, TS = 9; SC + HF group: WT = 9; TS= 8. Two-way ANOVA, genotype effect π $p<0.05$; diet effect ωω <0.01; ωωω < 0.001.

4.2 Monoaminergic profile in brain regions related to feeding behavior in Ts65Dn mice in basal and obesogenic conditions

Since brain monoamines are key modulators of feeding behavior, we used HPLC-ED in hypothalamus, striatum and prefrontal cortex, brain structures related to food intake and reward, of Ts65Dn and their respective WT mice to determine the monoaminergic profile under obesogenic conditions.

Hypothalamus

We did not detect genotype-dependent differences in monoamine levels in the hypothalamus. Long term access to energy-dense food also did not lead to significant changes in the monoamine profile in hypothalamus (Table 3) in either genotype, suggesting that the mild feeding behavior differences between WT and Ts65Dn mice in non-obesogenic conditions are not dependent on this mechanism.

Region	genotype	diet	(ng/mg)								
			NA	AD DOPAC	DA	5HIAA	5HT	HVA	HVA/DA	5HIAA/5HT	NA/DA
HT	WT	SC	37.47±19.10	4.08±2.83	5.16±4.54	22.54±14.99	31.34±17.45	0.00	0.00	0.87±0.55	11.64±9.01
		SC +CM	48.22±922.19	3.96±1.94	6.61±4.46	29.83±12.71	64.35±35.17	4.09±10.02	1.97±4.81	0.50±0.12	8.94±3.78
		SC+HF	51.65±12.67	4.13±2.38	3.69±3.04	24.32±9.00	35.08±19.10	0.00	0.00	0.74±0.37	18.72±8.26
	TS	SC	45.82±20.73	3.70±2.21	5.45±4.37	28.67±13.03	57.20±33.52	0.00	0.00	0.56±0.19	139.19±291.44
		SC +CM	32.36±7.86	2.27±0.64	5.07±2.59	24.73±10.43	43.76±12.92	2.77±6.79	1.18±2.90	0.55±0.08	7,55±3.32
		SC+HF	38.08±13.03	3.57±1.47	7.63±4.21	32.63±10.14	38.26±13.98	0.00	0.00	0.93±0.30	5.89±3.21

Table 3. Levels of monoamines in the hypothalamus of wild-type (WT) and Ts65Dn (TS) mice. NA: noradrenaline; AD: adrenaline; DA: dopamine, DOPAC: 3,4-Dihydroxyphenylacetic acid, a metabolite of DA; 5HT: serotonin; HVA: Homovanillic acid, a metabolite of DA; 5HIIA: 5-Hydroxyindoleacetic acid, a metabolite of 5HT. The ratios between the neurotransmitters and their metabolites are also presented. Data are presented as mean ± SEM, of ng/mg total tissue. WT: SC (n = 6), SC + CM (n = 6), SC + HF (n = 5), TS: SC (n = 4), SC + CM (n = 6), SC + HF (n = 5).

Striatum

The striatum is a key area for goal directed behavior and for assignation of the correct value of a reward, and thus, has a major role in seeking behaviors (Kessler et al. 2016). Again we did not observe genotype-dependent differences in striatal monoamines. Mice

Region	genotype	diet	(ng/mg)								
			NA	AD DOPAC	DA	5HIAA	5HT	HVA	HVA/DA	5HIAA/5HT	NA/DA
ST	WT	SC	6.23±5.82	42.70±8.75	194.43±66.63	44.88±13.31	35.48±8.60	5.58±6.36	0.04±0.04	1.39±0.74	0.05±0.06
		SC +CM	4.58±2.26	53.00±20.01	294.95±116.86	89.24±42.23	43.49±27.49	32.06±26.78	0.12±0.11	3.01±3.08	0.02±0.01
		SC+HF	13.05±12,17	71.09±21.30	295.88±44.98	72.23±18.43	42.06±12.71	13.34±13.38	0.04±0.04	2.04±1.48	0.04±0.03
	TS	SC	11.44±12.42	45.10±15.18	193.80±73.58	62.89±21.62	57.33±28.88	5.88±9.15	0.02±0.04	1.39±0.60	0.06±0.06
		SC +CM	8.35±5.90	55.16±15.48	249.90±71.72	67.36±20.20	48.35±8.32	33.29±28.60	0.16±0.14	1.45±0.63	0.04±0.04
		SC+HF	8.98±5.12	46.61±10.24	200.66±32.03	54.33±9.30	36.22±21.58	13.47±8.01	0.06±0.04	1.65±0.58	0.05±0.03

having access to energy-dense diets showed higher levels of DA and HVA, a metabolite of DA, in the striatum as compared to SC mice (Two-way ANOVA, diet effect, DA, $F(1,2) = 3.46, P<0.05$; HVA, $F(2,34) = 6.94$, $P<0.01$). Consequently, diet also changed the HVA/DA ratio (Two-way ANOVA, diet effect, $F(2,34) = 6.15$, $P<0.01$; Table 4). These changes were detected in both WT and Ts65Dn mice, with no differences between genotypes.

Table 4. Tissue levels of monoamines in the striatum of wild-type (WT) and Ts65Dn (TS) mice. NA: noradrenaline; AD: adrenaline; DA: dopamine, DOPAC: 3,4-Dihydroxyphenylacetic acid, a metabolite of DA; 5HT: serotonin; HVA: Homovanillic acid, a metabolite of DA; 5HIIA: 5-Hydroxyindoleacetic acid, a metabolite of 5HT.The ratios between the neurotransmitters and their metabolites are also presented. Data are presented as mean ± SEM, of ng/mg total tissue. WT: SC (n = 6), SC + CM (n = 6), SC + HF (n = 5), TS: SC (n =4), SC + CM (n = 6), SC + HF (n = 5).

Prefrontal cortex

The prefrontal cortex (PFC) is a key region for executive and reward processes (Ballard et al. 2011). Altered monoaminergic input and/or alteration in the monoamine receptors in the PFC could lead to disinhibited eating associated to executive dysfunction and thus to obesity (Cohen et al. 2011).

A genotype x diet interaction was observed in PFC for dopamine (DA) levels (Two-way ANOVA, diet x genotype interaction, $F(2,33) = 5.02$, $P<0.05$). In non-obesogenic conditions, Ts65Dn mice had lower DA concentration than WT mice (Bonferroni as a Post-hoc $P<0.01$, Table 5). In SC+CM group, the DA levels in PFC were higher in Ts65Dn mice (Bonferroni as a Post-hoc $P<0.01$), whereas in SC+HF group were lower compared to their respective WT mice (Bonferroni as a Post-hoc $P<0.05$). As a consequence of the changes in DA in PFC, the NA/DA ratio, a measure of DA turnover, also showed a significant diet x genotype interaction (diet x genotype interaction, $F(2,33) = 7.55$, $P<0.01$, Table 5). In non-obesogenic conditions, Ts65Dn mice showed a significant higher NA/DA ratio than WT mice (Bonferroni as a Post-hoc $P<0.05$). A similar increase was observed in SC+HF Ts65Dn mice (Bonferroni as a Post-hoc $P<0.01$). On the contrary, in SC+CM group, the NA/DA ratio was lower in Ts65Dn than in WT mice (Bonferroni as a Post-hoc $P<0.05$).

Regarding serotonin and its metabolites, mice having access to energy-dense food showed lower concentrations of HIAA as compared to SC mice (Two-way ANOVA, diet effect, $F(1,33) = 4.07$, $P<0.05$), but not significant changes in serotonin (5HT) were observed. This change was independent of the genotype.

Region	genotype	diet	(ng/mg)								
			NA	AD DOPAC	DA	5HIAA	5HT	HVA	HVA/DA	5HIAA/5HT	NA/DA
PFC	WT	SC	40.10±18.41	6.94±4.65	6.2±1,41	37.33±9.45	81.78±33.13	0.00	0.00	0.56±0.36	7.70±6.47
		SC +CM	54.29±14.12	6.38±3.88	2.30±0.99	27.91±6.97	96.15±41,92	16.59±19.83	6.20±8.09	0.33±0.15	29.97±14.63
		SC+HF	35.10±11.25	3.55±2.11	4.82±1.21	29.09±8.67	61.74±20.33	0.00	0.00	0.56±0.38	7.84±3.79
	TS	SC	43.76±17,28	5.23±2.80	2.27 ±0.56	36.98±10.05	63.03±19.54	0.00	0.00	0.65±029	24.52±21.15
		SC +CM	34.70±15,50	6.02±2.89	5.37±2.38	29.88±15.45	70.01±26.61	16.78±21,50	2.96±5.46	0.53±0.43	7.52±4.67
		SC+HF	36.05±5.93	5.03±1.88	1.68±1.36	22.03±4.19	55.45±15.77	0.00	0.00	0.41±0.09	36.97±28.80

Table 5. Tissue levels of monoamines in the prefrontal cortex of wild-type (WT) and Ts65Dn (TS) mice. NA: noradrenaline; values for AD: adrenaline and DOPAC: 3,4-Dihydroxyphenylacetic acid, a metabolite of the neurotransmitter dopamine (DA) were given together, since 2 species could not be quantified separately due to similar elution times; 5HT: serotonin; HVA: Homovanillic acid, a metabolite of DA; 5HIIA: 5-Hydroxyindoleacetic acid, a metabolite of 5HT. The ratios between the neurotransmitters and their metabolites are also presented. Data are presented as mean ± SEM, of ng/mg total tissue. WT: SC (n = 5), SC + CM (n = 6), SC + HF (n = 5), TS: SC (n = 5) SC + CM (n = 6), SC + HF (n = 5).

4.3 Principal component analysis and correlation of the monoaminergic changes with behavioral variables

The monoamine measurements in the brain regions related to feeding behaviors did not reveal significant differences between genotypes besides from the lower dopamine levels in PFC of Ts65Dn mice. However, it is well known that the simplification of the variables of the analysis in a complex system reduces the chances to capture existing differences (Catuara-Solarz et al. 2015). For this reason, and taking advantage of the physical and behavioral data, we had from the same mice used for monoamine quantification, we performed principal component analysis to identify the relationship between the obesity phenotypes and the neurochemical changes upon energy-dense food free access in wild-type and Ts65Dn mice.

Principal component analysis (PCA) using behavioral data

We performed a principal component analysis (PCA) using the neurochemical data and behavioral variables that defined the obesity phenotypes to explore what were the variables explaining most of the variance between animals. The first principal component (PC1) explained 32.22% of the variance and was separated mainly the diet. “SC group” of both genotypes had negative values whereas free choice energy-dense foods groups showed positive values, having the “SC+HF group” the most extreme

phenotypes. On the other hand, wild-type and Ts65Dn mice were mainly separated by the second principal component (Figure 25).

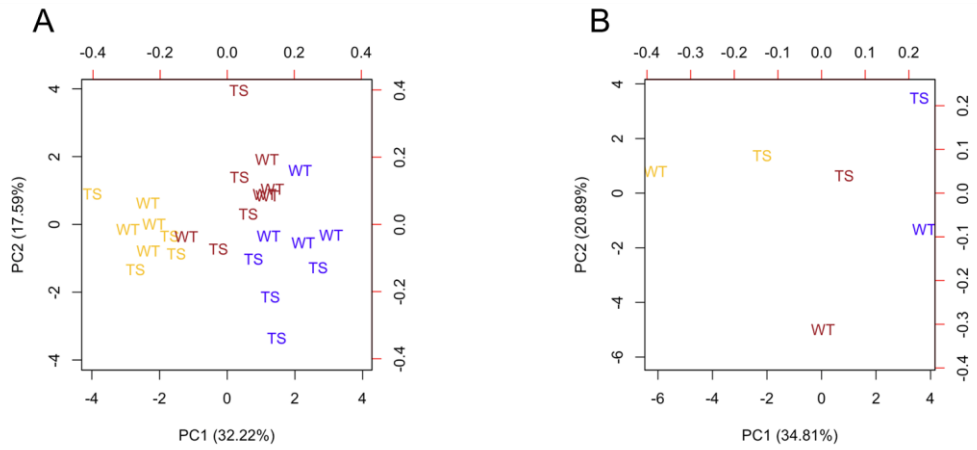

Figure 25. Physical, behavioral and neurochemical data are mainly different between diets, but also explain difference associated with the trisomy. (A) Principal component analysis (PCA) using as variables physical, behavioral and neurochemical data of all conditions. (B) Same as (A) but using the mean value of each group of animals instead of the individual values. WT = wild-type; TS = Ts65Dn. SC mice in yellow, SC+CM mice in brown, SC+ HF mice in blue.

When dissecting the differences between both genotypes upon different diet conditions, in non-obesogenic conditions (SC), a clear separation of genotypes was observed by the PC1 (Figure 26A), contributed by the serotonin, noradrenaline and dopamine content of different brain areas and behavioral variables related to flexibility (energy intake upon starvation and energy intake of adulterated chow during the quinine adulteration; Figure 26B).

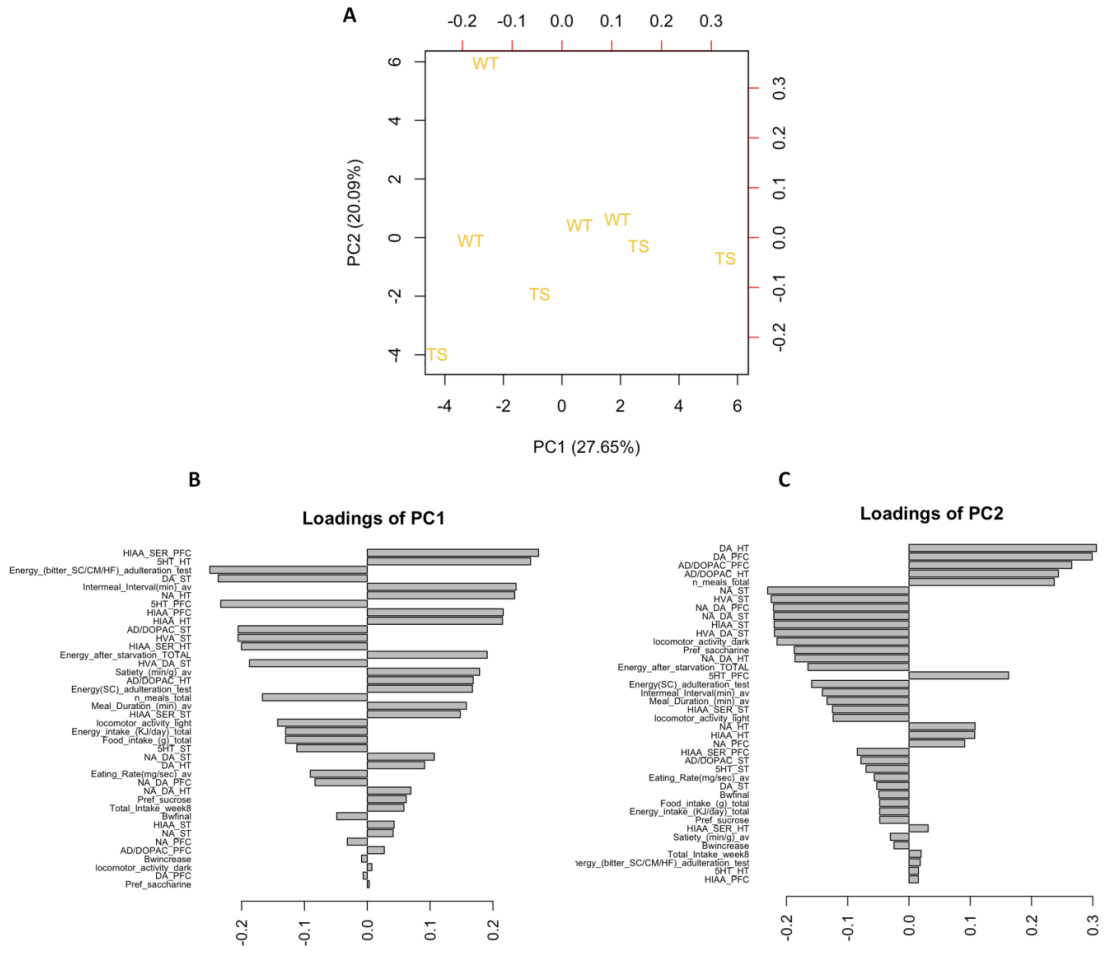

Figure 26. Physical, behavioral and neurochemical data are able to explain difference between wild-type and Ts65Dn mice in SC condition. (A) Principal component analysis (PCA) with physical, behavioral and neurochemical data. WT = wild-type; TS = Ts65Dn. (B) Loading for principal component 1. (C) Loadings for principal component 2.

Upon long-term access to CM diet, the genotypes separated along the PC1 (Figure 27A) mainly contributed by the monoaminergic profile in the hypothalamus and behavioral parameters related to satiety (intermeal interval, satiety ratio, number of meals (Figure 27B).

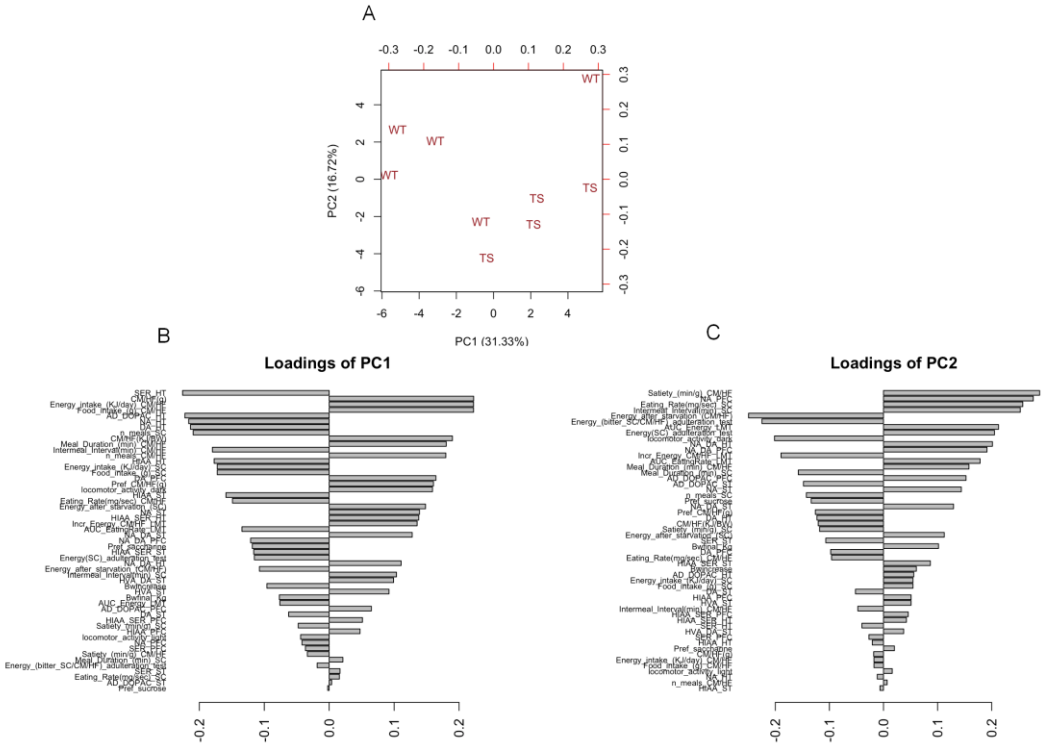

Figure 27. Physical, behavioral and neurochemical data are able to explain difference between wild-type and Ts65Dn mice in the SC+CM condition. Principal component analysis (PCA) using as variables behavioral and neurochemical data of SC +CM mice. (A) Principal component analysis (PCA) with physical, behavioral and neurochemical data. WT = wild-type; TS = Ts65Dn. (B) Loading for principal component 1. (C) Loadings for principal component 2.

Finally, in SC+HF group, genotypes were separated by a principal component (Figure 28A) contributed by dopamine levels in prefrontal cortex, and behavioral parameters such as food intake during the quinine adulteration test (Figure 28B).

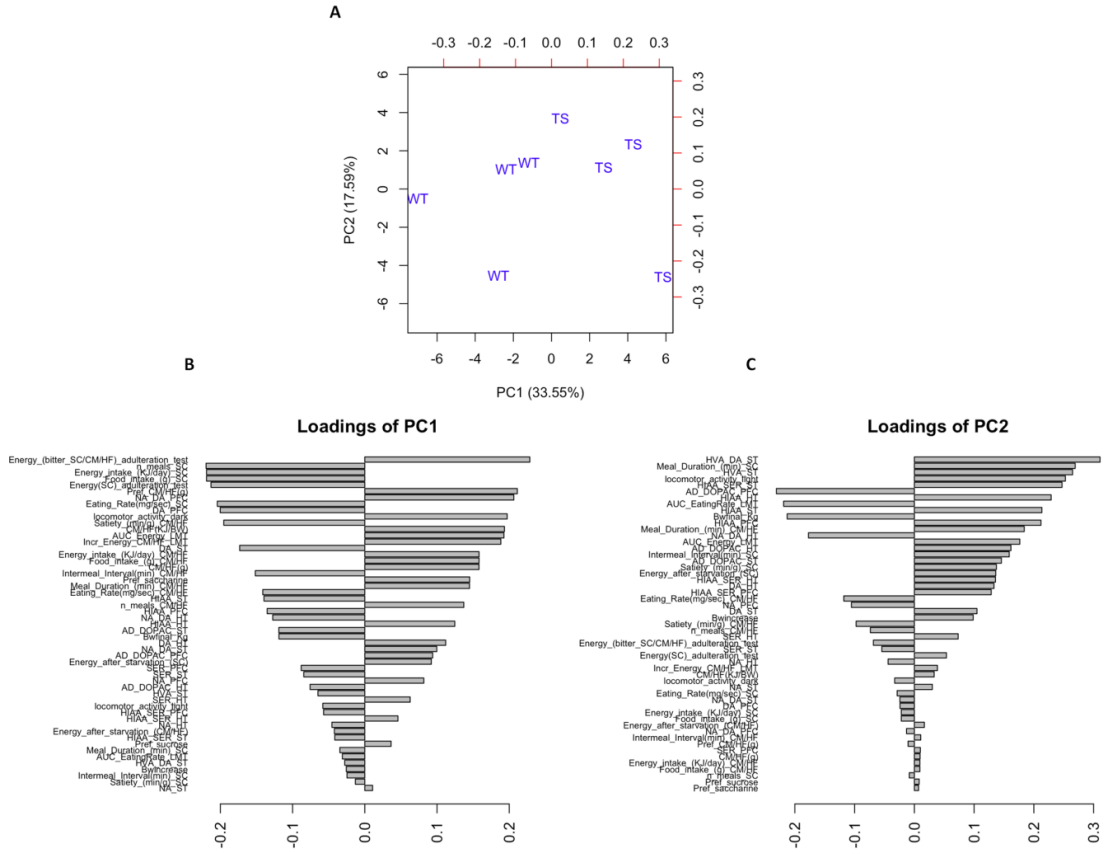

Figure 28. Physical, behavioral and neurochemical data are able to explain difference between wild-type and Ts65Dn mice in the SC+HF condition. Principal component analysis (PCA) using as variables behavioral and neurochemical data of SC +HF mice. (A) Principal component analysis (PCA) with physical, behavioral and neurochemical data. WT = wild-type; TS = Ts65Dn. (B) Loading for principal component 1. (C) Loadings for principal component 2.

Correlations of behavioral variables with neurochemical measurements

The most informative variables were selected from the PCA analysis. Then, we performed a correlation matrix that revealed different phenotypic sets strongly correlated with monoamine composition in the three different brain regions in all our samples or in a cluster separated by diet. Here, we show for each group, some selected examples for their biological and statistical significance (p-value < 0 upon z fisher transformation).

Our matrix of correlating variables revealed that in SC group the energy intake of adulterated SC in the quinine adulteration test strongly correlated with body weight increase (Spearman's rho of 0.6, Figure 29A) which suggest that behavioral inflexibility might account for body weight gain. Among the different variables strongly correlating in mice with free access to energy-dense diets, we observed that those mice that had higher preference for CM and HF had also higher intake of those diets during quinine

adulteration test (Spearman's rho of 0.9, Figure 29B). When we studied the free-choice groups separately, we observed a strong negative correlation between the CM intake (g) and the serotonin levels in hypothalamus (Spearman's rho of - 0.9) in SC+CM group which may be related to satiety (Figure 29C). However, in SC+HF mice, we found several behavioral variables related to inflexibility strongly correlated with dopamine levels. For example, low dopamine levels in prefrontal cortex correlated with the intake of bitter HF during the quinine adulteration test (Spearman's rho of and - 0.8, Figure 29D).

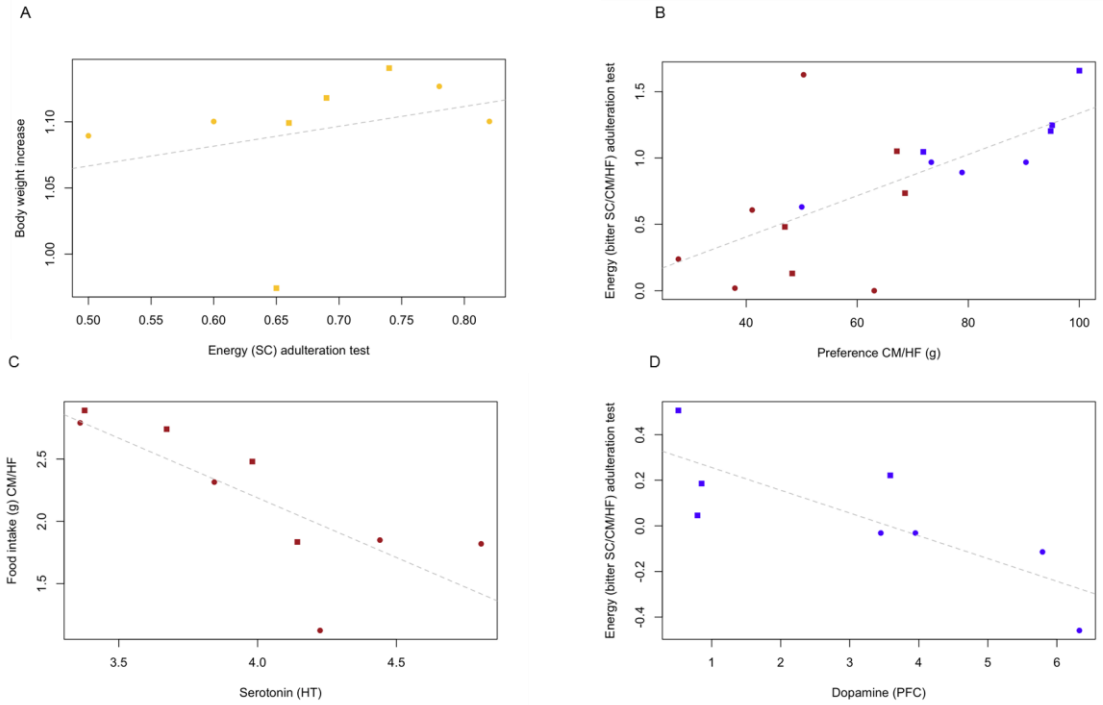

Figure 29. Some behavioral variables strongly correlated with neurochemical measurements showing a diet dependency. (A) Dot plot showing the relationship between energy intake of adulterated SC in the quinine adulteration test on the x-axis and the body weight increase on the y-axis in SC group. (B) Dot plot showing the relationship between Preference CM/HF (g) on the x-axis and Energy (bitter SC/CM/HF) adulteration test on the y-axis in free-choice mice. (C) Dot plot showing the relationship between serotonin levels in the hypothalamus on the x-axis and food intake on the y-axis in SC+CM group. (D) Dot plot showing the relationship between bitter HF consumed during the quinine adulteration test on the x-axis and dopamine levels in PFC on the y-axis in SC+HF. SC group in yellow, SC+CM group in brown and SC+HF group in blue. WT mice are depicted as dots and Ts65Dn mice as squares.

4.4 Glucose homeostasis and pancreatic function in Ts65Dn mice

Besides the behavioral component, a disrupted control of food intake through metabolic mechanisms, such as glucose homeostasis or pancreatic pathology could also play a role in the higher prevalence of obesity in DS population (Fonseca et al. 2005). In this part

of the Thesis, we explored the glucose homeostasis and pancreatic phenotypes in non-obesogenic conditions (standard chow, SC).

Glucose homeostasis and insulin levels

Circulating blood glucose levels were higher in Ts65Dn than in wild-type mice (Ts65Dn vs. WT, Mann-Whitney-U-test, z = -3.15, P = 0.01, Figure 30A) after 5 hours of fasting, while plasma insulin levels were non-significant reduced (Figure 30B). We used the oral glucose tolerance test (OGTT), a widely used procedure to classify carbohydrate tolerance measuring plasma glucose and insulin responses upon a glucose load. OGTT revealed that Ts65Dn mice showed high glucose levels during longer time upon glucose load, indicating impaired recovery of basal glycaemia (Repeated measures ANOVA, $F(1,11) = 7.25$; $P<0.05$; Bonferroni as a post-hoc at 60 and 90 min after glucose administration, $P<0.05$ Figure 30C). Concomitantly, insulin levels in Ts65Dn mice were markedly lower compared to WT mice (Repeated measures ANOVA, $F(1,8) = 9.24$; $P<0.05$; Bonferroni as a post-hoc; Figure 30D). These results suggested an impaired regulation of glucose homeostasis probably due to a dysfunction at the pancreas level.

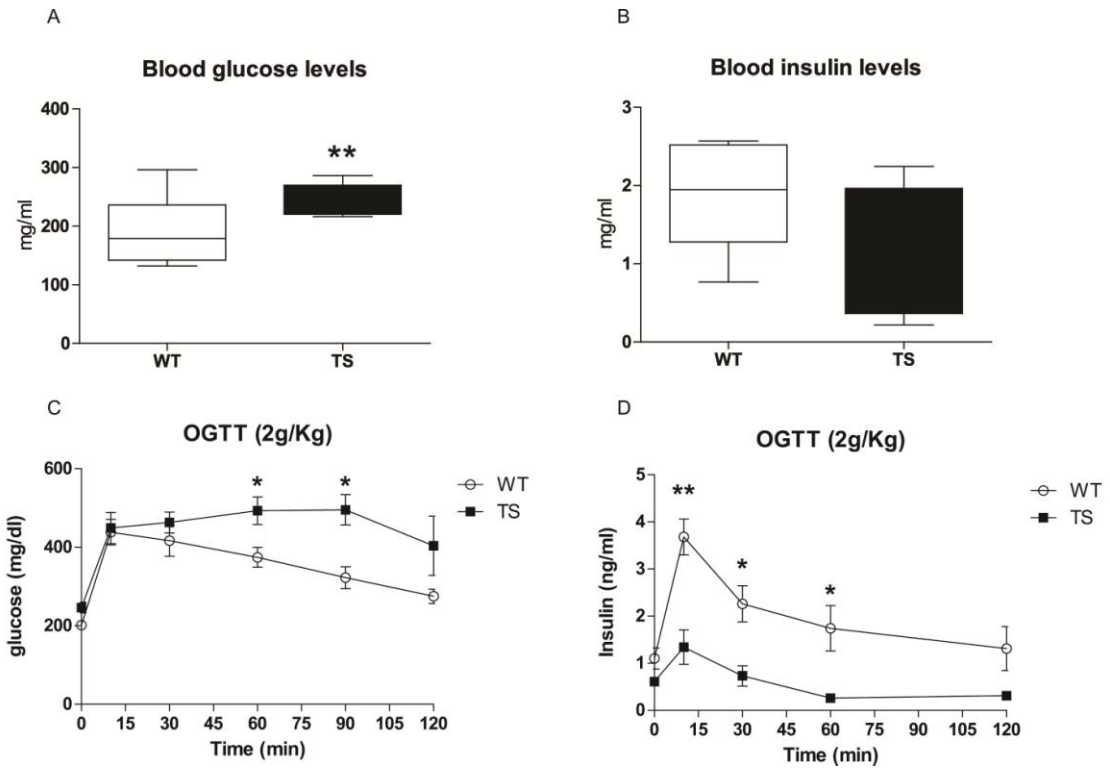

Figure 30. Glucose and insulin levels in starving conditions and upon a glucose load. (A) Blood glucose and (B) insulin concentrations in fasted wild-type (WT) and Ts65Dn (TS) mice at 5 months old. (C) Glucose and (D) insulin levels during oral glucose tolerance test (OGTT). Data are shown as the mean ± SEM. (A-B) WT = 11, TS = 9. Mann-Whitney-U-test for independent samples ** P<0.01. (C-D) WT = 5, TS = 5; ANOVA Repeated measures, Bonferroni as a post-hoc; * P<0.05; P **<0.01.

Morphological and functional studies of islets of Langerhans

To analyze pancreatic function, we focused on the morphology and functionality of pancreatic beta cells involved in insulin production. Immunostaining for insulin revealed less insulin-positive cells in Ts65Dn than in WT pancreas (Figure 31A). The lower insulin content in Ts65Dn pancreases along with the lower plasma levels could be a consequence of a secretory problem in the islets of Langerhans. To confirm that possibility, we evaluated glucose-induced insulin secretion in islets isolated from WT and Ts65Dn mice under basal (2.8 mM glucose) and stimulating conditions (16.7 mM glucose). Basal insulin secretion was significantly decreased in Ts65Dn islets compared to WT (Mann-Whitney-U-test, z = -2.65, P< 0.01; Figure 31B). In stimulating conditions, incubating with 16.7mM of glucose, islets of both genotypes increased insulin secretion. However, although non-significant, the glucose-induced insulin secretion in Ts65Dn was lower compared to WT islets (Figure 31B).

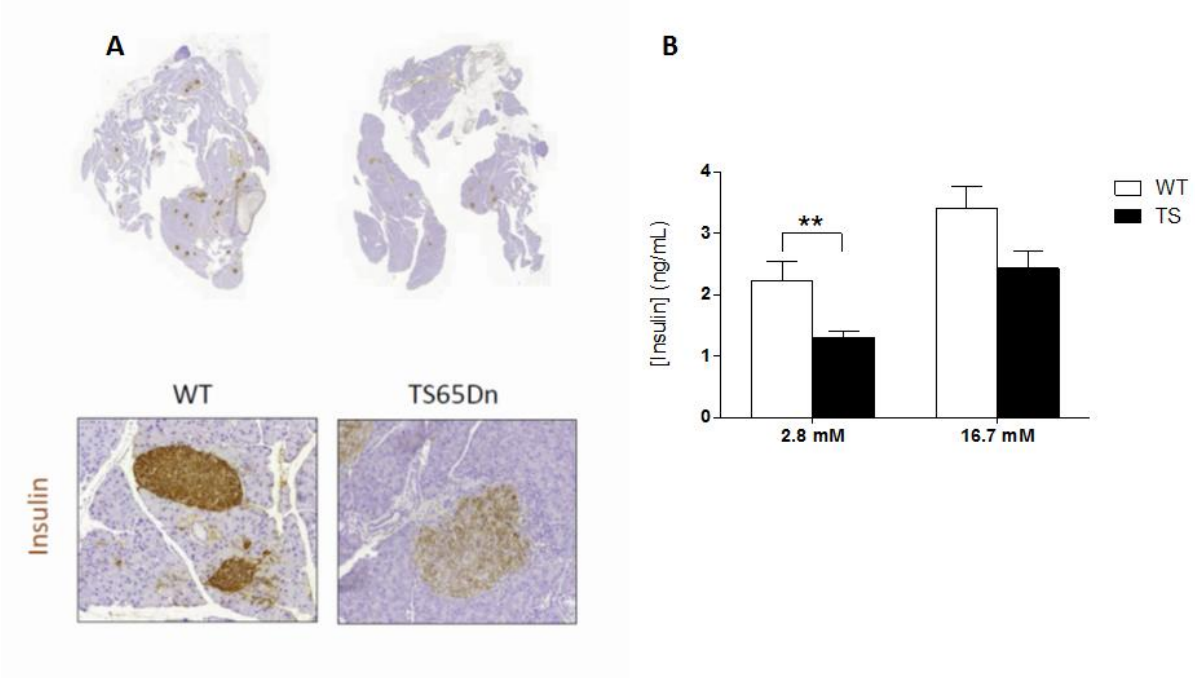

Figure 31. Representative images of sections of pancreases and *in vivo* insulin secretion in 5 months-old wild-type (WT) and Ts65Dn (TS) mice. (A) Representative images of sections of pancreases immunostained against insulin. The lower panel shows magnifications of insulin islets. (B) For the in vitro insulin secretion study, pools of 50 islets from 3 experimental replicates were used. Insulin secretion

was measured under basal (2.8 mM glucose) and stimulating conditions (16.7mM glucose). Data are shown as the mean ± SEM. Mann-Whitney-U-test for independent samples **P<0.01.

4.5 Role of DYRK1A in the Down syndrome obesity phenotypes

In the first part of this Thesis we showed that Ts65Dn recapitulates obesity-related DS phenotypes, such as high adiposity, feeding behavior perturbations and higher intake of energy-dense foods. However, using a trisomic model does not allow to establishing the genetic dependence of these phenotypes.

Among the hundreds of genes triplicated in DS individuals and Ts65Dn mice, the tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A) is one of the best candidates to explain brain alterations and cognitive impairment. Moreover, previous studies have reported its implication in obesity-related phenotypes. In mice, its overexpression resulted in reduced fat mass along with reduced expression of adipogenic proteins, including peroxisome proliferator-activated receptor γ (Song et al. 2015) and increased pancreatic β-mass leading to improved insulin sensitivity (Rachdi et al. 2014b). Based on previous data showing the *Dyrk1A* dosage sensitive influence on the nigrostriatal dopaminergic system (Martinez de Lagrán et al. 2007), we propose that *Dyrk1A* overexpression could affect the sensitivity to natural rewards that could lead to compensatory overeating. Also, that the implication of *Dyrk1A* in craniofacial development (Arron et al. 2006) could be involved in the altered feeding observed in Ts65Dn mice (see above).

We have used mBACTgDyrk1A mice (Guedj et al. 2012) to explore whether the sole overexpression of *Dyrk1A* is sufficient to recapitulate the feeding behavior perturbations associated to the trisomy and the compulsive-like phenotypes in an obesogenic environment

4.5.1 Somatometric characteristics and feeding behavior in mBACTgDyrk1A mice

No differences were detected in body weight, nor body length of mBACTgDyrk1A (TG) mice fed with SC compared to their WT littermates (Figure 32A-B).

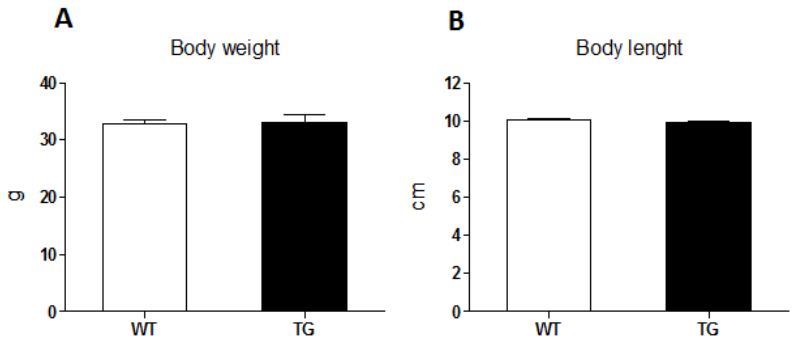

Figure 32. Somatometric characteristics of mBACTgDyrk1A mice. (A) Body weight (grams), (B) body length (cm) in mice fed with balanced diet (standard chow) in mBACTgDyrk1A (TG) and wild-type (WT). Data expressed as mean ± SEM. WT (n = 13), TG (n = 12). Mann-Whitney-U-test for independent samples.

Meal pattern analysis

Meal pattern analysis upon SC consumption revealed no differences in average meal duration, eating rate, meal number or intermeal interval (Figure 33A-D). No differences in total food intake were observed (Figure 33E). Drinking behavior was also similar between genotypes (not shown). These results indicate that *Dyrk1A* overexpression does not recapitulate Ts65Dn feeding behavior disturbances.

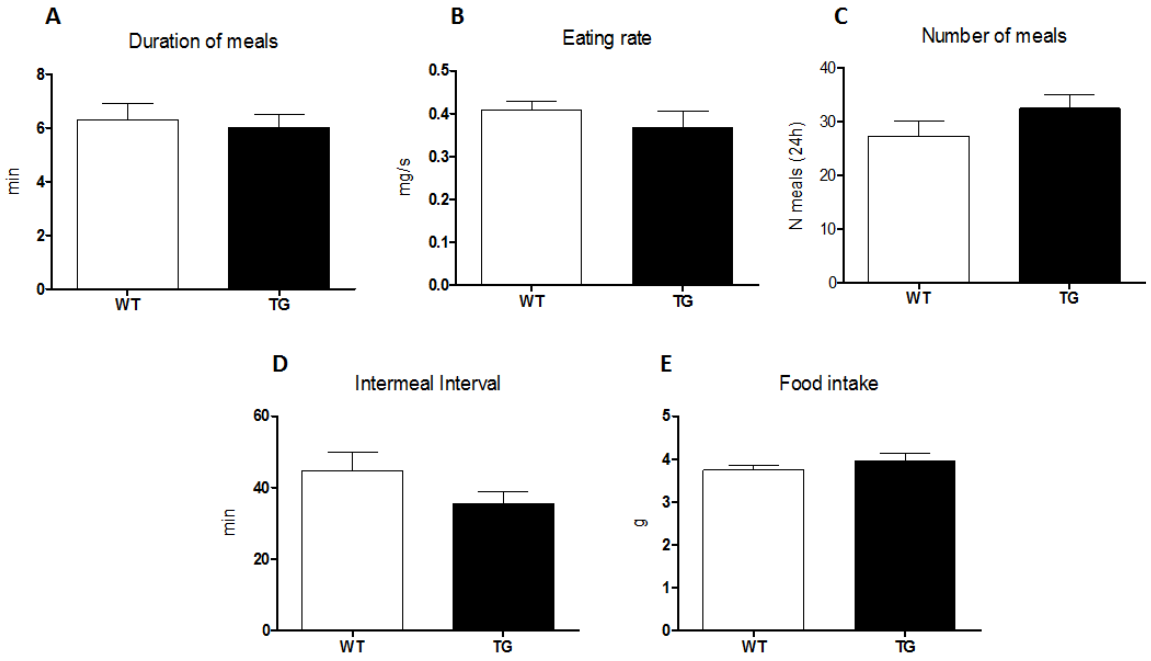

Figure 33. Meal pattern analysis of mBACTgDyrk1A and wild-type (WT) mice fed with standard chow (SC). (A) Bar plot shows the average duration (min), (B) eating rate (mg/s), (C) the number of meals, (D) the average intermeal duration (min) and (E) the total SC consumed (in grams) in 24 hours. Wildtype (WT) n = 5; mBACTgDyrk1A (TG) n = 4. Data are expressed as mean ± SEM. Mann-Whitney-U test for independent samples.

4.5.2 Changes in feeding behavior upon exposure to free access to energy-dense food in mBACTgDyrk1A mice

To determine if overexpression of *Dyrk1A* has an effect on behavioral phenotypes driving overeating, we used the same experimental diet-induced obesity model based on free choice of SC, and CM or HF used in Ts65Dn mice. After long-term access to CM or HF we also evaluated the appearance of compulsive and inflexible feeding responses in stand-alone laboratory tests (starvation, limited access to energy-dense food, quinine adulteration and preference for sweet solutions) to determine the *in vivo* role of *Dyrk1A* on those traits.

Body weight gain

Upon free choice access to energy-dense food, two-way ANOVA revealed a significant effect of genotype and diet on body weight gain (genotype effect: Two-way ANOVA F (1,42) = 14.47, P<0.001, and diet effect: Two-way ANOVA, F(2,48) = 101.68; P<0,001; Figure 34A). We also observed a non-significant trend for a diet x genotype interaction (Two-way ANOVA F (2,47) = 3.154, P = 0.053). In WT mice, free access to both CM and HF lead to significant body weight increase compared to mice fed with SC. Conversely, mBACTgDyrk1A mice showed a slight, though not significant, body weight reduction upon free access to CM, but upon free access to HF, transgenic mice gained significantly more weight than mice receiving SC only. In SC+HF mice, although the percentage of increase of body weight was not different between genotypes, WT mice gained slightly more weight than mBACTgDyrk1A (34% and 26% body weight increase, respectively, Figure 34B).

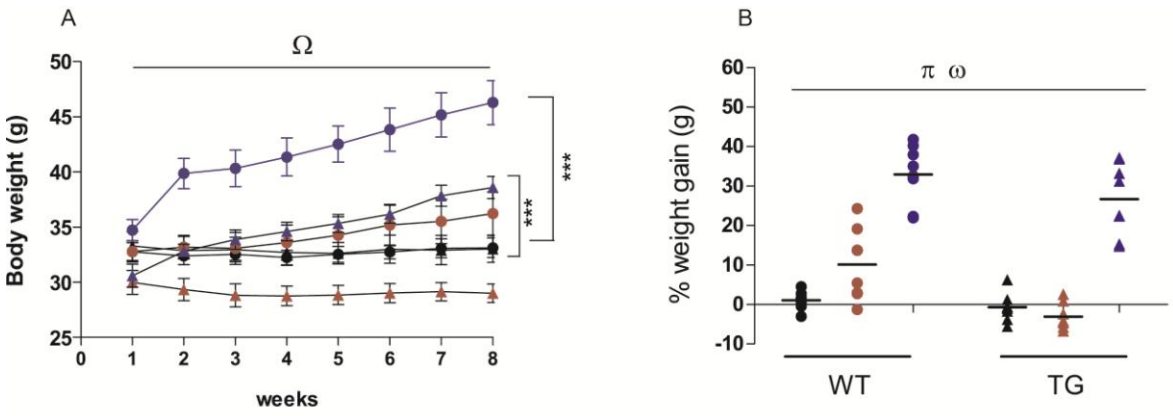

Figure 34. Body weight increase upon energy-dense diets in wild-type (WT) and mBACTgDyrk1A (TG) mice. (A) Time course of body weight increase along the 8 weeks of exposure to energy-dense food. (B) The plot represents the percentage of body weight gain at the end of the experiment. Genotypes

are represented as dots (WT) and triangles (TG), diet conditions are shown as colors (SC = black; SC+CM = brown and SC+HF = blue). (A) Depicts the mean of the group whereas in (B) each dot/triangle represents one individual. SC group: WT n = 8, TG n = 8; SC+CM group: WT n = 8, TG n = 8; SC + HF group n = 8, TG n = 8. Repeated measures, Ω for genotype x diet interaction, Bonferroni as a post-hoc *** P<0.001; Two-way ANOVA genotype effect π P<0.001; diet effect ω P<0.001.

Feeding behavior and locomotor activity changes

At the end of the obesity development phase, energy intake increased in mice receiving SC+CM and SC+HF compared to SC mice (diet effect ANOVA $F(1,48) = 58.65$, $P<0.001$; Bonferroni as post-hoc: $P<0.001$, Figure 35A) regardless of the genotype. Two-way ANOVA revealed a significant genotype effect, with mBACTgDyrk1A mice exposed to CM showing the highest energy intake (ANOVA $F(2,48) = 5.65$, $P<0.01$; Bonferroni as post-hoc: $P<0.001$, Figure 35A). The increased energy intake was not only due to the higher energy content of both CM and HF (Table 2), but to the increase in absolute food intake (paired Mann-Whitney-U-test, CM vs. SC, $z = -2.38$, $P<0.01$; paired Mann-Whitney-U-test, HF vs. SC $z = -2.51$, $P<0.01$, in WT; paired Mann-Whitney-U-test CM vs. SC, $z = -2.52$, $P<0.01$; paired Mann-Whitney-U-test, HF vs. SC, $z = -5.21$, $P<0.01$ in TG; Figure 35B). mBACTgDyrk1A mice had higher net intake of CM than WT mice (CM intake, TG vs. WT, Mann-Whitney-U-test, $z = -2.486$, $P<0.05$). Interestingly, while mBACTgDyrk1A consumed more KJ of CM than WT mice (TG vs. WT Mann-Whitney-U-test, $z = -2.84$, $P<0.01$), no differences were detected in the KJ of HF consumed (Figure 35C). Regarding energy expenditure, no changes in locomotor activity between genotypes were found (Figure 35D).

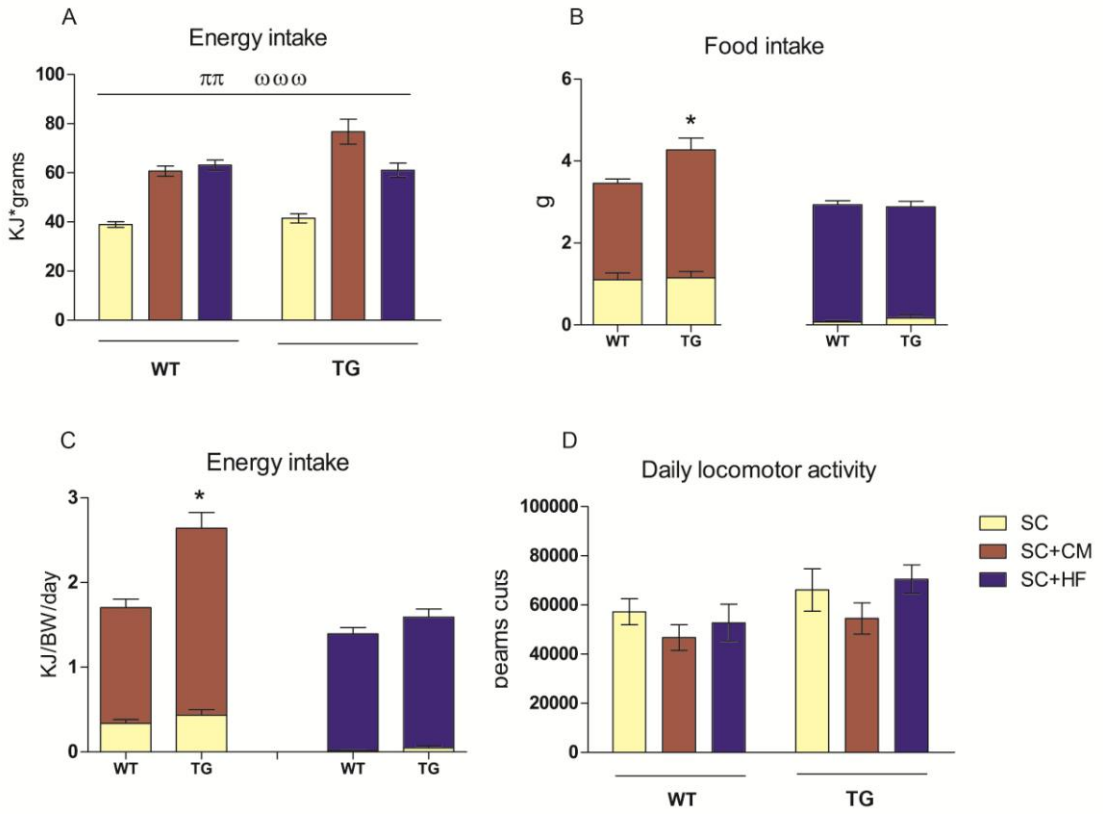

Figure 35. Energy and net intake of energy-dense foods in wild-type (WT) and mBACTgDyrk1A (TG) mice. (A) Bar plots represent the gross daily energy intake (KJ) of the SC (yellow bars), SC+CM (brown bars) or SC+HF (blue bars) groups of mice. (B) Bar plots show separately the daily food intake (g) of SC (yellow segment of the bar) and CM (brown segment of the bar) in SC+CM mice of either genotype (left panel) and of SC (yellow segment of the bar) and HF (blue segment of the bar) in SC+HF mice of either genotype. (C) Bar plots show separately the daily intake (KJ/g body weight (BW) of SC (yellow segment of the bar) and CM (brown segment of the bar) in SC+CM mice of either genotype (left panel) and of SC (yellow segment of the bar) and HF (blue segment of the bar) in SC+CM mice of either genotype. (D) No differences in activity were found between genotypes. Data are expressed as mean ± SEM. SC group: WT n = 8, TG n = 8; SC + CM group: WT n = 8, TG n = 7; SC + HF group n = 8, TG n = 8. Two-way Repeated measures ANOVA genotype effect ππ $P<0.01$; diet effect ωωω $P<0.001$, Bonferroni as a post-hoc ***$P<0.001$. (B, C) Mann-Whitney-U-test for TG vs. WT comparisons, * $P<0.05$, ** $P<0.01$.

In both genotypes, we detected preference for the energy-dense food respect to SC. However, mBACTgDyrk1A mice preferred CM more than WT (Figure 36) while the preference for HF (Figure 36B-D) was similar.

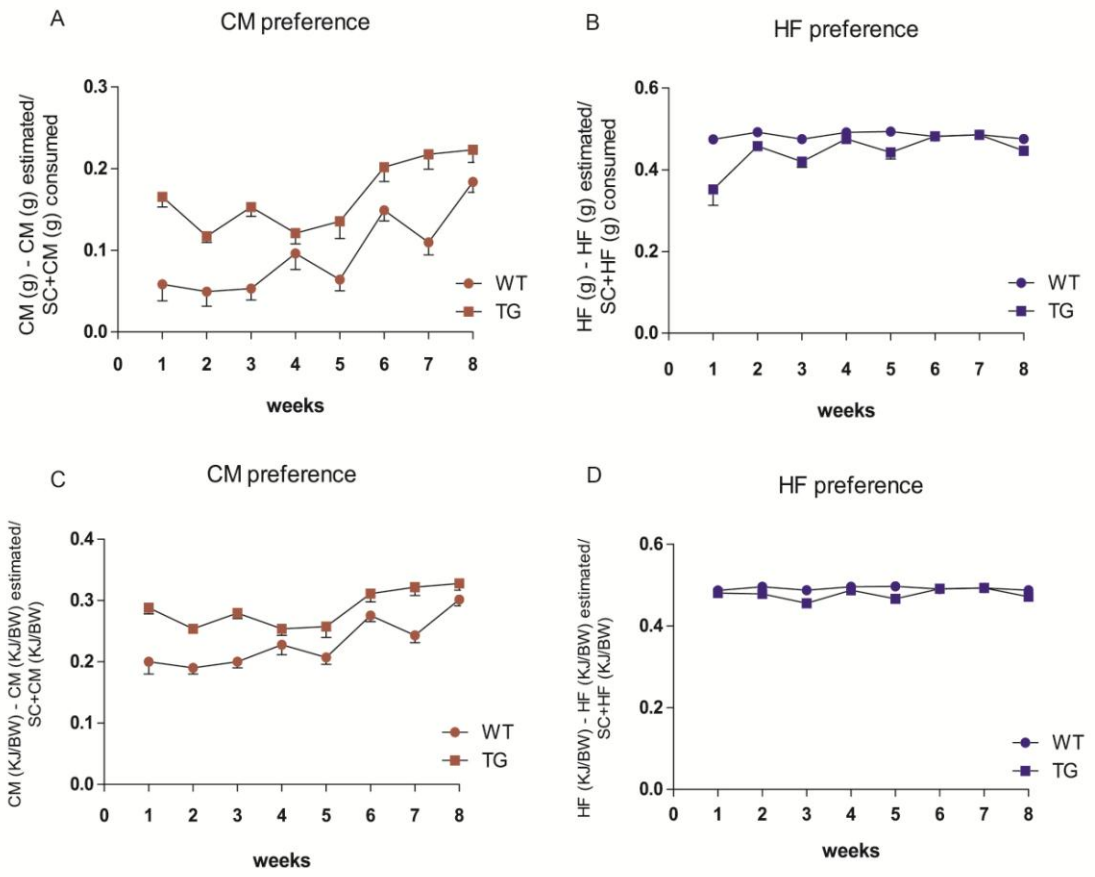

Figure 36. Preference for energy-dense foods in wild-type (WT) and mBACTgDyrk1A (TG) mice. (A) Time course of the CM and (B) HF preference in grams across the experimental time. (C) Time course of the preference for CM and (D) HF relative to the energy intake (KJ) per grams of body weight. Data are expressed as mean ± SEM. in (A-C) WT mice are depicted as brown dots and mBACTgDyrk1A mice as brown squares. In (B-D) WT mice are depicted as blue dots and mBACTgDyrk1A mice as blue squares. SC + CM group: WT n = 8, TG n = 8; SC + HF group WT n = 8, TG n = 8. ANOVA Repeated measures.

Meal pattern analysis

As occurred in Ts65Dn mice, free choice access to CM lead to changes of feeding behavior in both genotypes (Figure 37A-E). SC meals were longer than CM meals (paired Mann-Whitney-U-test, average meal duration CM vs. SC, $z = -2.03$; $P<0.05$ in WT and $z = 4.71$; $P<0.05$ in TG, Figure 37A) and CM was consumed quicker than SC (paired Mann-Whitney-U-test, eating rate CM vs. SC, $z = -3,03$; $P<0.05$ in WT and $z = -5.66$; $P<0.05$ in TG, Figure 37B). The total number of meals and the time between meals was similar for SC and CM in both WT and mBACTgDyrk1A mice (Figure 37C-D). Even though the satiety ratio of SC was not different from CM in any genotype (Figure 37E), there was a trend for lower CM satiety only in mBACTgDyrk1A mice.

For this experiment, due to a breeding problem in our colony, the sample size of SC+CM group is small and we could not include a SC+HF group.

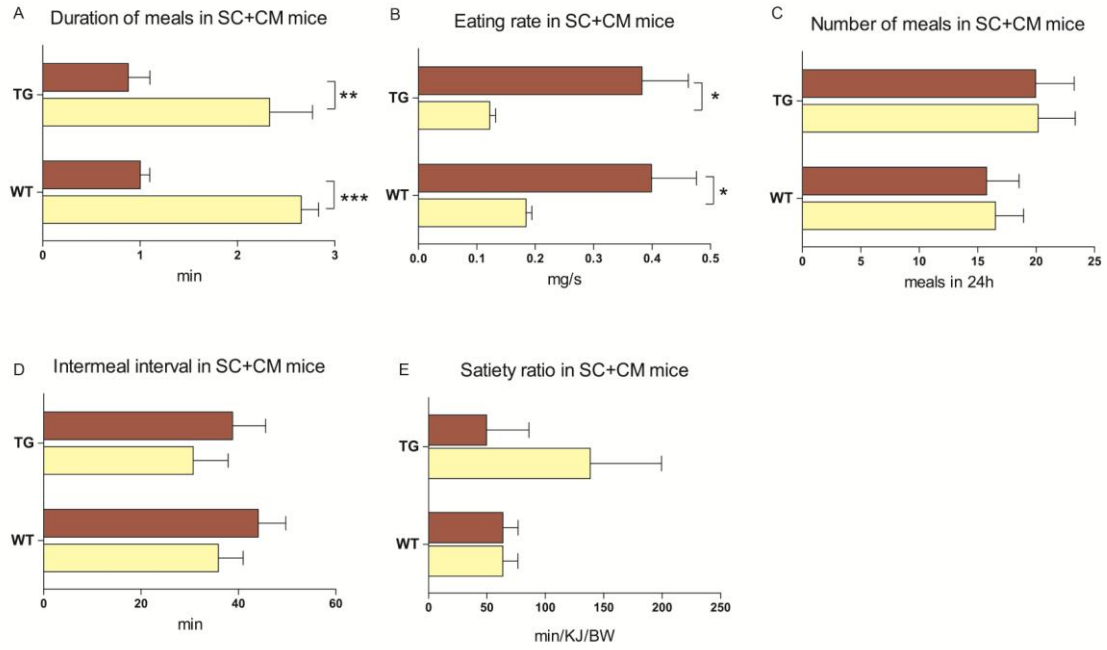

Figure 37. Meal pattern changes upon free access to CM in wild-type (WT) and mBACTgDyrk1A (TG) mice. Bar plots represent the meal patterns for each type of food for each experimental group: yellow (SC) and in brown (CM). The upper panel shows TG mice values, and lower panel results of WT. (A) The average duration (min) of meals. (B) The eating rate (mg/s). (C) The number of meals. (D) The intermeal interval (min). (E) Satiety ratio after correcting for body weight (BW) of the animal (g) for CM and SC (min/KJ/g). Data are expressed as mean ± SEM. WT = 5, TG = 4. Paired Mann-Whitney-U-test for within genotype comparisons (SC vs. CM) * P<0.05, ** P<0.01, *** P<0.001.

Circadian feeding pattern

Similar to what we did with Ts65Dn mice, we used Pergola (http://cbcrg.github.io/pergola) to visualize food intake and the temporal distribution of feeding events along time at the single mouse level. Figure 38 depicts a simplified output of 3 weeks. The 3 intervals depicted were selected as a representative scenario of the immediate (1st week), the middle time (4th week) and late changes (8th week) in circadian feeding activity. We have discussed above that feeding behavior is subjected to well-controlled daily oscillations with feeding bouts mainly accumulated over the dark (active) phase (see the SC group behavior Figure 38). However, energy-dense foods are usually also consumed during the light (inactive) phase. CM leads to a subtle increase of feeding events during the inactive phase in both genotypes from the first week of access and was maintained across the experimental time (Figure 38).

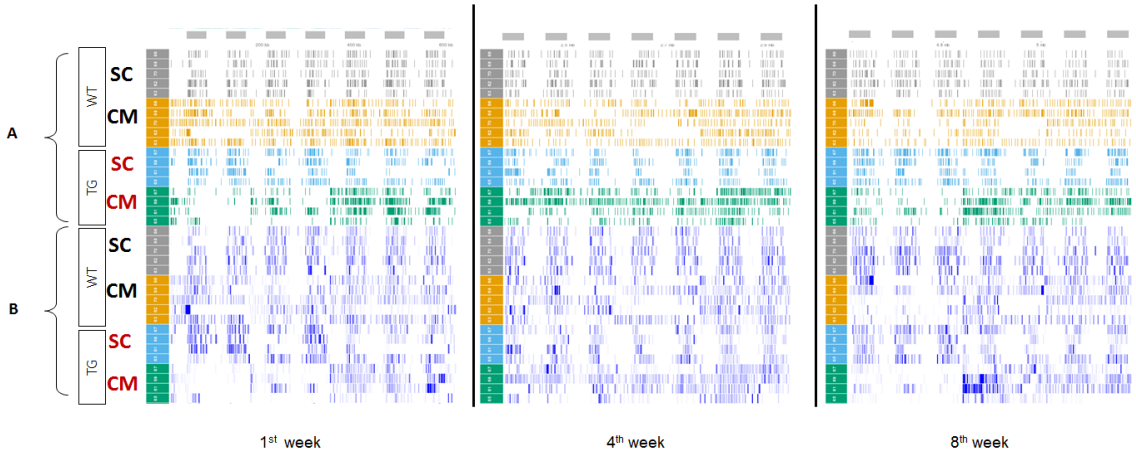

Figure 38. Longitudinal behavioral recordings from the 1st, 4th and 8th week of free access to CM in wild-type (WT) and mBACTgDyrk1A (TG) mice. Snapshots Pergola representations of SC+CM mice feeding bouts along time (WT n = 5, TG n = 4). Each row corresponds to one animal and food channel except the last track that represents light (white bar) and dark (grey bar) periods of the light/dark cycle. Metadata included in the figure are (i) identifiers for each mouse. SC + CM mice WT (grey for SC and orange for CM), TG (blue for SC and green for CM) (ii) Dark (active) periods (light-dark phase track) represented as grey rectangle on the top of the figure. (iii) The weeks of the longitudinal recordings are written on the bottom of the figure. (A) In the upper part of the figure, each bar represents a feeding bout and its size corresponds to meal duration. WT mice are depicted in the upper panel (gray SC channel and orange CM channel) and those corresponding to mBACTgDyrk1A (TG) mice in lower panel (blue SC and green CM). For the same mice, in (B) each line shows the accumulated intake (g) of each individual during a period of 30 minutes. The intensity of the blue indicates the value of the accumulated food intake (white= 0 grams, blue ≥ 0.5 grams).

To quantify this circadian disruption, we calculated the number of accumulated meals in 30 min windows for each week of the obesity development phase (Figure 39). The diet (SC/CM) and the phase (active/inactive) had a different effect on the number of meals in each genotype (Three-way ANOVA (genotype, diet and phase), $F(7,44) = 6.37$). During the active (dark) phase, the number of SC meals was significantly higher than the CM meals in WT mice (Bonferroni as a post-hoc, $P < 0.001$) whereas mBACTgDyrk1A showed equivalent number of SC and CM meals (Figure 39). Although non-significant, mBACTgDyrk1A showed an increased number of CM meals compared to WT (Bonferroni as a post-hoc, $P = 0.09$). During the inactive (light) phase, the number of CM meals was equivalent in both genotypes. However, WT showed similar number of SC and CM meals whilst mBACTgDyrk1A mice showed a non-significant higher number of CM meals (Bonferroni as a post-hoc, $P = 0.07$).

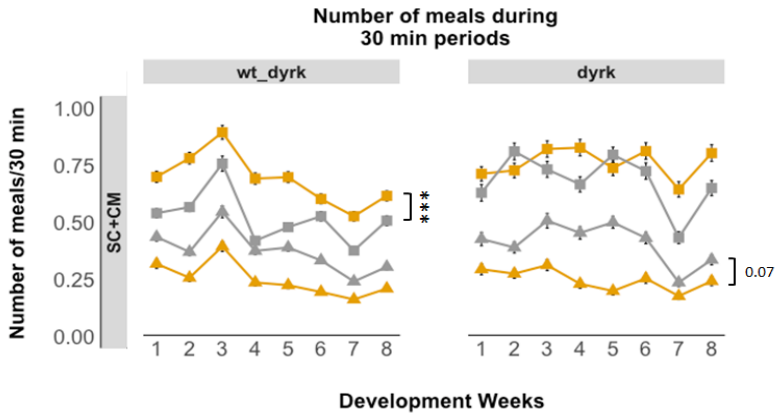

Figure 39. Quantitative measure of the circadian feeding activity for day and nights upon CM free access in wild-type (wt_dyrk) and mBACTgDyrk1A (dyrk) mice. The figure depicts the mean of the accumulated number of meals in time windows of 30 min across the whole experiment during the inactive (triangles) and during the active (squares) phases for WT and mBACTgDyrk1A (TG) mice. SC is represented in orange and CM in grey. Three-way ANOVA (diet x genotype x phase); Bonferroni as a post-hoc *** P< 0.01.

4.5.3 Testing phase

After 8 weeks of free access to CM/HF, mice underwent the same test battery as Ts65Dn mice to understand the contribution of the overexpression of *Dyrk1A* on compulsivity, inflexibility and reward.

Starvation

24h starvation lead to increased energy intake (KJ) upon refeeding in all mice compared to their energy in free feeding conditions (genotype x diet interaction, ANOVA F (2,42) = 6.43, P<0.01). In the SC group, upon refeeding mBACTgDyrk1A compensated more their energy intake than WT mice (TG vs. WT, Mann-Whitney-U-test, z = -2.63, P<0.001, Figure 40A).

In SC+CM mice the increase of SC intake upon refeeding was similar between genotypes, but WT mice tended to consume more SC whereas mBACTgDyrk1A tended to consume more CM (Figure 40B), although the difference did not reach statistical significance. In SC+HF mice, starvation preferentially increased HF intake in both genotypes (Figure 40C) but the increase was only significant in mBACTgDyrk1A mice (paired Mann-Whitney-U-test, SC vs. HF z = 0.40, P<0.01, in TG). Starvation led to a significant higher SC energy intake in WT mice (TG vs. WT, Mann-Whitney-U-test, z = -2.93, P<0.01).

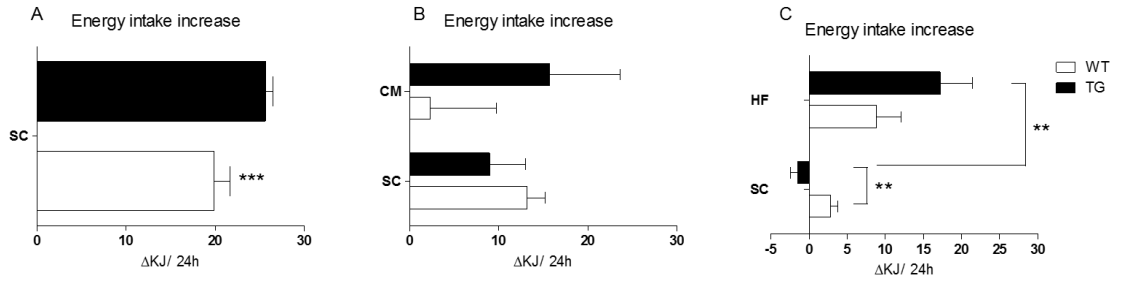

Figure 40. Energy intake upon 24 hours of starvation in wild-type (WT) and in mBACTgDyrk1A (TG) mice. Bar plots show the increase in energy intake calculated as the KJ consumed 24h after refeeding - KJ consumed in 24 hours in free feeding conditions. (A) Increase in daily energy upon refeeding in the SC group (B) the SC+CM group, and (C) the SC+HF mice. Data are expressed as mean ± SEM. SC group: WT = 8, TG = 8; SC + CM group: WT n = 8, TG n = 8; SC + HF group n = 8, TG n = 8. Mann-Whitney-U-test for genotype comparisons, Paired Mann-Whitney-U-test for within genotype comparisons (SC vs. CM and SC vs. HF), ** P<0.01; *** P<0.001.

Limited access to energy-dense food

Access to HF or CM diet was restricted to 1 hour during the light (resting) phase of the light-dark cycle (from 14:00 to 15:00h) for 3 consecutive days in overweight mice (SC+CM and SC+HF; Figure 41A). SC and water were provided unrestrictedly *ad libitum*.

During the periods of CM/HF restriction (Figure 41B), the SC energy intake (KJ/body weight) was increased as compared to the SC intake in *ad libitum* feeding condition (SC+CM, SC+HF; $F(1,27) = 224.716$; $P<0.001$). However, the SC energy intake during the periods of restriction of energy-dense foods was different between SC+CM and SC+HF groups (two-way ANOVA, diet effect, $F(1,30) = 57.50$; $P<0.001$), being higher in mice from SC+CM group. When we compared the SC intake during the periods of energy-dense foods restriction with the total energy intake calculated as the energy intake from SC, was lower (paired Mann-Whitney-U-test, $z = -2.66$; $P<0.01$ in WT; $z = -2.70$; $P<0.01$, in TG) in both groups. However, both genotypes of SC+CM group showed a tendency for increase SC intake during limited HF/CM access phase that may reflect energy compensation (Figure 41C).

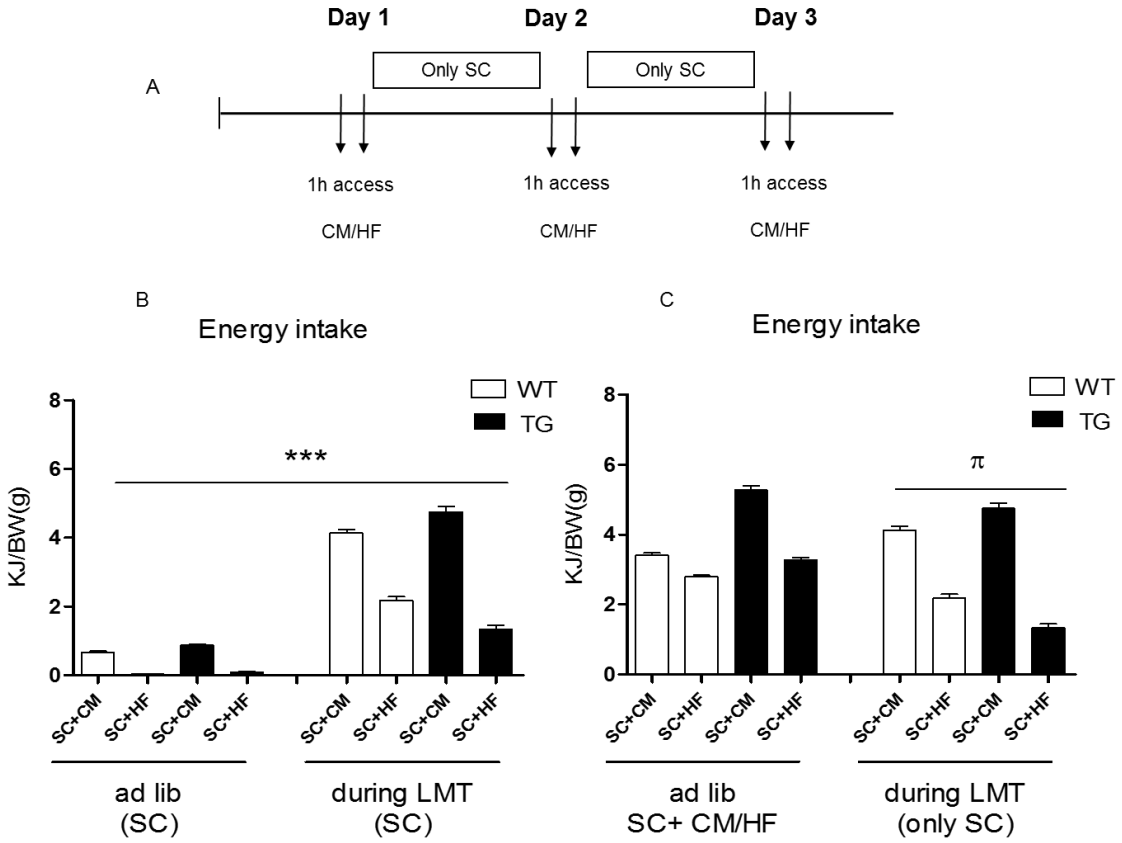

Figure 41. SC energy intake during the periods of absence of energy-dense foods in wild-type (WT) and in mBACTgDyrk1A (TG) mice. (A) Schedule of the three days of limited access experiment. (B) The bars plots show the mean values of energy intake of SC in *ad* libitum feeding conditions (when CM and HF are freely available, "*ad lib*") and when energy-dense foods are only available for 1 hour each day (limited, "LMT").(C) The bars plots show the mean values of total energy intake in *ad libitum* feeding conditions (SC+CM or SC+HF according to the experimental group) and when access to energy-dense food is limited considering only the SC intake (LMT). WT are depicted in white and TG in black. Data are expressed as mean ± SEM. SC + CM group: WT n = 8, TG n = 8; SC + HF group: WT n = 8; TG n = 8. ANOVA Repeated measures, factor (test day) *** P< 0.001. Two-way ANOVA, genotype effect π P<0.05.

We studied the presence of binge-like episodes during the 1h access of CM and HF to analyze the compulsive behavior.

In SC+CM group, we found a significant increased consumption of CM during the limited access periods along the test days in mBACTGDyrk1A mice (Repeated measures ANOVA, F (1,14) = 22.27, P<0.001; Figure 42A). Moreover, although no significant, energy increase for CM between the *ad libitum* conditions and during the third day of the CM limited access was higher in mBACTGDyrk1A compared to WT (Figure 42C). On the contrary, *Dyrk1A* overexpression did not lead to a HF escalation across the days (Figure 42B).

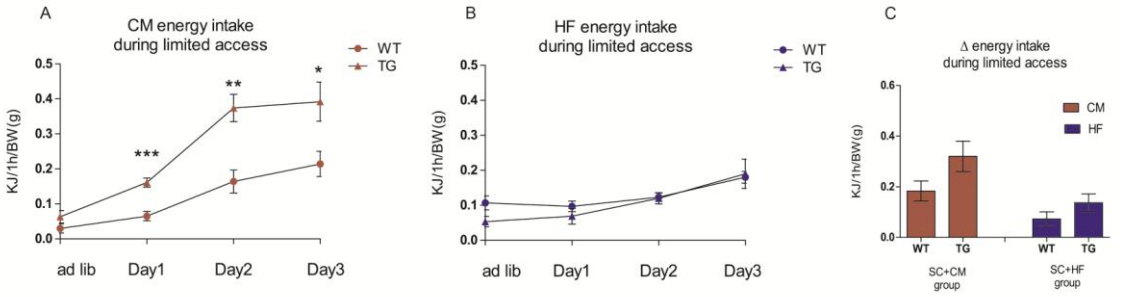

Figure 42. Energy intake during limited access to energy-dense food in wild-type (WT) and in mBACTgDyrk1A (TG) mice. (A) Time course of the energy intake of CM (A) and (B) HF during the periods of access. (C) CM/HF energy increase between the *ad libitum* conditions and during the third day of the limited access. *ad lib* = *ad libitum*; Day 1, Day 2 and Day 3 are the three consecutive days of restricted access (1h) to CM/HF. Data are expressed as mean ± SEM. SC + CM group: WT n = 8, TG n = 7; SC + HF group n = 8, TG n = 8. Repeated measures ANOVA, Bonferroni as a post-hoc * P<0.05 ** P<0.01 *** P<0.001.

Since mice have continuous access to chow, rather than a hunger effect, the increased energy intake of CM during limited access compared to *ad libitum* conditions, suggest an increased compulsive like intake in mBACTGDyrk1A compared to WT mice.

Food adulteration with quinine hydrochloride

Food was manipulated with quinine hydrochloride to address if mBACTgDyrk1A mice still consumed energy-dense foods in spite of their aversive taste as a sign of inflexibility (Heyne et al. 2009).

In the SC group, mice were given free choice between SC and SC adulterated with quinine hydrochloride and both genotypes showed reduced their intake of adulterated SC as compared to non-adulterated chow (paired Mann-Whitney-U-test, z = -2.52; P<0.01 in WT and z = -2.52; P<0.01 in TG, Figure 43A, 44A) suggesting adequate bitter detection. Moreover, both genotypes increased their SC intake for the SC non-adulterated compared to their 24h average SC energy intake in non-adulterated conditions (paired Mann-Whitney-U-test, z = -2.52; P<0.01 in WT and, z = -2.51, P<0.01 in TG, Figure 43B-C).

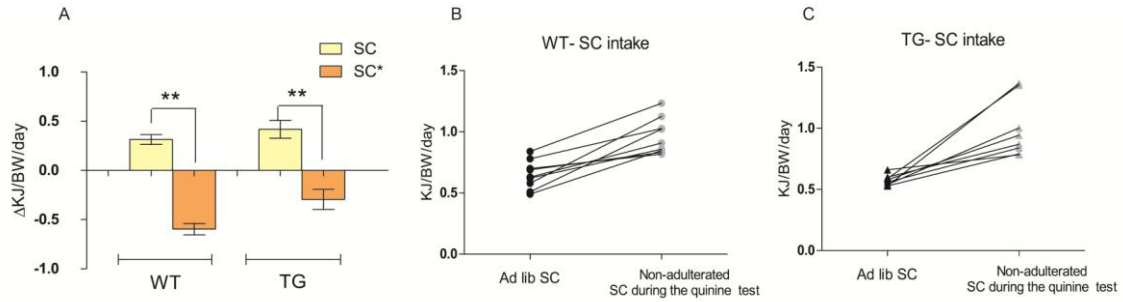

Figure 43. Bitter detection in non-obesogenic conditions in wild-type (WT) and in mBACTgDyrk1A (TG) mice. (A) Percentage of reduction of adulterated and non-adulterated SC consumption (percentage of KJ/g of body weight/day) in WT and Ts65Dn (TS) mice. (B-C) Individual values of 24 hours intake of non-adulterated SC (KJ/g of body weight) in basal conditions (black symbols) and upon presentation of quinine adulterated SC (grey symbols) in WT (B) and in TG (C). Data are expressed as mean ± SEM. SC group, WT n = 8, TG n = 8. Paired Mann-Whitney-U-test ** P<0.01.

In the SC+CM group, CM intake was reduced upon quinine hydrochloride adulteration in WT mice (paired Mann-Whitney-U-test, intake of bitter CM vs. CM intake, z = -2.51; P<0.001). However, 38 % of the mBACTgDyrk1A mice still ate bitter CM (Figure 44B).

In the SC+HF group, quinine adulteration reduced HF intake in both WT and mBACTgDyrk1A mice compared to free feeding conditions (paired Mann-Whitney-U-test, intake of bitter HF vs. HF intake, z = -2.52; P<0.01 in WT; and paired Mann-Whitney-U-test, intake of bitter HF vs. HF intake, z = -2.37; P<0.01 in TG, Figure 44C).

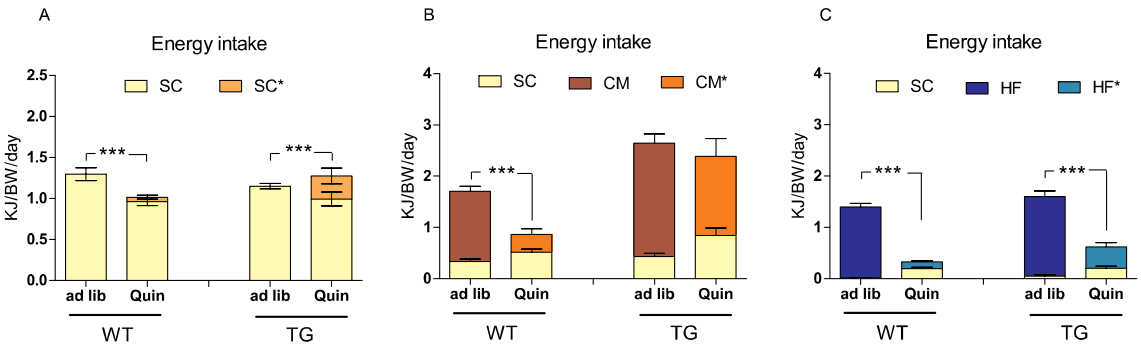

Figure 44. Energy intake of quinine-adulterated food in wild-type (WT) and mBACTgDyrk1A (TG) mice. Bar plots show food intake (KJ/g of body weight) in 24 hours in non-adulterated conditions (*ad libitum*, "*ad lib*") and the energy intake after quinine hydrochloride adulteration (Quin) of (A) SC , (B) CM (C) and HF. Adulterated food is marked by "*" . In (A) light orange SC* = bitter SC; in (B) dark orange CM* = bitter CM and in (C) light blue HF* = bitter HF. (A) SC adulteration in SC group. (B) CM adulteration in SC+CM group. (C) HF adulteration in SC+HF group. Data are expressed as mean ± SEM. SC group: WT = 8, TG = 8; SC + CM group: WT = 8, TG = 8; SC + HF group: WT = 8; TG = 8. Paired Mann-Whitney-U-test ** P<0.01.

Preference for sweet solutions

The two-bottle test was used for assessing reward and taste perception. The overexpression of *Dyrk1A* had no effect on the preference for sweets solutions in non-obesogenic conditions (Figure 45).

The preference for saccharin was similar to SC mice (Figure 45A). Energy-dense diets only had a significant effect on the preference for sucrose solution, being lower for SC + HF mice (diet effect ANOVA F (2,46) = 4.15; P<0.05, Figure 45B).

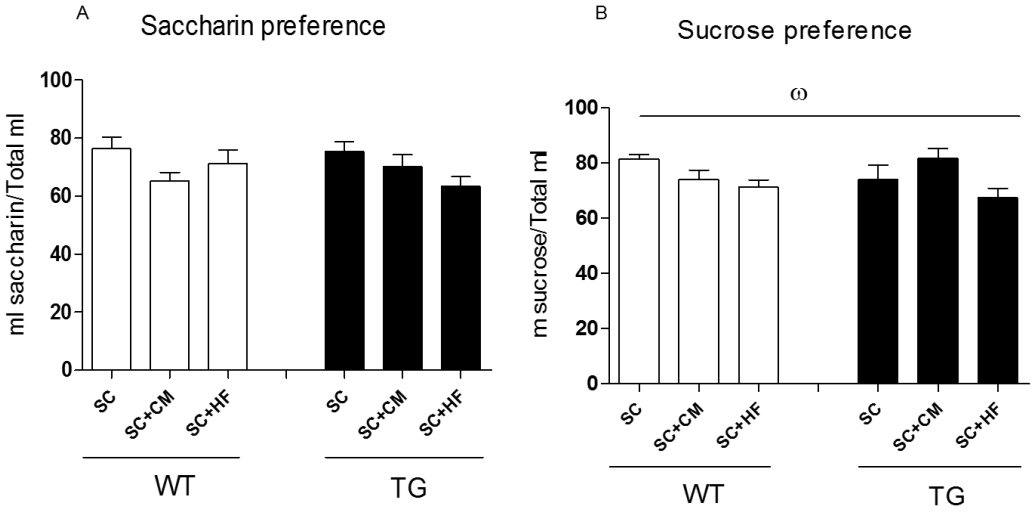

Figure 45. Preference for sweets solutions upon long-term access to energy-dense diets in wild-type (WT) and in mBACTgDyrk1A (TG) mice. Bar blots show the preference for (A) saccharin and (B) sucrose against water [ml sweet solution/ total liquid intake (water + sweet solution)] in SC, SC+CM and SC+HF mice. Data are expressed as means ± SEM. SC group: WT n = 8, TG n = 8; SC + CM group: WT n = 8, TG n = 8; SC + HF group = WT n = 8, TG n = 8. Two-way ANOVA, diet effect ω <0.05.

DISCUSSION

5. DISCUSSION

The prevalence of obesity in DS individuals is higher than in general population (Bell and Bhate 1992; Melville et al. 2005; Must et al. 2014). However, the underling mechanisms and factors behind are not well understood. The main goal of this Thesis was to determine the contribution of abnormal feeding behavior to obesity development and investigate the possible underlying mechanisms. We also explored peripheral obesity phenotype, analyzing glucose metabolism and pancreatic phenotypes. To explore these aspects, we used Ts65Dn mice, a mouse model of DS that has been extensively validated for the study of diverse features of DS related to learning and memory (Escorihuela et al. 1995; Escorihuela et al. 1998; Catuara-Solarz et al. 2016), attention (Moon et al. 2010), impulsivity (Whitney and Wenger 2013) and motor dysfunction (Hampton et al. 2004; Altafaj et al. 2013), among others. The present work confirmed the face validity of the Ts65Dn mice for the investigation of DS obesity phenotypes. Concretely, Ts65Dn mice showed a tendency to adiposity, and meal pattern disturbances. When exposed to an obesogenic environment, we detected increased preference for energy-dense foods and Ts65Dn mice gained more weight than WT when exposed to HF. Part of these phenotypes may be due to a deficient dopaminergic tone in prefrontal cortex, which is a risk factor for overeating. At the peripheral level, we observed that Ts65Dn pancreas had reduced β-cell mass along with diminished insulin secretion, which is in agreement with the increased risk of autoimmune diseases associated to trisomy of 21, like diabetes mellitus type 1. We also detected increased levels of circulating signals associated to immunologic and oxidative damage suggesting that obesity development in DS could be facilitated by a sustained inflammatory state.

In the second part of this thesis, our goal was to narrow down the genetic dependency of the behavioral and metabolic abnormalities, and thus we used the mBACTgDyrk1A mice, overexpressing only *Dyrk1A* since previous works suggested that the encoded kinase could be involved in reward related mechanisms and behavioral control. However, the majority of the obesity phenotypes were opposite to those found in Ts65Dn mice. Although our results suggest that DYRK1A may have a role on energy-dense foods preference and overeating, it is possibly not involved in DS-related obesity phenotypes.

5.1 Face validity of the Ts65Dn mice for the study of Down syndrome obesity

5.1.1 Ts65Dn mice recapitulate obesity-related Down syndrome metabolic phenotypes

Body weight, adiposity and leptin resistance

Adults with DS are more likely to be obese than euploid population. Proposed causes of their obesity risk are reduced physical activity (Phillips and Holland 2011), a reduced basal metabolic rate (Luke et al. 1996), and increased risk of subclinical hypothyroidism (Murray and Ryan-Krause 2010). More generally, though, weight management issues in most individuals with DS are due to the intake of too many calories in relation to the level of physical activity.

Here we did not detect increased body weight in trisomic mice as compared to their WT, when they were maintained in non-obesogenic conditions (standard chow), and supporting this finding, also in the PCA analysis body weight was not contributing to genotype differences in baseline conditions. The lack of overweight in trisomic mice fed with standard chow could be seen as opposite to the human phenotype, since a high percentage of individuals with DS present overweight and obesity, but we propose that this might simply reflect the fact that current human diets are obesogenic by definition since they contain high amounts of fat and sugar (Williams 2001), and thus in humans we are studying obesity phenotypes in an obesogenic environment. In support of this assumption, previous investigations reported that community-dwelling individuals with DS experience greater prevalence of obesity than do those living in institutions. Compared to institutionalized population, community-dwelling individuals met less the current guidelines for fruit and vegetable intake (Braunschweig et al. 2004), presenting higher intake of soft drinks, precooked meals, and meals often not adjusted to the reduced energy needs (Nordstrøm et al. 2015). Our results thus reinforce the concept that a balanced diet would result in normal body weight in trisomic individuals.

Even though trisomic mice were not overweighed in non-obesogenic conditions (standard chow), Ts65Dn displayed increased percentage of body fat compared to WT mice. This increased adiposity observed in Ts65Dn mice was accompanied by higher leptin levels compared to WT mice (Annex III). Again, this is consistent with accumulating data showing that DS individuals display abnormally high levels of total and regional fat mass than their counterparts without DS (Gonzalez-Agüero et al. 2011;

Loveday et al. 2012). Also increased leptin levels (Magni et al. 2004; Tenneti et al. 2017) have been detected in this population that correlate with adiposity in obese people with DS (Proto et al. 2007). Leptin is a proteo-hormone encoded by the "ob" gene, and secreted in adipocytes that acts in the hypothalamus to suppress appetite and regulate body weight (Considine et al. 1996). In our work, this increased level of leptin was observed in lean Ts65Dn mice which is in agreement with the findings of a recent study in non-obese DS children (Tenneti et al. 2017). This increased leptin might be interpreted as a predictor of higher obesity risk, as shown in a prospective population-based human study in which baseline leptin levels significantly predicted the development of obesity and metabolic syndrome (Franks et al. 2005). In fact, in the general population leptin concentrations correlate with adiposity and body mass index in both lean and obese subjects (Ahima and Flierer 2000).

Thus, we can speculate that chronic hyperleptinemia in Ts65Dn mice could reflect diminished leptin sensitivity that, when exposed to an obesogenic environment, might increase the risk of obesity development. In support of this hypothesis, Ts65Dn gained more weight than WT when given free-choice access to high-fat. According to our results, DS is a case of leptin resistance phenotype and thus healthy dietary habits could be a key strategy in preventing obesity development of obesity in DS despite their higher propensity to adiposity.

Glucose homeostasis and diabetes-like pancreatic phenotypes

Obesity induces insulin resistance and involves a plethora of molecules that predispose individuals to an inflammatory state and metabolic complications. In fact, a direct relationship between BMI and diabetes has been demonstrated, which pathogenesis has been demonstrated to be a resistance to insulin action in peripheral tissues. DS is associated to a higher prevalence of diabetes compared to general population (Anwar et al. 1998; Bergholdt et al. 2006; Lammer and Weimann 2008; Rohrer et al. 2010). However, the mechanisms remain understudied. Reduced insulin secretion, reduced insulin sensitivity, or both characterize diabetes. Type 1 diabetes (T1D) is commonly associated to immunological problems leading to deficient insulin production and β-cell death, whereas type 2 diabetes (T2D) is associated with insulin deficiency and insulin resistance. The majority of studies of diabetes in DS did not differentiate between T1D and T2D (Rohrer et al. 2010), but those that specifically were done in type 1 diabetes,

showed that DS has a prevalence of 0.38-0.7%, compared with 0.17% in the general population (Bergholdt et al. 2006).

Trisomy 21 mouse models, Ts65Dn and Ts16 (a complete mouse chromosome 16 trisomy) are hyperglycemic, and Ts65Dn mice have impaired glucose tolerance (Peiris et al. 2016). Here we confirmed this previous study, showing higher levels of plasma glucose in Ts65Dn mice compared to WT in starved conditions. Moreover, the postprandial glucose homeostasis was altered, so that Ts65Dn mice were unable to recover the basal glycaemia upon oral administration of glucose in the tolerance test (OGTT) similar to the previous reports (Peiris et al. 2016).

In our study, hyperglycemia in Ts65Dn mice occurred concomitantly with low insulin plasmatic levels during the OGTT, suggesting pancreatic β-cell dysfunction that would lead to a deficient insulin production or to reduced secretory capacity of islets of Langerhans in Ts65Dn. In fact, we detected less insulin-positive cells and reduced total insulin pancreatic content (insulin (ng/ml)/mg of pancreatic tissue), along with reduced β-cell mass calculated as the number of β-cell per mg of pancreas (work by Dr. Rachdi). Our results in Ts65Dn mice suggest that β-cell loss could be a mechanism leading to diabetes type 1 in DS. However, we did not find any report quantifying β-cell mass in DS humans, although several studies have reported increased levels of insulin autoantibodies, which is an indication of autoimmune damage of pancreatic β-cells (Steck et al. 2015). Further experiments would be needed to explore this possibility as a mechanism underlying β-cell death in Ts65Dn.

Besides the deficient β-cell mass in Ts65Dn pancreas, hyperglycemia could also be related to secretory problem of the islets of Langerhans. To confirm this aspect, we evaluated insulin secretion in isolated islets under basal (2.8 mM glucose) and stimulating (16.7 mM glucose) conditions, and observed decreased basal and glucose-induced insulin secretion in Ts65Dn islets. Our results are in agreement with the study of Helguera and collaborators showing that basal insulin secretion by fetal human pancreatic cells with DS was drastically decreased compared to non-DS cells (Helguera et al. 2013).

Taken together, these results suggest that both β-cell expansion and secretory mechanisms are altered in Ts65Dn mice compared to WT. Previous studies have suggested that these phenotypes arise from the overexpression of chromosome 21 genes such as *RCAN1* and DYRK1A, which are expressed in pancreas where they have

functional roles. A microarray analysis in islets from Ts65Dn and WT mice (work by Dr. Rachdi) confirmed that both genes contained were upregulated in Ts65Dn (*RCAN1* = 1.2; DYRK1A = 1.3 with respect to WT). In fact, the overexpression of *RCAN1* in mice causes the reduction on the glucose-stimulated insulin secretion along with a mitochondrial dysfunction in β-cells (Peiris et al. 2012; Peiris et al. 2016), but overexpression of DYRK1A in transgenic mice promotes β-cell mass expansion (Rachdi et al. 2014b), opposite to what we detected in Ts65Dn. Of course, the combined effect of the overexpression of these two proteins could be different to the impact of their single overexpression in transgenic mice, since RCAN1 activity is regulated by DYRK1A protein-mediated phosphorylation (Jung et al. 2011).

Besides the predicted overexpression of HSA21 genes, we also found a significant downregulation of *HSPA1A/Hsp72* in trisomic islets of Langerhans as compared to WT (Dr. Rachdi, personal communication). *HSPA1A/Hsp72* encodes for a heat shock protein that prevents the aggregation of multiple proteins. Among those, the misfolded human islet amyloid polypeptide hormone (h-IAPP, also called amylin), which forms toxic aggregates, destroys pancreatic β-cells (Rosas et al. 2016). On the other hand, Hsp72 blocks apoptosis primarily by inhibiting Bax activation and thereby preventing the release of proapoptotic factors from mitochondria (Stankiewicz et al. 2005). Thus, we could speculate that the net effect of reduced levels of HSP1A/Hsp70 in Ts65Dn islets could be an increase of apoptosis through increased aggregation of amylin along with reduced inhibition of Bax (Figure 46). Accordingly, the pancreatic duodenal homeobox 1 (Pdx1) transcription factor, which plays a central role in pancreatic β-cell function and survival (Fujimoto and Polonsky 2009) was reduced in Ts65Dn β-cell compared to WT (Dr Rachdi, personal communication). Downregulation of *HSPA1A* has been reported before in brain of DS fetus (Seidl et al. 2001), and in DS astrocytes cultures (Helguera et al. 2013) suggesting that altered antiapoptotic mechanisms in DS could play a role in the pathogenesis of diabetes.

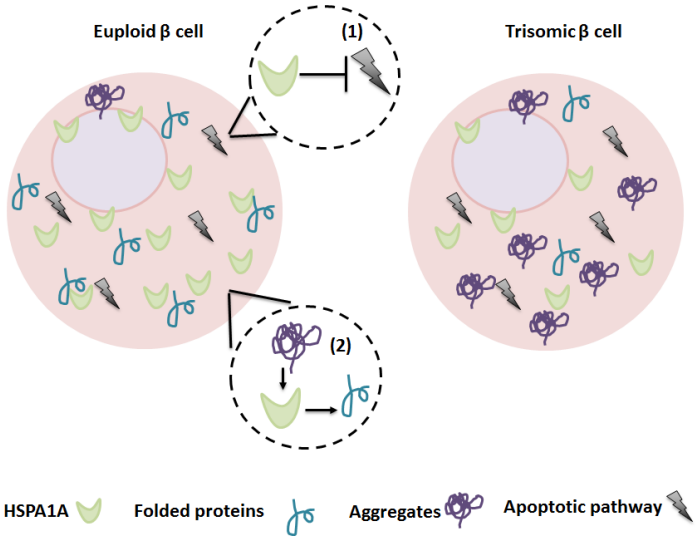

Figure 46. Proposed model of the effects of HSPA1A downregulation in DS diabetes. Lower levels HSPA1A might contribute to the impaired glucose homeostasis (1) by interfering in the antiapoptotic β-cell program or (2) through impaired regulation of protein folding that might cause aggregate accumulation.

5.1.2 Ts65Dn mice recapitulate Down syndrome-related feeding problems

Our meal pattern analysis in mice given standard chow revealed that Ts65Dn mice present slower and longer meals as compared to WT mice, confirming a previous observation (Turner et al. 2001). In humans, DS is associated with high incidence of feeding abnormalities. Between 31% and 80% of children with DS have eating or drinking difficulties (Pipes and Holm 1980; Field et al. 2003) with abnormal-tongue/jaw function and difficulty in initiating and maintaining the feeding sequence (Kumin and Bahr 1999; Cooper-Brown et al. 2008). Some of these feeding abnormalities, such as reduced chewing frequency and increased mastication time, persist to adulthood (Hennequin et al. 2005; Smith et al. 2014) leading to prolonged meals. The slower and longer meals observed in Ts65Dn mice thus reproduce the DS phenotype, and may result of the complex interactions between anatomical, medical, physiological, and behavioral factors.

One important factor could be related to mastication and deglutition alterations that depend on the coordination of mouth and tongue movements. Poor coordination and sequencing of oral-motor movements, weak lip closure and weak and reduced tongue movements have been reported in DS patients (Faulks et al. 2008a-b; Smith et al. 2014). We did not explore these biomechanic phenotypes in the context of the present work. However, recently Glass and collaborators found significantly lower expression of

myosin heavy chain subunit 2 (MyHC-2b) protein levels in the anterior digastric and posterior digastric muscles, which are involved in jaw movement, in Ts65Dn mice compared to WT. In wild-type mice, MyHC-2b expression in the tongue (Maejima et al. 2005) and in digastric muscles (Yoshii et al. 2008) is required for the correct transition from suckling to swallowing solid food. However, despite this reduced MyHC-2b expression, Ts65Dn mice did not show swallowing or inter-swallow intervals (Glass and Connor 2016), suggesting that the developmental aspects leading to reduced tongue movements and swallowing difficulties in DS humans may not be attributed to MyHC-2b. Even though the authors did not directly evaluate meal duration, this result may suggest that swallowing difficulties are not contributing significantly to prolonged meals in Ts65Dn mice. Other factors such as the relative macroglossia and reduced mandibular volume found in DS (Cooper-Brown et al. 2008) may be also important for explaining swallowing difficulties, but could be only partially replicated in Ts65Dn embryos (Billingsley et al. 2013). Taken together, these results suggest that factors other than these oromotor deficits should be involved.

Anatomical constraints such as retrognathia have been recognized as important in feeding biomechanics, as acknowledged for diverse syndromes with craniofacial alterations that lead to feeding problems (Cooper-Brown et al. 2008). DS patients present a small jaw (micrognathia) or a chin jaw posteriorly displaced (retrognathia) causing the tongue to fall backward toward the posterior pharyngeal wall (glossoptosis) (Levine and Simpser 1982; Limbrock et al. 1991; Guimaraes et al. 2008). Some of these phenotypes are recapitulated in Ts65Dn mice that show craniofacial dysmorphology and mandible hypoplasia (Richtsmeier et al. 2000), results that were also replicated in our laboratory (Annex II). Briefly, compared to euploid mice, trisomic mice showed smaller, thicker and more curved mandibles, with smaller and less pronounced coronoid and condylar processes, which correspond to the most common craniofacial traits in DS individuals. Given the role of the mandible in mastication and deglutition, we speculate that the observed craniofacial alterations may have functional implications in feeding behavior of Ts65Dn mice.

5.1.3 Ts65Dn mice present brain monoamine alterations involved in feeding behaviors

In the brain, the initiation, termination, and choice of meals is controlled by distinct and interconnected neural circuits that use a variety of neurotransmitters and neuropeptides

and that receive input on the status of energy balance via endocrine factors and peripheral nerves (Lutter and Nestler 2009). Among those, the involvement of brain monoamines in the control of food intake is well established, although how monoamines such as dopamine, serotonin, and noradrenaline are involved in energy balance is only partly understood. Through massive projections to the entire cortex and hippocampus, the monoaminergic systems exert a powerful modulatory effect on brain regions vitally important for cognition, motivation and behavioral control. Thus, it is assumed that monoamines specifically participate in the modulation of reward aspects of food intake.

We first had to define how the monoaminergic status in our mouse model was. Under standard chow diet, we did not find statistical differences between genotypes for monoamine concentrations in the hypothalamus and the striatum. These results do not replicate previous experiments in Ts1Cje mice, another segmental trisomy 16 mouse model (Sago et al. 1998), that had higher levels of dopamine and serotonin in striatum than their wild-type littermates (Shimohata et al. 2017). One possible cause of this discrepancy is technical, since Sago and collaborators used microdialysis along with other complementary techniques such as western blot, and immunohistochemistry, while here we exclusively used HPLC analysis in postmortem tissues. Also, the different genetic background and age of the animals could be confounding factors.

Interestingly, in our experiments we detected lower levels of dopamine (DA) in the prefrontal cortex (PFC) of Ts65Dn mice compared WT, a result that replicates previous DS human studies (Yates et al. 1983; Risser et al. 1997; Whittle et al. 2007) showing reductions in dopamine and its acidic metabolites DOPAC and HVA, in fetal and adult postmortem brains of individuals with DS. The PFC hypodopaminergic phenotype detected in Ts65Dn mice is possibly linked to the executive dysfunction detected in mouse behavioral studies (Coussons-Read and Crnic 1996; Escorihuela et al. 1998) and in DS individuals (Pueschel et al. 1991; Jernigan et al. 1993; Ball 2008). In a previous study of the laboratory, in young adults with DS, we found that genotypes conferring higher DA availability, such as Met carriers and VNTR-DAT1 10-repeat homozygotes, resulted in improved performance in executive function tasks that require mental flexibility (Del Hoyo et al. 2016). This deficit could also underlie a tendency toward perseveration and impulsive behaviors that could be a predisposing factor for compulsive overeating, so that reduced dopamine in the PFC, strongly involved in

inhibitory control, may contribute to behavioral disorders of loss of control such as overeating (Volkow et al. 2008).

Different mechanisms could explain the low DA concentration detected in the PFC of trisomic mice. The most obvious could be alterations in dopaminergic nuclei. We found one report (Mann et al. 1987) showing that DS individuals have reduced number of surviving thyroxin hydroxylase positive cells in the ventral tegmental area (VTA) which is the main region containing the dopaminergic neurons projecting to the PFC (Oades and Halliday 1987). Even though this was not seen in Ts65Dn mice that have similar number of dopaminergic cells in this region compared to WT (Megías et al. 1997), the dopamine synthesis, transport or the number of projections from VTA-dopaminergic cell bodies to cortical areas have not been studied and could be different. However, in our previous studies, although we detected important alterations in cAMP accumulation upon dopaminergic (D1) receptor stimulation with the D1 agonist SKF 38393 in postmortem cortical human samples of individuals with DS, these alterations were not detected in the hippocampus of trisomic mice (Baamonde et al. 2011).

Given that PFC-DA is implicated in self-control behaviors and reward sensitivity (Kehagia et al. 2010), the reduced DA tone detected in Ts65Dn PFC might impair inhibitory control of food intake. In DS people, this would imply a high risk of overeating driven by the availability of energy-dense foods in our obesogenic environments.

5.2 Obesogenic environments lead to genotype-specific obesity phenotypes and monoaminergic changes in Ts65Dn mice

In this Thesis, we selected a validated diet induced obesity paradigm that provides free access to energy-dense diets (chocolate-mixture diet, CM; or high-fat diet, HF), while maintaining standard rodent chow (SC) availability, thus mimicking in a more naturalistic way the human situation than forced diets (La Fleur et al. 2014). This paradigm has been previously validated as a powerful tool to induce obesity in mice and to provoke inflexible and compulsive behaviors associated to overeating (Heyne et al. 2009; Espinosa-Carrasco et al. submitted). Given that both sweets and fatty diets have the ability to induce overeating in both humans and mice (Avena et al. 2009; Volkow et

al. 2011) and DS show a marked tendency to consume energy-dense foods, would also allow to studying behavioral changes promoted by energy-dense diets.

5.2.1 Ts65Dn mice show increased preference and overeat energy-dense foods

In both WT and Ts65Dn mice body weight was significantly increased upon energy-dense diets, though it was more marked upon free-choice access to HF than to CM even though the digestible energy of both energy-dense diets is similar (20.6 MJ kg^{-1} for CM and 22 MJ kg^{-1} for HF). However, HF diet has a 60% of fat content and 6.4% simple sugars whereas CM contains 24% fat carbohydrate and approximately 44% simple sugars (Table 2). Whilst carbohydrate overfeeding produces progressive increase in carbohydrate oxidation and total energy expenditure, fat overfeeding has minimal effects on fat oxidation and total energy expenditure leading to adipocytes hyperplasia (Surwit et al. 1995). This different composition and metabolism may have an effect on weight gain, as has been demonstrated in both humans and mice excess of fat leads to greater fat accumulation than excess of carbohydrate (90-95% versus 75-85%) (Surwit et al. 1995; Buettner et al. 2007). Moreover, carbohydrate stimulates sympathetic nervous system activity and thermogenesis, while fat inhibits this effect by decreasing hypothalamic corticotropin releasing factor release (Surwit et al. 1995), which could also impact weight gain.

Ts65Dn mice gained significantly more weight than WT upon free access to HF. This effect is driven by the amount of HF consumed, since Ts65Dn showed higher intake of HF (1.42 ± 0.06 KJ/body weight) than WT mice (1.12 ± 0.05 KJ/body weight) in our free access paradigm. Interestingly, compared to their WT counterparts, trisomic mice gain significantly more weight than what would be expected from the increase of energy intake. This finding is in accordance with human reports showing that for the same amount of energy consumed, individuals with DS are more prone to present positive energy balance and increased fat storage (Wong et al. 2014). This effect may partially be driven by the high frequency of hypothyroidism that is a common clinical comorbidity in DS (Lavigne et al. 2017). Although we found no data on thyroid function in Ts65Dn mice, in the Ts1Cje E15.5 brains there is an up-regulation of *Slc7a5* (*Lat1*), involved in the transport of thyroid hormones T3 and T4 across the cell membrane (Guedj et al. 2015).

Upon free access to CM, also both genotypes gained weight, though less markedly than upon HF. When comparing both diets, we detected that mice of both genotypes tend to consume less chocolate than HF. These differences between HF and CM diets support previous observations that mice learn quickly to prefer flavors associated with high-energy content and select high-fat foods when available (Johnson et al. 1991; Sclafani et al. 2007; Rapp et al. 2009), but when it comes to sweet foods, the preference follows an inverted U-shaped curve with a sensory optimum, and both lower and upper concentrations reduced the hedonic response (Drewnowski 1995).

Ts65Dn consumed significantly more energy from the chocolate-mixture than WT mice (1.29 ± 0.10 and 0.84 ± 0.09 KJ/BW, respectively) also indicating a clear preference for sweet foods. However, opposite to what we observed in HF conditions, the body weight gain was similar in both genotypes, even though Ts65Dn mice consume more chocolate-mixture. We cannot explain this effect, but we could speculate that this less important than expected weight increase upon free access to CM could be related to abnormal carbohydrate metabolism, with impaired glucose tolerance, as observed in DS patients (Yasuda et al. 1979).

In both experiments trisomic mice showed increased preference for energy-dense (HF and CM) foods. This preference would parallel the human phenotype. DS individuals consume considerable amounts of highly processed foods such snacks and sweets beverages, especially when the meals depend on their choices (Nordstrøm et al. 2015). In a study conducted in DS teens (aged between 11 and 18 years old), 58% of the participants chose foods high in fat and sugar when asked about favorite foods and 81% reported to consume those foods every day.

A number of studies strongly argue in favor of the involvement of the brain reward circuit in the preference for energy-dense diets and in compulsive overeating. Those have led to the concept of Reward Deficiency Syndrome (RDS) which is a genetic and epigenetic phenomenon leading to impairment of the brain reward circuitry resulting in a hypo-dopaminergic function (Blum et al. 2000) that results in abnormal food preferences and in craving behavior (Blum et al. 2012). Thus we speculate that the trisomic phenotypes could be driven by the reduced dopamine levels detected in the prefrontal cortex (PFC) of Ts65Dn mice. Geiger and collaborators showed that obesity-prone rats present lower extracellular dopamine levels and lower evoked dopamine release in the nucleus accumbens, the dorsal striatum and PFC. They suggest that

obesity-prone rats have a global presynaptic deficit in central dopamine likely due to differences in the expression of genes that regulate dopamine exocytosis (Geiger et al. 2008). Thus, the hypodopaminergic state of trisomic mice would lead to overconsumption of energy-dense foods altering food inhibition responses to obtain stimulatory effects on otherwise sub functional reward circuits (Johnson et al. 2011; Leigh and Morris, 2016).

5.2.2 Obesogenic environments induce similar meal pattern changes in Ts65Dn and WT mice

When comparing CM and HF diet to standard chow (SC), we observed that Ts65Dn mice increased eating rate and reduced meal duration for both HF and CM. Similar effects were observed also in WT mice and have been reported before in previous studies (Espinosa-Carrasco et al. submitted). Above, we suggested that the slower and prolonged meals detected in Ts65Dn mice might be partially explained by mechanical difficulties due to the orofacial characteristics linked to trisomy 21. That fact that CM and HF foods are softer would certainly contribute to this finding, as in DS humans, viscoelastic foods, like cheese, need fewer cycles of mastication than brittle foods, like bread (Hennequin et al. 2005). Therefore, the increased feeding rates observed for HF and CM, could at least partially depend on the physical characteristics of energy-dense foods.

5.2.3 Ts65Dn mice are less vulnerable to high-fat diet induced circadian rhythm changes

It has been demonstrated that free access to energy-dense food promote changes in circadian activity characterized by an increased food intake during the inactive periods (Oike 2017), by advancing the phase of the liver molecular clock and altering daily rhythms of eating behavior and locomotor activity (Arble et al. 2009; Honma et al. 2016). In our experiments, consumption of HF or CM diet disrupted this temporal coordination in WT and in Ts65Dn mice, but with a diet- and genotype-specific pattern. Here we defined the loss of rhythmicity as the increase of the number of meals during the inactive period of the light/dark cycle.

In conditions of free access to CM, independently of the genotype, mice showed only subtle circadian rhythmicity changes. This result is in agreement with Espinosa and collaborators that also observed only a slight impact of chocolate diet in promoting circadian rhythm changes (Espinosa-Carrasco et al. submitted).

Instead, under free access to HF, we detected an important loss of circadian rhythmicity in WT mice that did not show the expected reduction of meals during the resting (light) phase, as shown by the similar number of HF meals in the light and dark phase cycle. This phenotype has already been described by other studies (Pendergast et al. 2013; Kohsaka et al, 2007; Espinosa-Carrasco et al. submitted) with *ad libitum* access to only HF. Those paradigms showed overeating of HF both during the active and inactive periods. In our experiments we extend these observations, demonstrating that not only forced HF regimes, but also free access to HF and SC lead to circadian rhythm disturbances.

Interestingly, no major changes in feeding behavior were detected in Ts65Dn, which concentrated their meals during the dark (active) phase, suggesting that high-fat diet affects less the central clock and/or eating behavior in Ts65Dn than in WT mice. We lack an interpretation for this finding, as on the basis of the obesity phenotype of Ts65Dn such differences could be hardly justified. In fact, previous reports provided evidence that mice fed a high-fat diet only during the 12-hour light phase gain significantly more weight than mice fed only during the 12-hour dark phase (Arble et al. 2009), but our result suggests that light phase feeding is not a critical component of weight gain in trisomic mice. It could be speculated that circulating satiety hormones, such as leptin, that we found significantly increased in Ts65Dn mice, may have a circadian variation independent of meal timing and be contributing to obesity as seen with humans on a circadian misaligned schedule. Also, several HSA21 genes encode for several proteins involved in circadian clocks (Kurabayashi et al. 2010), but their putative effect in the control of the circadian feeding behavior and the resistance to high-fat induced loss of feeding circadian rhythm in trisomic mice needs to be defined.

5.2.4 Ts65Dn mice show an increased risk for developing inflexible and compulsive-like overeating

Starvation promotes similar responses in WT and Ts65Dn mice

Food restriction has been shown to increase the risk of compulsive eating in non-clinical populations as well as to prolong compulsive eating in individuals with eating disorders (Stice et al. 2000). In our experiments, we used a starvation protocol of 24h fasting to analyze the compensatory responses to a mild energy deficient state but also to evaluate the hunger effect on energy-dense foods seeking.

To understand the effect of the trisomy in energy compensation, we first analyzed the energy intake in mice fed only with SC. In non-obesogenic conditions, WT mice fed similar amount of SC after starvation compared to their energy intake in free feeding conditions, similar to what has been previously reported in C57Bl/6 mice following alternate-day fasting (Anson et al. 2003). However, we observed Ts65Dn mice slightly increased their energy intake during the day after refeeding suggesting that mice overcompensate for the energy deficit.

It has been shown that glucose is a potent meal inducer when the levels in plasma are low. However, according to our results, Ts65Dn have impaired glucose buffering from blood both in starved and feeding conditions, suggesting circulating glucose may not explain this behavior. Indeed, the concentration of many hormones and peptides change upon starvation and also modulate feeding responses. Among them, the gut peptide ghrelin is reduced on feeding and has been implicated in the regulation of feeding behavior and energy balance. We have shown that circulating ghrelin is low in Ts65Dn compared to WT (Fructuoso et al. submitted). Similar to what we found, obese subjects have lower fasting ghrelin concentrations than do lean subjects (Tschöp et al. 2001). Ghrelin is increased after weight loss in obese subjects, and this change has been proposed to be related to weight regain (Heilbronn et. 2005). Although we here did not measure the circulating levels after this test, we speculate that differences in ghrelin could potentially mediate energy imbalance and thus increase food intake after a period of food restriction in Ts65Dn mice.

In obesogenic conditions, both WT and Ts65Dn mice showed similar amount of consumed energy the day of refeeding compared to a day on free-feeding conditions. This result suggest that overweight mice had not major problems in adapt their energy balance upon a mild food restriction. Although we have not previous data about the

hormone concentration in our free choice mice, from our result, it seems that trisomy don't further compromise energy compensation in overweight mice.

It has been shown that starvation increases the desire for palatable and energy-dense foods (Di Segni et al. 2014) (Berridge et al. 2010). Upon starvation, we found a similar increase of HF, but not of CM intake in both genotypes with respect to non-starved conditions. This result was contrary to our expectations. Indeed, we hypothesized that Ts65Dn would show increased CM/HF intake against SC than WT since Ts65Dn mice already showed higher preference for both energy-dense foods in free feeding conditions.

In mice compulsive-like intake after food deprivation occurs immediately after refeeding and decreases along time (Bake et al. 2014). For this reason, we analyzed energy intake during the first hour of refeeding after starvation. We observed that during this first hour Ts65Dn mice showed a compulsive-like intake of energy-dense foods. However, a similar phenotype was observed also in WT, suggesting that the trisomy does not have major effects in promoting the rebound consumption of palatable foods.

We did not found reports in DS humans for compulsive eating episodes upon a short period of food restriction. However, according to our results, a mild energy-deficit would thus not be an important contributor to energy-dense foods overeating in Ts65Dn mice. However, we don't know if the hunger-induced seeking effect would be different in a protocol using repeated cycle of food restriction, which indeed is a better approach to model dieting in humans.

Limited access to energy-dense food promotes increased compulsive-like overeating and reveals inflexibility in Ts65Dn mice

Dieting and food restriction have been demonstrated to increase the risk of compulsive eating in non-clinical populations as well as to prolong compulsive eating in individuals with eating disorders (Stice et al. 2000). In mice, compulsive-like intake after food deprivation occurs immediately after refeeding and decreases along time (Bake et al. 2014). Since mice have continuous access to standard chow, the increased energy intake of energy-dense foods during the 1h access compared to *ad libitum* conditions, when they have both SC and CM/HF access are indicators of a compulsive like intake rather than a hunger effect.

Limited access to CM and HF increased intake of energy-dense food in both genotypes though less than what is reported in binge-eating models (Avena 2010; Bake et al. 2014) suggesting that our schedule only induced moderate compulsive-intake. This is probably due to the fact that, opposite to classical methods, in our experiment mice were not food deprived (Corwin and Buda-Levin 2004). We purposely chose this design to avoid the hunger-based seeking bias of classical paradigms. Our model measured reward-driven intake since mice had always access to SC. With these conditions, CM induced stronger compulsive-like responses than HF. This was expected since only rodent models having intermittent sugar have shown strong binge-behaviors (Avena 2008; Avena 2010), which would agree with our observations (CM diet has much higher sugar content (44%) than HF (6.4 %).

Even though, as discussed, compulsive-like eating behavior was not very strong, Ts65Dn mice showed a more marked rebound consumption of energy-dense foods after restrictive access to energy-dense diets than WT, in both CM and HF groups. This rebound effect was only detected at the end of the experiment. This increased compulsiveness in trisomic mice could result from the hypodopaminergic phenotype we detected in trisomic prefrontal cortex. Although compulsive overeating has not been studied systematically in people with DS, the intensity of routinized and compulsive–like behaviors is higher in individuals with Down syndrome than in healthy population (Glenn and Cunningham, 2007; Glenn et al. 2012), and is not related with psychiatric diagnosis (Glenn and Cunningham 2015) suggesting that it is a DS personality trait.

Our protocol also allows to measuring flexibility, here defined as the ability to compensate the energy intake by increasing standard chow consumption during limited access. Neither WT nor Ts65Dn mice compensated the intake of energy coming from CM or HF by increasing the SC energy intake. This lack of adaptation reflects a lack of flexibility and has been previously reported in mice and rats using similar paradigms (Heyne et al. 2009). Even though we did not find significant genotype-dependent differences, the fact that DS humans, especially in childhood, consume a limited variety of foods and express strong likes and dislikes for food could be considered as an inflexible behavior (Marchi et al. 1999).

Thus, we also assessed inflexibility using an adulteration paradigm that allows to test ability to modify behaviors, such as avoiding the preferred food when is unpleasant, which is considered a sign of inflexibility. Briefly, quinine hydrochloride is used to

adulterate the organoleptic properties of energy-dense foods that become bitter (Sclafani 1976). Flexible mice would neglect or significantly reduced the intake of the craved food (CM/HF) when is bitter, while inflexible individuals would still consume these foods in spite their unpleasant taste (Heyne et al. 2009).

In most of the mice adulteration with quinine hydrochloride reduced the CM intake (Bruinsma and Taren 1999) (Patrono et al. 2015). However, independently of the genotype, some 30% of mice still consumed biter CM in spite of the lack of the rewarding effects (sweetness) but the amount of intake varied, in agreement with previous reports (Heyne et al. 2009).

HF adulteration with quinine hydrochloride lead to a stronger reduction of HF consumption in both genotypes compared to adulterated CM. Interestingly, Ts65Dn mice consumed equivalent amounts of bitter HF than non-adulterated in spite of its unpleasant taste, indicating that trisomy predisposes to more inflexible behavior that is only revealed for HF diets. It could be argued that, instead of inflexibility, the taste and/or olfactory deficits described in DS humans (McKeown et al. 1996) and the olfactory deficits reported in Ts65Dn mice (de Souza et al. 2011) could account for this effect. However, Ts65Dn and WT mice avoided equally adulterated SC and CM, and we observed similar preference for sweets solutions in the two-bottle preference test. Thus, rather than a gustatory dysfunction, this lack of avoidance of HF reflects in fact inflexible behavior in Ts65Dn mice.

Our results show that overeating of high-fat foods might be more detrimental for behavioral control (e.g. flexibility, impulsivity management) in Ts65Dn mice.

5.2.5 Energy-dense foods induce neurochemical changes that may participate in the observed behavioral abnormalities

In baseline conditions, we could not detect significant changes in brain monoamines in Ts65Dn mice. This is in contrast with previous studies showing monoaminergic deficits in DS (Yates et al. 1983; Risser et al. 1997; Whittle et al. 2007). However, we found low dopamine levels in prefrontal cortex. Interestingly those were correlated with the amount of HF eaten during the quinine adulteration test. Specifically, we observed that trisomic mice were those having lower DA values and the lower the PFC DA levels, the higher the amount of bitter HF intake and that those Ts65Dn fed with adulterated CM,

that did reduce consumption, increased the DA levels. In WT mice both long-term access to HF and CM, reduced DA levels in PFC in agreement with other studies (Carlin et al. 2013) (Wakabayashi et al. 2015). Again, those WT that showed inflexible behavior also presented the strongest reduction in the PFC-DA levels. These results strongly support the conclusion that DA-PFC levels modulate inflexibility, and that inflexible behavior is a manifestation of the hypodopaminergic syndrome.

Diet composition has an impact on the concentration of brain monoamines (Chalon et al, 1998; Kim et al, 2013; Fernández-Fernández et al. 2015). Therefore, we studied the neurochemical changes induced in hypothalamus, striatum and PFC by long-term access to CM and HF.

Comparisons of HPLC data between non-obesogenic and obesogenic conditions showed that the monoamine composition in hypothalamus was unaffected upon long-term CM and HF consumption. However, free choice access to CM lead to higher serotonin (5HT) levels in hypothalamus of WT mice but reduced the 5HT levels in Ts65Dn as compared to their respective SC controls. Even though the changes were not significant, possibly due to the low number of samples, we suggest that this result might be biologically relevant. Previous studies in rats have shown that serotonin arriving from raphe innervating to hypothalamus serves for meal termination (Leibowitz and Alexander 1998). Considering the inhibitory effect of serotonin on meal ingestion, a deficient hypothalamic serotoninergic tone could contribute to the increased CM intake of Ts65Dn mice while the increase 5HT levels in WT might be interpreted as a compensatory mechanism. In support of this hypothesis, we observed a strong negative relationship between the levels of 5HT in hypothalamus and CM intake (Spearman's rho of - 0.9). Interestingly, this neurochemical change was only detected in CM free access, while HF did not increase the concentration of 5HT in the hypothalamus. The specificity might be due to an increase of tryptophan, which is a precursor of serotonin but also a metabolite of carbohydrates that are present in higher concentrations upon CM diet.

In our experiments, energy-dense diets induced similar changes in DA content in striatum in both genotypes. Specifically, mice having access to CM and HF showed higher levels of striatal DA and HVA, one of its metabolites, as compared to SC mice. The striatum is involved in the assignation of reward value, and thus, has a major role in food seeking behaviors (Kessler et al. 2016). Accordingly, the increased DA tone in

striatal regions could be participating in the perseveration in consuming highly palatable and energy-dense foods beyond the energy needs (Wang et al. 2011) since elevations in ventral striatal dopamine positively reinforces actions to obtain food (Adamantidis et al. 2011). WT showed greater DA levels upon energy-dense long-term access than Ts65Dn mice that did not show important change in DA levels. The lack of rise in DA levels upon the consumption of HF in trisomic mice could indicate a depressed reward system.

In the PFC, free access to energy-dense food lead to a lower DA levels in WT mice, reproducing previous results from other groups (Carlin et al. 2013) (Wakabayashi et al. 2015). In the case of the Ts65D mice, HF and CM diets lead to opposite changes: while CM increased the DA levels, HF aggravated the hypodopaminergic state that we detected in non-obesogenic conditions. The DA levels of CM trisomic group were closer to the DA levels of the WT in non-obesogenic environment. The reduction of DA might have important behavioral consequences since it has been proposed that an inverted-U-type of response function for dopamine underpins the relationship with impulsivity (Nutt et al. 2015). In fact, we observed that low PFC-DA negatively correlated with higher intake of HF diet but also with adulterated HF consumption during the quinine adulteration test. Interestingly, Ts65Dn mice having the lower DA levels (SC+HF group) manifests higher inflexibility, reflected by a much stronger persistence of feeding bitter HF than bitter CM.

5.3 The involvement of DYRK1A in the Down syndrome obesity phenotypes

DS phenotypes are the result of a trisomy, and thus of the deregulation of an extensive number of genes. Among the triplicated genes, accumulating evidences suggest that the Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1A (DYRK1A), could be implicated in both homeostatic and non-homeostatic aspects of feeding behavior.

In this Thesis, we explored the specific involvement of DYRK1A in the feeding abnormalities contributing to DS obesity.

5.3.1 DYRK1A overexpression does not recapitulate Down syndrome behavior abnormalities

As in the Ts65Dn mice, we did not detect increased body weight in mBACTgDyrk1A mice when maintained in non-obesogenic conditions (standard chow), as compared to

their WT. In our experiments we could not reproduce previous reports showing that *Dyrk1A* transgenic mice exhibited increased food intake than wild type-mice (Hong et al. 2012). We neither observed the feeding behavior difficulties that in Ts65Dn mice manifested as longer and slower meals. DYRK1A overexpression in mBACTgDyrk1A mice did not lead to meal pattern changes compared to their WT, suggesting that DYRK1A overexpression alone is not sufficient for recapitulate this phenotype. Interestingly, we found that mandible hypoplasia is less pronounced in BACTgDyrk1A than in Ts65Dn mice (see Annex II), which could explain the absence of differences on meal duration and eating rate compared to their WT. This result could explain why the normalization of *Dyrk1A* overexpression to euploid levels by genetic deletion in Ts65Dn mice, did not significantly improve their mandibular dysmorphology (McElya et al. 2016).

One limitation of our interpretation is the possible differences derived from the genetic background, which is not the same in transgenic and trisomic mice. However, our PCA analysis confirmed that those did not influence the craniofacial alterations observed in Ts65Dn and mBACTgDyrk1A as WT mice from both models clustered together. On the other hand, the same PCA analysis revealed a complete overlap between Ts65Dn and mBACTgDyrk1A mice for the skull morphometric analysis, suggesting that *Dyrk1A* overexpression in mice is sufficient to recapitulate brachycephaly.

We conclude that DYRK1A overdosage is not sufficient for recapitulating DS feeding disturbances. However, our work adds a piece of evidence supporting the role of *Dyrk1A* in craniofacial morphogenesis. Our results do not support a role for *Dyrk1A* in DS obesity.

5.3.2 DYRK1A overexpression leads to diet-specific body weight gain upon free access to CM or HF

In our experiments, HF diet promoted weight gain in all mice independent of their genetic background. However, while Ts65Dn mice get more obese than their WT littermates, mBACTgDyrk1A gained similar body weight than their non-transgenic counterparts. This seems to contrast previous results claiming that mBACTgDyrk1A are protected against obesity (Rachdi et al. 2014a; Song et al. 2015) at least in HF conditions. In fact, mBACTgDyrk1A exposed to CM did not gain weight whereas

Ts65Dn and the wild-type mice from both models showed increased body weight gain compared to SC mice.

The discrepancy could be due to differences in the type HF diet used or in the age of the animals since Rachdi and collaborators used 4 weeks-old mice that have not yet reached a stable weight while in our study we purposely waited until mice reached a plateau in their body weight increase, at 5 months of age. In this same work, transgenic mice gained were shown to be glucose tolerant and hyperinsulinemic compared to their WT (Rachdi et al. 2014a). Also, Song et al related the leaner phenotype of mice overexpressing DYRK1A (Song et al. 2015) to DYRK1A phosphorylation of glycogen synthase kinase 3 (GSK-3), which would lead to the inhibition of adipogenesis.

Again, our results suggest that the overweight in Ts65Dn mice cannot be explained by the single overexpression of DYRK1A. Indeed, according to our results and based on the literature, DYRK1A might rather have a positive effect in body weight regulation. The increased body weight in HF free access conditions in Ts65Dn mice suggest that other genes might be implicated in adiposity and glucose control.

5.3.3 DYRK1A overexpression leads to an increased preference for high-carbohydrate meals (CM)

Independently of the genotype, we found that HF was highly preferred against SC in all mice, which is supported by accumulating studies showing that the majority of the mouse strains always prefer fatter meals over any other nutritional combination (West 1991) (Levine 2003). However, the most salient result of these experiments was the higher preference for the chocolate-mixture (CM) detected in mBACTgDyrk1A as compared to WT and Ts65Dn mice. One limitation to the interpretation of this result in the context of the DS phenotype is that the WT littermates of mBACTgDyrk1A mice also consumed more CM than the WT littermates of Ts65Dn, suggesting a strain effect (see Annex II). However, contrary to Ts65Dn mice, mBACTgDyrk1A mice consumed almost the same amount of CM than of HF indicated a clear preference for CM that was not present in trisomic mice thus reinforcing the implication of *Dyrk1A* in this phenotype, and suggesting that the impact of DYRK1A overexpression may be different in a disomic or in a trisomic background. Several studies have shown that neuropeptide Y (NPY) expression in the hypothalamus stimulates the ingestion of carbohydrates, having little effect on low-carbohydrate meals or fats (Beck 1992). Given that *Dyrk1A* is

expressed in hypothalamus and promotes NPY expression, the increased expression level and activity of DYRK1A recently reported in the hypothalamus of BACTgDyrk1A mice (Hong et al. 2012; London et al. 2017) could promote this preferential effect of NPY on carbohydrate consumption (Stanley 1989) in transgenic mice. In this same line, the fact that mRNA levels of NPY in the hypothalamus of Ts65Dn mice by qPCR were downregulated compared to WT, could explain their reduced CM preference. It is unclear what would be the mechanism underlying NPY downregulation in the trisomic model, which overexpresses DYRK1A, suggesting that other genes might be probably inhibiting NPY production. We propose that *Dyrk1A* overexpression might have a role in food preferences that might be compensated by other molecular pathways that are disturbed in the trisomic context.

5.3.4 DYRK1A overexpression leads to inflexibility and increased binge-like responses

When access to energy-dense food was limited, WT and mBACTgDyrk1A mice from SC+HF group were unable to compensate their energy intake by increasing their consumption of SC, indicating inflexible behavior. Conversely, in the SC+CM group, both mBACTgDyrk1A and their WT littermates increased their SC intake close to their energy intake of free feeding conditions. This pattern is different from trisomic mice that showed inflexibility both upon HF and CM exposure. However, the different behaviors showed by WT mice of both strains, that paralleled that of their mutant counterparts, suggest that C57BL/6J strain is more flexible to adapt to the available food sources than B6EiC3H. This strain effect makes impossible to discern the contribution of DYRK1A in the trisomic context.

Even so, in the quinine adulteration experiment, both Ts65Dn and mBACTgDyrk1A showed persistent consumption of adulterated foods compared to their respective WTs, suggesting that DYRK1A could had a role in DS compulsive and inflexible behaviors. However, this inflexible response was associated with specific diets. As such, both WT and mBACTgDyrk1A reduced the consumption of adulterated SC and HF, but the consumption of bitter CM, which is well inhibited in WT, was not reduced in mBACTgDyrk1A mice. This result again suggests that *Dyrk1A* overexpression promotes inflexible behavior.

This CM-specific inflexible response could be related to altered reward-related mechanism. In fact, long-term access to CM promoted reduced preference for sucrose in WT mice but not in mBACTgDyrk1A that showed a non-significant increase of their preference for sucrose (diet x genotype integration ANOVA $F(2,47) = 2.57$; $P = 0.09$). Accumulating evidence has shown that obesogenic diets lead to reward hypofunction. Consequently, mice usually show a reduced preference for sweets solutions upon long-term access to palatable treats like HF diets (Carlin et al. 2013; Rabasa et al. 2016). In agreement with these studies, it has been suggested that obesity might arise from a basal reward hyposensitivity where the higher consumption of energy-dense reflects an anhedonic state. Similarly, in mouse models both a reduction and an increase in the preference for sucrose consumption are thought to reflect aberrant responsiveness to reward (Mourlon et al. 2010). The increased sucrose preference in mBACTgDyrk1A mice could be an indicator of an increased seeking for sweet rewards, also observed in DS patients (Nordstrøm et al. 2015). Interestingly preliminary results of a study performed in collaboration with Dr Elena Martín García from the University Pompeu Fabra, suggest that mBACTgDyrk1A are more impulsive in a progressive ratio paradigm to receive chocolate pellets, which is in line with higher sensitivity for food rewards and thus increased craving (Dr Elena Martín, personal communication).

The involvement of *Dyrk1A* in reward related mechanism has not been addressed before but several studies suggest that DYRK1A influences the dopaminergic system (Martínez de Lagrán et al. 2007; Barallobre et al. 2014). Specifically related to reward, it has been shown that *Dyrk1A* dosage participate in the control of the dopaminergic neurons survival during development (Barallobre et al. 2014) an effect possibly mediated through modulation of the caspase-9, that is phosphorylated by DYRK1A, which prevents the subsequent activation of the intrinsic apoptotic pathway (Laguna et al. 2008). Although the number of dopaminergic cells is not altered in the ventral tegmental area (VTA) of adult mBACTgDyrk1A mice (Barallobre et al. 2014), it was proposed that synaptic wiring of the dopaminergic system may be altered upon *Dyrk1A* overexpression, with a potential functional implication in adulthood.

DYRK1A seems to be related specifically to the modulation of sweet preference and could be implicated in food craving and reward seeking. This may explain in part why DS patients over-consume sweets and high-fat diets. However, the comparison with the

Ts65Dn model suggests that in the context of the trisomy some of its effects are diluted, probably due to the impact of the genetic background.

5.3.5 Possible confounding influence of genetic background on obesity related behavioral and metabolic phenotypes

Table 6 shows that *Dyrk1A* overexpression only recapitulates some of the obesity phenotypes described in Ts65Dn mice. However, the association of the phenotypes with the overexpression of DYRK1A could not be directly inferred from the similarities between the two DS models since the genetic background of mBACTgDyrk1A and Ts65Dn mice are different and affect the penetrance of the explored phenotypes (see Annex II).

Preference for sweets solutions is strongly affected by the genetic background (Lush 1999; Pothion et al. 2004; Lewis et al. 2005). In fact, here again we detected a strain effect since wild-type counterparts of DYRK1A transgenic mice (WT(TG) mice showed reduced intake for adulterated foods compared to WT(TS) (see Annex II). In fact, it has been shown that although both C57BL/6J and C3HeB/FeJ mice are able to detect low concentrations of quinine, C3HeB/FeJ (C3) are clearly less sensitive than C57BL/6J mice (Boughter 1992), but in our experiments, in non-obesogenic conditions, independently of the genotype and the model, mice of both strains had similar preference for saccharin and sucrose for the doses tested.

Table 6. DS obesity phenotypes in Ts65Dn (TS) and mBACTgDyrk1A (TG) mice.

OBESITY PHENOTYPES	Ts65Dn (TS) vs. mBACTgDyrk1A (TG)
Body weight gain	Long-term HF and CM free access promoted body weight gain in TS mice whereas TG mice maintained their body weight (SC+CM group) or gained less weight (SC+HF group).
Body fat mass	TS mice showed increased body fat mass whilst it has been shown that in TG mice the visceral epidydimal fat pads and perirenal fat pads are lower by 60% and by 30%, respectively compared with WT mice (Song et al. 2015).
Glucose homeostasis	TS mice are glucose intolerant whilst it has been described that TG mice are glucose tolerant and hyperinsulinemic (Rachdi et al. 2014b).
Feeding behaviors in non-obesogenic conditions	Compared to WT mice, TS have longer and slower meals whereas the feeding behaviors were unchanged in TG mice.
Feeding behaviors in free access to CM	TS and TG (and their relative WT) showed a reduction of the average duration and an increased eating rate for energy-dense foods compared to SC
Food preference vs. SC: Fat food Carbohydrate- rich food	Independently of the genotype HF was highly preferred against SC. TS mice showed higher HF intake than their WT whereas TG did not. The preference for CM against SC was less strong than HF against SC. In TS model, the preference was moderate as compared to the TG model.
Energy compensation	24 hours of starvation lead to higher hyperphagia upon refeeding in both TS and TG mice. When HF was restricted to 1h/day during the limited access test TS and TG mice did not increased their SC energy intake. When CM was restricted TG did.
"Binge-like events"	Both TS and TG showed increased CM intake than WT when is offered in a limited access schedule. However, the energy consumed and the pattern of escalation in TG mice was more pronounced that in TS mice.
Inflexibility and impulsivity	Inflexibility in TS mice was revealed though bitter CM and HF intake whereas in TG mice was revealed by bitter CM intake.
Preference for sweets solutions	In both genotypes, SC+CM groups had reduced preference for sucrose compared to SC mice. In SC+CM groups, the preference for sucrose was reduced in TS mice and increased in TG mice.

CONCLUSIONS

6. CONCLUSIONS

1. The Ts65Dn mouse model recapitulates some phenotypes of Down syndrome obesity including increased adiposity and feeding behavior abnormalities. However, trisomic mice do not show increased body weight or BMI in basal conditions, suggesting that obesity manifests only in obesogenic environments and thus an adequate nutrition could significantly prevent obesity in this population.

2. In obesogenic environments, Ts65Dn mice show more propensity to body weight gain and increased intake and preference for energy-dense diets suggesting that both impaired control of food intake and motivational aspects might contribute to overeating in Down syndrome.

3. In spite of the differential behavioral adaptations detected between Ts65Dn and WT mice in obesogenic environments, our neurochemical analysis revealed similar diet-induced variations in brain monoamines, suggesting that changes on the composition would not be the major contributor to the behavioral abnormalities.

4. We detected increased inflexibility and food abnormal craving in Ts65Dn mice that could be partially due to a reward deficient profile, since we found correlation with prefrontal hypo-dopaminergic function. This might bias food election towards energy-dense foods in Down syndrome.

5. Ts65Dn mice present impaired fasting glycaemia and impaired glucose tolerance along with hypoinsulinemia that could be due to impaired insulin secretion but also to a deficient insulin output since Ts65Dn mice had reduced β-cell mass.

6. The reduction in pancreatic β-cell mass could be related to the finding of increased circulating levels of several inflammatory and oxidative markers that are increased in obesity, and may reflect autoimmune pancreatic β-cell destruction.

7. Ts65Dn had a significantly higher acute leptin response to oral glucose administration and non-significant higher steady-state leptin levels, replicating the leptin resistance phenotype of Down syndrome.

8. The single overexpression of *Dyrk1A* cannot explain the overweight observed in Ts65Dn mice upon exposure to an obesogenic environment. Indeed, our results confirm previous reports showing that DYRK1A might rather have a positive effect in body weight regulation.

9. The increased preferences and compulsive responses to carbohydrate-rich foods detected in mBACTgDyrk1A mice suggest a specific role of DYRK1A in those phenotypes.

10. *Dyrk1A* overexpression in mice is sufficient to recapitulate the brachycephalic phenotype associated to Down syndrome, without altering mandible morphology. Our work adds a piece of evidence supporting the role of DYRK1A in craniofacial morphogenesis but discard its involvement in Down syndrome feeding behavior alterations.

In summary, our work sheds light to some important mechanisms involved in Down syndrome obesity, and suggests that prefrontal cortex-derived traits such as impulsivity and reward sensitivity are involved in the propensity to overeating in this population. This adds to a certain leptin resistance, and possibly to autoimmune pancreatic β-cell destruction thought inflammatory and oxidative stress mechanisms. We propose that *Dyrk1A* overexpression might be related to some of the behavioral abnormalities associated to overeating, especially for carbohydrate-rich foods, but its overdosage is not sufficient to recapitulate Down syndrome obesity.

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ANNEX

ANNEX

ANNEX I: ABREVIATIONS

AD	adrenaline
AgRP	agouti-related protein
ARC	arcuate nucleus
BW	body weight
BMI	body mass index
BF	body fat
CART	cocaine- and amphetamine-regulated transcript
°C	Celsius
CM	chocolate-mixture diet
cm	centimeter
CNS	Central Nervous System
DA	dopamine
DS	Down syndrome
DYRK1A	Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1A
DOPAC	3-4dihydroxyphenylacetic acid
g	gram
s	second
HT	hypothalamus
HF	high-fat diet
HSA21	Homo sapiens autosome 21
h	hour
HPLC	High- performance Liquid Chromatography
HVA	homovanillic acid
mBACTgDyrk1A	Transgenic mice overexpressing Dyrk1A
MHPG	3-methoxy- 4-hydroxyphenylglycol
min	minute
ml	milliliter
mM	millimolar

mm	millimeter
mg	milligram
MRI	Magnetic resonance imaging
MMU16	Mus musculus chromosome 16
MMU17	Mus musculus chromosome 17
NA	noradrenaline
NAc	nucleus accumbens
NMR	nuclear magnetic resonance
NPY	neuropeptide Y
n	number
OGTT	oral glucose tolerance test
PBS	Phosphate Buffered Saline
PET	Positron emission tomography
PFC	prefrontal cortex
POMC	proopiomelanocortin
Quin	quinine hydrochloride
SC	Standard chow
SC+CM	standard chow + chocolate-mixture diet
SC+HF	standard chow + high-fat diet
SEM	Standard Error of the Mean
ST	striatum
SPECT	Single-photon emission computed tomography
TG	Transgenic mice overexpressing Dyrk1A
Ts65Dn	Trisomic mice
TS	Trisomic mice
VTA	vental tegmental area
WT	Wild Type
5-HIAA	5-hydroxy-3-indolacetic acid
5-HT	5-hydroxytryptamine, serotonin
µl	microliter
µm	micrometer
µCT	X-ray micro computed tomography

ANNEX II: SUPPLEMENTARY RESULTS

1. Increased levels of inflammatory plasma markers and obesity risk in a mouse model of Down syndrome.

Manuscript in second revision in Free Radical Biology and Medicine.

Increased levels of inflammatory plasma markers and obesity risk in a mouse model of Down syndrome

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Abstract

Down syndrome (DS) is caused by the trisomy of human chromosome 21 and could lead to multiple endocrine and nervous system alterations. Those might account for the higher prevalence of obesity in DS compared to the general population. Obesity usually is accompanied by a sustained inflammatory state. However, the link between adiposity and inflammation has not been explored in DS.

Here we used the Ts65Dn mice, a validated DS mouse model, for the study of obesity and obesity-related inflammatory status in lean mice (maintained in standard chow) and in obese mice (under high-fat diet). Compared to wild type (WT), lean Ts65Dn mice had altered plasma levels of some anorexigenic and orexigenic signals like leptin and ghrelin that might affect feeding behavior and energy balance. Independently of the diet, Ts65Dn mice have increased body adiposity along with increased energy consumption and energy expenditure compared to WT. Concomitantly, Ts65Dn mice had increased plasma galectin-3 and HSP72 levels which are markers of inflammation and oxidative damage. Our results suggest that an altered immune response in Ts65Dn mice could potentially contribute to obesity propensity in DS.

Highlights

In non-obesogenic conditions, Ts65Dn mice present increased body adiposity, reduced plasma ghrelin in starved conditions and higher leptin levels upon re-feeding than WT mice. The levels of galectin-3 and HSP72 in plasma are higher in Ts65Dn mice compared to WT suggesting increased inflammatory and oxidative stress in trisomic mice.

Keywords

Down syndrome, inflammation, obesity, leptin, ghrelin, resistin, interleukin-6, galectin-3, HSP72

Abbreviations

DS: Down syndrome, gal-3: galectin-3, GIP: glucose dependent insulinotropic polypeptide; GLP-1: glucagon-like peptide-1(7-36) amide, HF: high-fat, HSP72: heat shock protein HSP (72), IL-6: interleukin-6, ROS: reactive oxygen species, PAI-1: Plasminogen activator inhibitor 1.

1. Introduction

Down syndrome (DS) is caused by trisomy of chromosome 21 and is associated to multiple endocrine and nervous system alterations [1]. Individuals with DS have high prevalence of obesity possibly due to metabolic complications, insufficient exercising and poor eating habits [2] [3] [4]. DS is associated with high body adiposity [5] [6] suggesting that a propensity for fat accumulation could be a major contributor of DS obesity.

In the general population, high adiposity is associated with inflammation and oxidative stress markers [7] [8]. Indeed, visceral fat accumulation contributes to pro-oxidant and pro-inflammatory states, as well as to alterations in glucose and lipid metabolism [8]. Among the mechanisms involved, hormones directly implicated in food intake regulation such as leptin, a hormone secreted by the adipose tissue, increase the secretion of inflammatory cytokines and stimulates the production of reactive oxygen species (ROS), leading to increased inflammation and oxidative stress [9] [10] [11]. Recently, it has also been shown that ghrelin levels are negatively correlated with nitric oxide concentration in plasma of obese patients [12].

Conversely, a deficient immune response might increase the vulnerability to obesity development. Modulators typically related to immune process such as interleukin-6 (IL-6) galectin-3 and extracellular heat shock protein 72 (eHSP72), are positively correlated with obesity and inflammation in the general population [13] [14] (Krause, Keane et al, 2014).

Increased susceptibility to autoimmune disorders, hypothyroidism, celiac disease and diabetes mellitus are more common in DS individuals than in non-DS individuals [15], all clinical conditions having in common inefficient immune responses or abnormally increased inflammation [16]. However, the potential link between adiposity and inflammation has not been explored. Here we used the Ts65Dn mice, a validated DS mouse model, for the study of the effect of trisomy 21 on adiposity, energy balance and inflammatory status in non-obesogenic (standard rodent chow) and obesogenic conditions (high-fat chow).

2. Methods

Animals and housing

4-6 moths-old male Ts(1716)65Dn (Ts65Dn) and wild-type (WT) mice used in this study were obtained through crossings of a B6EiC3Sn a/A-Ts (1716)65Dn (Ts65Dn) female to B6C3F1/J males purchased from The Jackson Laboratory (Bar Harbor, ME). Genotyping was performed by amplifying genomic DNA obtained from the mice tail as previously described in [17]. The mouse colony of Ts65Dn mice was bred in the Animal Facilities if the Barcelona Biomedical Research Park (PRBB, Barcelona, Spain, EU) in controlled laboratory conditions with the temperature maintained at 22 ºC ± 1oC and humidity at 55 ± 10% on a 12 hours light/dark cycle (lights off 20:00 hours). Food and water were available *ad libitum* except during food deprived experimental sessions. All experimental procedures were approved by the local ethical committee (Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB); procedure number MDS-12-1464P3) and the institutional animal care and use committee of the Paris Diderot University (CEEA40), met the guidelines of the local (law 32/2007) and the Standards for Use of Laboratory Animals n° A5388-01 (NIH). Both Institutes are authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14).

Diets

Ts65Dn and WT mice were given standard chow, SC (SDS, UK) for the assessment of the genotypic differences in body weight, body composition, metabolic phenotyping and plasma biomarkers in non-obesogenic conditions. Those parameters were also studied in obesogenic conditions, by given Ts65Dn/WT mice *ad libitum* access for 8 weeks to a high-fat diet (High-fat/DIO, Test Diet, USA). Supplementary Table 1 shows the composition of the different experimental diets.

Indirect calorimetry and metabolic phenotyping

Metabolic phenotyping was performed using calorimetric cages (Labmaster, TSE Systems GmbH, Bad Homburg, Germany) located in the Functional & Physiological Exploration Platform (FPE) of the Functional & Adaptive Biology (BFA) Unit of the University Paris Diderot-Paris 7, CNRS UMR 8251. The apparatus combine a set of highly sensitive feeding and drinking sensors for automated online measurements, and a frame with an infrared light beam based activity monitoring system, allowing measurement of total locomotion. The sensors for gases and detection of movement

operate efficiently in both light and dark phases, allowing also continuous recording. Ratio of gases was determined through an indirect open circuit calorimeter [18] [19]. This system monitors O2 and CO2 concentration by volume at the inlet ports of a tide cage through which a flow of air (0.4 l/min) is being ventilated and compared regularly to a reference empty cage. Whole energy expenditure is calculated according to the Weir equation respiratory gas exchange measurements [20]. Sensors were previously calibrated with a mixture of known concentrations of O2 and CO2 (Air Liquide, Paris, France).

Mice were individually housed in the calorimetric cage in controlled laboratory conditions with the temperature maintained at 22 ºC ± 1oC on a 12 hours light/dark cycle (lights off 19:00 hours). Depending of the experimental condition, Ts65Dn/WT mice had *ad libitum* access to water and standard or high-fat chow diet (SC: WT, n = 10; Ts65Dn, n = 8; HF: WT, n = 6; Ts65Dn, n = 6). All animals were acclimated for 48 h and energy intake (KJ/ mouse body weight), locomotor activity (infrared breaks/h), oxygen consumption/carbon dioxide production (VO2, VCO2; ml/kg of lean mass/h), respiratory exchange rate (RQ ¼ VCO2/VO2) and energy expenditure (KJ/h) were established within the next 72 h. Representative 24 h measurements were the mean of the three consecutive experimental days. Data were collected every 40 min during the experimental times and analysis were performed on excel XP using extracted raw value of VO2 consumed, VCO2 production (expressed in ml/h), energy expenditure (KJ/h). Subsequently, each value was expressed either by total body weight or whole lean tissue mass extracted from the EchoMRI analysis (see below).

Whole-body composition analysis

Non-invasive analysis of body composition were estimated in a cohort of 5 months-old male Ts65Dn /WT mice feed with standard chow (WT, n = 10; Ts65Dn, n = 8) and a cohort of mice fed with high-fat fed mice for 8 weeks (WT, n = 6; Ts65Dn, n = 6) by nuclear magnetic resonance (NMR) technology via a scanner EchoMRI 900 (Echo Medical Systems, USA), that creates contrast between soft tissues taking advantage of the differences in relaxation times of the hydrogen spins and/or hydrogen density (http://www.echomri.com/). Lean mass is equivalent to muscle mass and organs. Body fat does not distinguish between different types of fat and fat depositions in every organ including the brain, muscles, other organs, bone marrow, and circulating blood lipids, and fatty acids.

Briefly, the mouse is placed in a specially sized, clear plastic holder without sedation or anesthesia and the holder is then inserted into a tubular space in the side of the EchoMRI™ system for 1 minute. In our experiment, lean and fat body composition was directly obtained from EchoMRI™ measurements in grams. From this data, body composition was calculated for each mice and corrected for body weight.

Bio-Plex Pro Mouse Diabetes Assay

The evaluation of the incretins activity and the response to glucose of related metabolic parameters, was performed in 5 months-old male Ts65Dn/WT mice on a standard chow diet prior to food deprivation and after a glucose load. Ghrelin, glucose dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1(7-36) amide (GLP-1), glucagon, insulin, leptin, Plasminogen activator inhibitor-1 (PAI-1) and resistin were measured in Ts65Dn (n = 7) and WT (n = 11) mice, using suspension bead array immunoassay kits following manufacturer's specifications (Bio-Plex Pro Mouse Diabetes Assay 8-plex, Biorad, France). Mice were food deprived for 5 hours prior to an oral administration of 2g/Kg of 30% glucose (Lavoisier, France). Blood was collected from the tail vein before the glucose administration (T0) and 30 minutes after (T30) to mimic the postprandial levels. Upon centrifugation of the blood, plasma was stored in presence of Linagliptin, a DPP-4 inhibitor (Selleckchem, USA) and Protease inhibitor (Roche, France) to inhibit the degradation of incretins. The samples were measured using a Luminex 200 apparatus (Luminex, Austin, TX).

Hormonal and metabolic determinations

Galectin-3, HSP72 and interleukin-6 were measured from blood in a cohort of 4-5 months-old male Ts65Dn /WT mice feed with standard chow (WT, n = 6; Ts65Dn, n = 5) and a cohort of mice fed with high-fat fed mice for 8 weeks (WT, n = 8; Ts65Dn, n = 4). Samples were obtained by retro-orbital sinus sampling with heparinized capillaries into the tubes containing a 1/10 volume of 3.8% sodium citrate. Plasma was isolated by centrifugation at 2500 x g for 15 min at 4°C. Plasma galectin-3 (Abcam ELISA kit, France), HSP72 (Enzo Life sciences ELISA kit, Germany) and IL-6 (Enzo Life sciences ELISA kit, Germany) were assessed using sandwich ELISA. After removal of unbound conjugates, bound enzyme activity was assessed by use of a chromogenic substrate for measurement at 450 nm by a microplate reader (Flex Station 3, Molecular Devices, Ltd., UK). Alanine aminotransferase (ALT) level was assayed using the Alanine

Aminotransferase Activity Assay Kit (Sigma-Aldrich, France), based on the pyruvate generated. All assays were performed in duplicate.

Statistical analyses

Sample size was estimated based on our previous experience with similar experiments. Differences between genotypes and diet groups were determined using 2-tailed unpaired Student's t test. Two-way ANOVA with Bonferroni post hoc test to correct for multiple comparison was used to test diet and genotype interaction. Levels of statistical significance were set at $P<0.05$. The statistical analyses were performed using the by using Statistical Package for Social Science software (SPSS version 17.0, IBM Corporation, USA).

3. Results

Increased adiposity and positive energy balance in Ts65Dn mice

The body weight and mass index of 5 months-old Ts65Dn mice fed with standard chow was similar to WT littermates (Figure 1 A-B), but Ts65Dn mice presented a significant reduction of body length ($t(1, 16) = 4.23$, $P< 0.01$, Figure 1C).

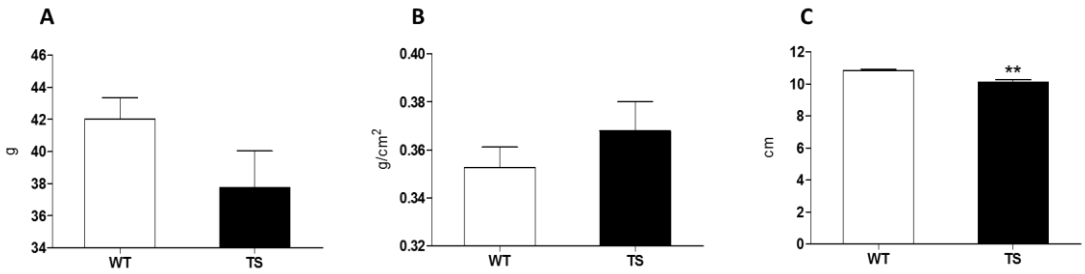

Figure 1. Somatometric characteristics of wild type (WT) and Ts65Dn (TS) mice fed with standard chow (SC). (A) Body weight (grams), (B) body mass index (BMI; grams/cm^2) and (C) body length (cm) in WT mice (n=10) and TS mice (n=8) fed with standard chow (SC). Student's T test for independent samples ** $P<0.01$.

Using Nuclear Magnetic Resonance (NMR) we detected reduced lean body mass ($t(1, 16) = 2.92$; $P = 0.01$; Figure 2A) and a higher percentage of body fat per body weight in Ts65Dn as compared to WT ($t(1, 16) = -2.96$, $P< 0.01$; $P<0.05$; Figure 2B). These features are in agreement with DS human somatometric characteristics that have low height and high adiposity as compared to typical development peers [6] [21].

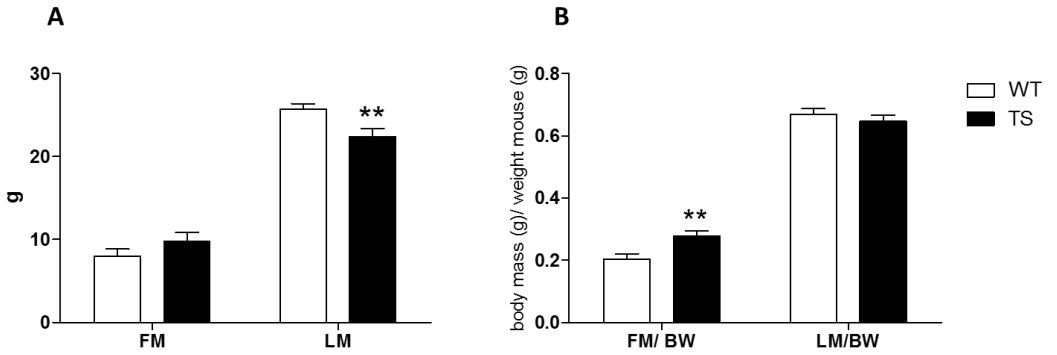

Figure 2. NMR non-invasive analysis of body mass composition in wild-type (WT) and Ts65Dn (TS) mice. Bar blots depicted (A) body composition in lean (LM) and fat mass (FM) represented in grams, and (B) body composition corrected for body weight (BW) in TS (n = 8) and WT (n = 10) mice. Data expressed as mean ± SEM. Student's T test for independent samples ** $P<0.01$.

We also measured the energy intake, and locomotor activity, energy expenditure and respiratory exchange ratio of Ts65Dn and WT mice. During the active (dark) phase but not during the resting (light) phase of the circadian cycle, Ts65Dn mice showed higher energy intake compared to WT ($t(1, 9) = -2.65$; $P<0.05$; Figure 3A). Locomotor activity was similar in both genotypes, but we detected hyperactivity in Ts65Dn mice during specific periods of the dark phase (Figure 3B). Even so, Ts65Dn mice still showed a significantly higher positive energy balance during the dark phase than WT mice ($t(1, 9) = -2.43$; $P<0.05$, Figure 3C), as measured by indirect calorimetry. To further analyze the effect of trisomy on whole-body energy homeostasis, we determined respiratory quotient. There were no significant differences between genotypes in total oxygen consumption (Figure 3D) or carbon dioxide production (Figure 3E) and consequently, the respiratory quotient ($RQ = VCO_2/VO_2$) was similar (Figure 3F).

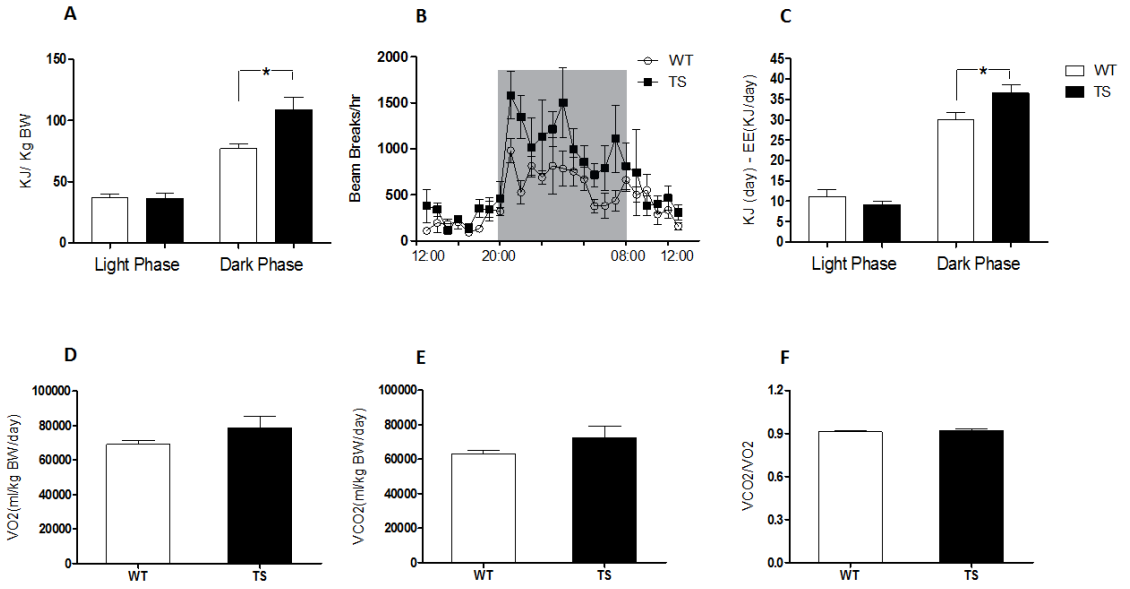

Figure 3. Indirect calorimetry in wild-type (WT) and Ts65Dn (TS) mice fed with standard chow. Bar blots depicted (A) energy intake (KJ/mouse body weight) during the inactive (light phase) and active (dark phase) period of the 12hours light/dark cycle. (B) Daily locomotor activity. (C) Energy balance (food intake – estimated resting energy). (D) Volume of total oxygen consumption, VO_2. (E) Volume of total carbon dioxide production, VCO_2. (F) Respiratory quotient in TS (n = 6) and WT (n = 5) mice. Data expressed as mean ± SEM. Student's T test for independent samples * P< 0.05.

Altered levels of metabolic and inflammatory biomarkers in Ts65Dn mice

To study the effect of trisomy 21 on inflammation and oxidative stress, and the trisomic basal and the postprandial phenotype, the concentrations of ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1 and resistin were measured in Ts65Dn and WT mice after a 5 hours starvation (T0) and 30 minutes after an oral glucose load (T30). We obtained the metabolic profile of selected markers (Table 1) in Ts65Dn and WT mice using the Bio-Plex Pro™ mouse diabetes immunoassay.

Upon starvation, glucose levels were higher in plasma from Ts65Dn mice ($t(1, 17) = -2.59$; $P<0.05$) compared with WT mice (Table 1), while the levels of insulin ($t(1, 17) = 2.40$; $P< 0.05$) and ghrelin ($t(1, 16) = 3.17$; $P<0.01$) were significantly reduced. Upon a glucose load, the levels of glucose in Ts65Dn mice remained significantly higher ($t(1, 17) = -2.26$, $P<0.05$) and the levels of insulin were lower than WT, although the difference did not reach statistical significance ($t(1, 17) = 2.07$; $P = 0.054$). Also, the levels of ghrelin were still slightly, though not significantly lower in Ts65Dn mice. While leptin levels were similar in both genotypes in starved conditions, after an oral glucose load Ts65Dn showed a non-significantly higher plasma leptin levels ($t(1, 17) = -2.09$; $P = 0.052$) than WT. GLP1 and GIP were not different, suggesting no incretin

dysfunction and the rest of the markers studied also exhibit similar profile in Ts65Dn and WT mice (Table 1).

		Ghrelin (pg/ml)	GIP (pg/ml)	GLP-1 (pg/ml)	Glucagon (pg/ml)	Insulin (ng/ml)	Leptin (pg/ml)	PAI-1 (pg/ml)	Resistin (pg/ml)	Glucose (mg/dl)
T_0	WT	5595.74 ± 360.27 **	518.16 ± 12.48	55.43 ± 2.28	387.07 ± 15.62	3658.11 ± 122.07 *	7193.91± 541.18	2876.12 ± 154.90	50159.32 ± 1696.01	195.5 ± 5.86 *
	TS	1540.14 ± 2.41.39	584.42 ± 30.78	54.53 ± 2	386 ± 19.51	2528.17 ± 135.78	10173.25± 848.14	2605.35 ± 221.52	48765.63 ± 1810.21	251.33 ± 4.55
T_{30}	WT	6382.22 ± 476.85	575.37 ± 11.42	121.15 ± 7.41	696.22 ± 37.63	6332.15 ± 200.37	12763.55 ± 1004.05	5155 ± 254.42	79604.79 ± 2515.79	340 ± 13.72 *
	TS	3421.21 ± 327.81	673.40 ± 31.77	135.86 ± 6.26	511.03 ± 22.28	4709.02 ± 193.55	20783.37 ± 1174.96	4416.92 ± 635.62	97005.72 ± 3480.49	470.67 ± 22.03

Table 1. Plasma levels of obesity biomarkers markers in wild type (WT) and Ts65Dn (TS) mice in starved conditions (T0) and 30 minutes after a glucose load (T30). Data are expressed as mean ± SEM. WT = 11, TS = 7. WT vs TS; Student's T test for independent samples * P< 0.05, ** P<0.01.

To see whether these mediators could explain the increased energy intake observed in Ts65Dn mice, we calculated the differences between genotypes for the changes of the hormone concentrations upon the glucose load (Figure 4). Compared to WT mice, Ts65Dn mice showed a significantly higher increase of leptin and resistin levels that have been previously associated with impaired glucose tolerance [22] and central leptin resistance [23]. No significant differences for the changes on the plasma concentrations of the rest of the markers were observed between genotypes after refeeding.

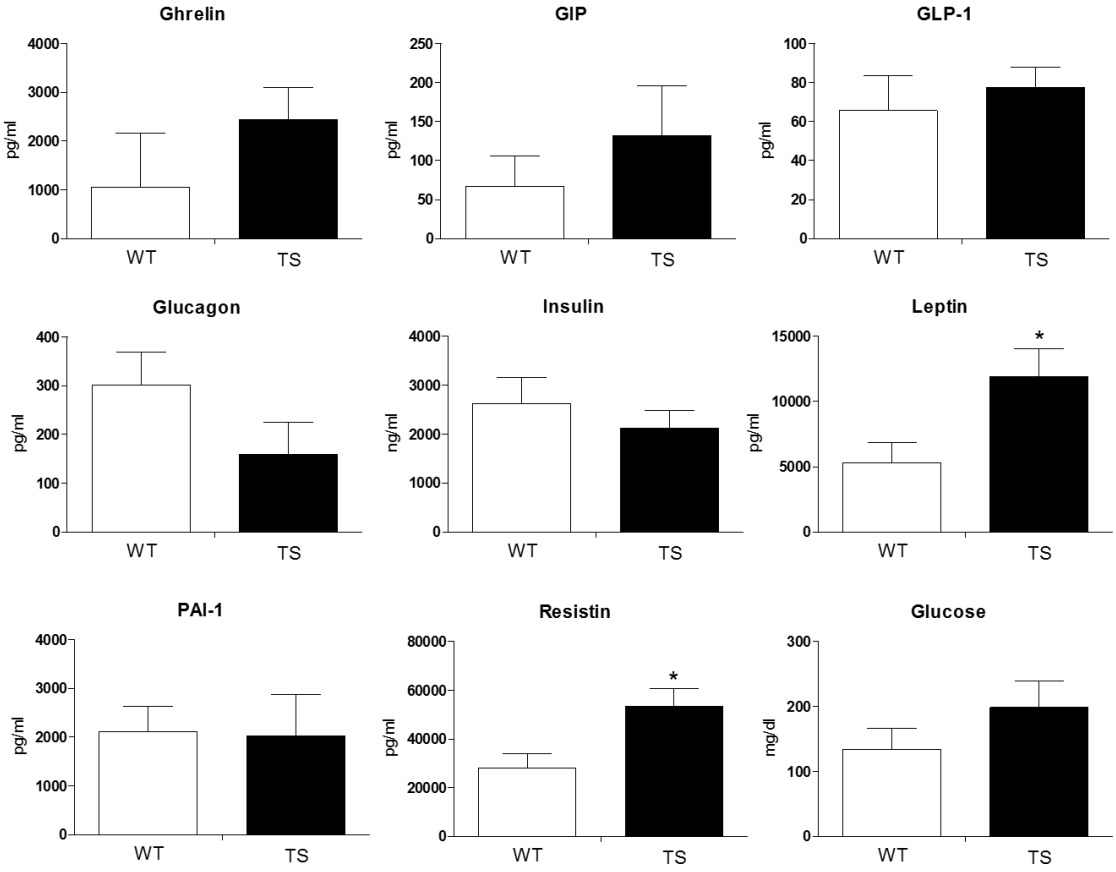

Figure 4. Changes in plasma levels of obesity biomarkers in wild type (WT) and Ts65Dn (TS) mice 30 minutes after a glucose load. Data are expressed as mean ± SEM. WT = 11, TS = 7. WT vs TS; Student's T test for independent samples * P< 0.05.

Body weight increase, body composition and indirect calorimetry upon HF diet access

8-weeks of access to high-fat (HF) diet promoted body weight gain in both genotypes being significantly higher in Ts65Dn mice ($t(1, 10) = -3.32$; $P < 0.01$; Figure 5A). We confirmed by NMR that both genotypes increased their body fat mass in a similar manner. However, when corrected by body weight, Ts65Dn again showed higher percentage of body fat compared to WT mice ($t(1, 10) = -3.40$; $P < 0.01$; Figure 5B), as we already observed in standard chow conditions.

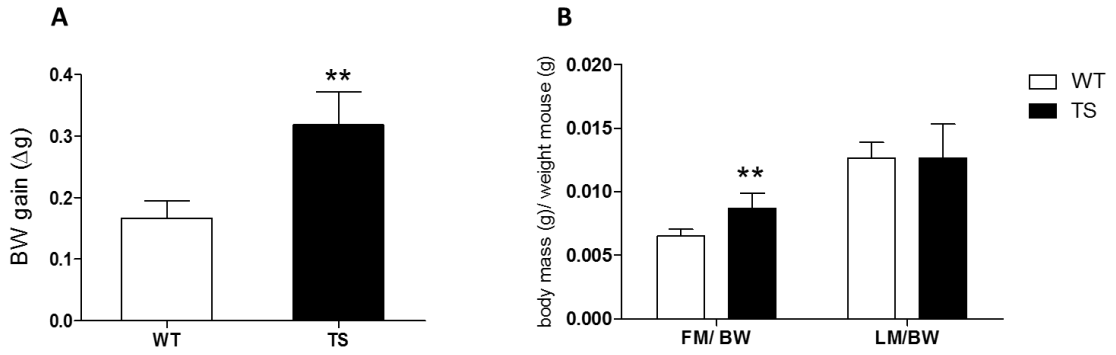

Figure 5. Body weight gain and fat body composition in obese wild type (WT) and Ts65Dn (TS) mice. (A) Body weight gain upon 8 weeks of HF. (B) Lean /body weight (g) and fat mass/ body weight (g) in the 8th week. WT = 6, TS = 6. Data expressed as mean ± SEM. Student's T test for independent samples ** P<0.01.

To assess if obesity development in Ts65Dn mice was associated to metabolic changes, we performed indirect calorimetry. Again, Ts65Dn mice showed higher energy intake (KJ consumed per mouse body weight; t (1, 8) = -3.51; P<0.01, Figure 6A) while the ambulatory activity was similar between genotypes (Figure 6B). Both genotypes had negative energy balance (during light phase and positive during the dark period (Figure 6C). Measurements of oxygen consumption and carbon dioxide production were increased for Ts65Dn mice fed a HF diet relative to WT (TS vs. WT (VO_2) t (1,8) = -3.7; P<0.01; TS vs. WT (VCO_2), t (1,8) = -3.81; P<0.01; Figure 6D-E). However, the metabolic exchange (RQ = CO_2/O_2) was similar between genotypes (Figure 6F).

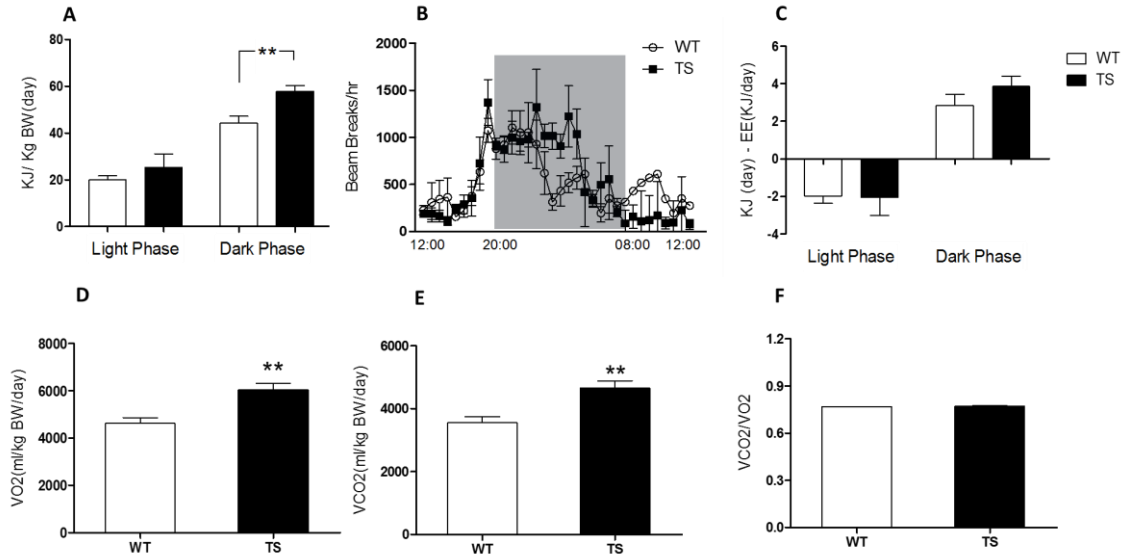

Figure 6. Indirect calorimetry in wild-type (WT) and Ts65Dn (TS) mice fed with high-fat diet (HF). Bar blots depicted (A) Energy intake (KJ/mouse body weight) during the inactive (light phase) and active

(dark phase) period of the 12hours light/dark cycle. (B) Daily locomotor activity. (C) Energy balance (food intake – estimated resting energy). (D) Volume of total oxygen consumption, VO_2. (E) Volume of total carbon dioxide production, VCO_2. (F) Respiratory quotient in TS (n = 5) and WT (n = 5) mice. Data expressed as mean ± SEM. Student's T test for independent samples, ** P<0.01.

Trisomy 21 in mice is associated with higher plasma levels of galectin-3 and HSP72

There is clear evidence that inflammatory signaling contributes to obesity-associated insulin resistance in peripheral tissues [24]. Hence, plasma levels of molecules responding upon an insult such as galectin-3, HSP72, and interleukin-6 (IL-6) have been shown to positively correlate with human obesity [13] [14].

We observed that Ts65Dn had higher levels of both galectin-3 and HSP72 independently of the diet compared to WT (genotype effect for galectin 3, ANOVA, F (1, 23) = 23.31, P<0.001; Figure 7A; and for HSP72 ANOVA, F (1, 23) = 7.2, P<0.05; Figure 7B). Upon 8 weeks of HF diet, we only observed in WT mice a non-significant increase for HSP72, along with unchanged levels of circulating galectin-3 (Figure 7B).

The plasma IL-6 levels of Ts65Dn SC fed mice were lower compared to WT mice, Figure 7C) reproducing some previous studies in DS patients [25]. However, both genotypes showed similar IL-6 levels upon HF diet that were higher than those of mice fed with standard chow (diet effect, ANOVA F (1, 23) = 18.25, P<0.001).

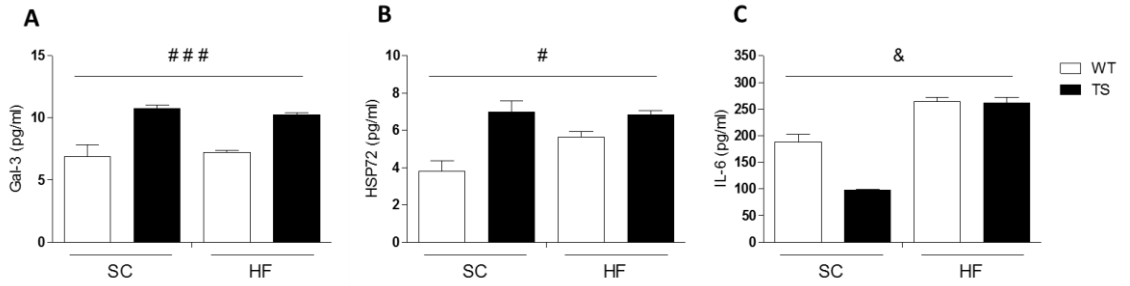

Figure 7. Measured level of inflammatory markers in wild type (WT) and Ts65Dn (TS) mice in non-obesogenic (standard chow, SC) and obesogenic conditions (high-fat diet, HF) fed conditions. Data are expressed as mean ± SEM. WT (SC) = 6, WT (HF) = 8; TS (SC) = 5, TS (HF) = 4. Two-way ANOVA. A-B) ANOVA # P< 0.05; ## P< 0.01; ### P< 0.01; C) & P< 0.001 genotype effect.

4. Discussion

DS obesity has been mainly attributed to insufficient exercising and reduced energy metabolism or to poor eating habits [26] [27]. However, there is ample evidence of the

involvement of oxidative stress and inflammation that are associated with increased propensity for fat accumulation in the general population [24] [28].

Here we showed that Ts65Dn mice, a mouse model of DS, had increased percentage of body fat compared to WT, indicating higher propensity to fat accumulation and reproducing human data [5] [6]. Ts65Dn had increased energy intake and a positive energy balance which supports hyperphagia, despite their significantly increased locomotor activity. The contribution of energy metabolism and energy expenditure in DS obesity is controversial. Some reports have shown that resting metabolism is reduced in DS [29] [30] [31], possibly due to defects on thyroid gland function, which is prevalent in DS [32]. DS neonates and children have significantly decreased VO_2, VCO_2, and resting energy expenditure (REE) when compared with controls [30] but the reduced REE was not explained by thyroid function or adiposity (Allison, Gomez et al. 1995). Moreover, having a lower REE at baseline, was not predictive of gain in fat mass over time [30]. Indeed, others have not found differences when compared to non-DS people [33] [34]. In our experiments, the similar positive energy balance in mice of both genotypes suggests that the hyperphagia observed in Ts65Dn mice is not a consequence of an increased metabolism.

Ts65Dn showed higher circulating glucose compared to WT both in starved conditions and upon glucose load, in agreement with previous studies in Ts65Dn mice, but also in Ts16 mice, a mouse model with complete trisomy of chromosome 16 [35]. We also detected reduced insulin plasma levels in fasting and glucose loaded conditions. The prevalence of type 1 diabetes in DS is higher compared to the general population [36] [37]. Therefore, the hypoinsulinemia of Ts65Dn mice could be due to the beginning of the autoimmune beta cell destruction leading to reduced beta cell mass, which are common pathological marks of the disease [38].

It has been shown that elevated serum leptin concentrations in human significantly predict the development of obesity and metabolic syndrome [39]. Ts65Dn had a significant higher acute leptin response to oral glucose administration and non-significant higher steady-state leptin levels. However, Ts65Dn mice exhibited hyperphagia, suggesting that they might be less reactive to the inhibiting appetite effects of leptin.

High plasma glucose levels in Ts65Dn mice could lead to increased leptin levels since in adipose tissue, mRNA levels of the ob gene, that encodes for leptin, are positively

correlated with glucose levels in lean animals after glucose loading, presumably leading to increased leptin secreted in plasma [40]. Greater leptin production per unit of adipose tissue has been proposed in DS humans [41] and could also explain this phenotype in Ts65Dn mice.

One important limitations of our study is that we did not explore directly the leptin resistance in our mice. However, the finding of hyperleptinemia along with hyperphagia in Ts65Dn mice lead to speculate that diminished leptin sensitivity might increase the risk of obesity development when exposed to an obesogenic environment. In support of this hypothesis, we detected a higher body weight gain and increased energy intake in Ts65Dn mice upon high-fat diet. It has been shown similar results in rats since those having greater absolute leptin response to intravenous glucose administration, also had increased energy intake and gained more weight upon 6 weeks of high-fat diet [42]. Moreover, although two studies have reported lower leptin levels in DS adults [43] and fetus [44] compared to non-DS controls, the majority of the existing DS human reports also support the leptin resistance phenotype. It has been shown that leptin levels correlates with adiposity in DS children and adolescents [45] [46] and free feeding leptin levels are higher in DS compared to unaffected siblings [41] [43].

In summary, according to our results, we propose that DS is a case of leptin resistance phenotype and thus healthy dietary habits could be a key strategy in preventing obesity development of obesity in DS despite their higher propensity to adiposity.

We here also detected significantly lower plasma ghrelin levels in Ts65Dn mice compared to WT mice. We only found one report of ghrelin levels in DS individuals showing no differences between typical developing peers [41]. In fact, in our experiments, the genotype-dependent differences were only significant in starved conditions. Interestingly, it has been shown that obese patients have usually lower plasma levels of ghrelin than lean individuals [47]. Besides the effect of leptin and ghrelin on appetite, several studies have shown that ghrelin acts also as an anti-inflammatory and antioxidant modulator [48] while leptin is implicated in the release of inflammatory cytokines and stimulates the production of reactive oxygen species (ROS), leading to increased inflammation and oxidative stress [49]. We could thus speculate that hyperleptinemia and hypoghrelinemia could be involved in the increased inflammation and oxidative damage associated to trisomy 21.

There are obviously other possible mechanisms related to obesity propensity in DS. Plasminogen activator inhibitor (PAI-1), which is regulated by the accumulation of visceral fat and associated with insulin resistance at high concentrations [50], has been shown to be reduced in blood of DS individuals compared with controls, probably thought action of DS-related proteins such as DYRK1A and RCAN1 that might attenuate the PAI-1 expression via the NGF-Calcineurin/NFAT pathway [51]. However, we here did not observe increased PAI-1 levels in Ts65Dn mice compared to WT.

The relationship between resistin and obesity is controversial. It has been shown that the levels of resistin in amniotic fluid of DS pregnancies are lower and it has been proposed that it could have an inhibitory effect on fat accumulation and to contribute to the tendency to childhood obesity in this population [52]. However, in general population other reports have also shown that high peripheral resistin levels are associated with impaired glucose tolerance, reduced insulin [22] and central leptin resistance [23] which is the case in Ts65Dn based on the measurement. Accordingly, we here found slightly higher levels in Ts65Dn mice, but even more important, a significant increase in resistin levels in Ts65Dn compared WT mice upon glucose overload. This may explain the paradox of Ts65Dn mice being lean but insulin resistant/hyperglycemic.

Incretins are also important regulators of feeding behavior. As a result, reduced GLP-1, GIP secretion and impaired incretin effect have been observed in obese subjects [53]. Glucagon has been also associated to increased inflammation in obese patients [54]. We did not find studies comparing the levels of GIP, GLP-1, and glucagon in plasma of DS and non-DS individuals. However, in our experiments, GIP, GLP-1, glucagon showed no difference between Ts65Dn and WT mice, suggesting no incretin dysfunction.

Although obesity and autoimmune diseases are common in DS (Giménez-Barcons, Casteràs et al. 2014), this relationship had not been addressed before in DS mouse models. Here we found that Ts65Dn mice had higher levels of pro-inflammatory and pro-obesogenic markers such as plasma galectin-3 and HSP72 compared to WT mice. Galectin-3 is a lectin which levels are highly increased during inflammation in both humans and obese mice [55]. Moreover, it has been showed that its administration causes insulin resistance and glucose intolerance in mice [56], possibly by diminishing monocyte to dendritic cell differentiation and T-cell antigen presentation [57]. Although we did not find previous reports on galectin-3 levels in DS individuals, it has been reported that it is significantly increased in patients with Alzheimer's disease [58].

Galectin-1, a lectin from the same family as galectin-3, is present in the placenta of DS pregnancies [59]. Therefore, our result suggest that galectin-3 could be a new interesting target for DS.

High levels of the stress heat shock protein 70 (HSP72) in plasma has been associated with autoimmunity [60] [61] and accumulating data show that HSP72 extracellular levels correlate with oxidative damage [62] [63]. According to our results, trisomy 21 seem the first example of a natural high level of plasma HSP72 which is in accordance with the high prevalence of autoimmune diseases in DS individuals.

Finally, Interleukin-6 (IL-6) is positively correlated with fat adiposity and inflammation in the general population and also in DS individuals in several studies. [64] [65] [66]. However, in our hands, Ts65Dn fed with SC had lower plasma IL-6 level compared to WT. We suspect that would parallel the depressed levels of IL-6 described before in DS children [25] [67]. As they proposed, that could favor the anti-inflammatory state and cause recurrent infection [25]. In support of this, it has been shown that mice lacking IL-6 expression develop systemic insulin resistance and late-onset obesity [68] [69].

Taken together our results suggest that Ts65Dn mice have a pro-obesogenic and pro-inflammatory phenotype. Accordingly to our prediction, when exposed to high-fat (HF) diet, although Ts65Dn mice showed similar metabolic exchange compared to WT, they showed higher energy intake and increased weight gain and fat mass. It has been shown in mice that high-fat diet increased the circulating galectin-3 and the effect was attenuated after macrophages depletion [56]. Higher circulating HSP72 levels have been also observed in medical conditions such as obesity and diabetes (Krause, Keane et al, 2014). We here found that upon HF, the levels of galectin-3 and HSP72 were higher in Ts65Dn mice compared to WT, similar to what observed in non-obesogenic conditions. Indeed, in our hands, diet intervention did not affect the levels of these inflammatory markers, probably due to a ceiling effect in Ts65Dn mice. Only the levels of IL-6 increased upon HF, which is in agreement with the literature [64] [65] [66].

In conclusion, in this study we found that lean Ts65Dn mice have increased body adiposity and altered inflammatory plasma profile compared to WT mice. We also observed altered plasma levels of some anorexigenic and orexigenic signals like leptin and ghrelin in Ts65Dn mice that might affect feeding behavior and energy balance. Similar to what has been shown in DS humans, Ts65Dn mice showed increased energy

consumption and energy expenditure along with increased leptin levels, which suggests leptin resistance. Concomitantly, we detected that Ts65Dn mice had altered plasma profile for some markers of inflammation and oxidative damage. Specifically, we here first described that Ts65Dn mice show increased plasma galectin-3 and HSP72 levels compared to WT that might explain their increased incidence of autoimmune disorders and potentially, obesity propensity.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness, 'Centro de Excelencia Severo Ochoa 2013-2017', the 'CERCA Programme / Generalitat de Catalunya' and the CIBER of Rare Diseases. We acknowledge DIUE de la Generalitat de Catalunya (Grups consolidats SGR 2014/1125), MINECO (SAF2013-49129-C2-1-R), CDTI ("Smartfoods"), and EU (Era Net Neuron PCIN-2013- 060).

We acknowledge EMBO for granting Marta Fructuoso with a short term EMBO fellowship for doing part of this work. The authors also acknowledge the technical platform Functional & Physiological Exploration Platform (FPE) of the Unit "Biologie Fonctionnelle et Adaptative", (Univ Paris Diderot, Sorbonne Paris Cité, BFA, UMR 8251 CNRS, F-75205 Paris, France) for metabolic analysis and Karine BAILLY of the Cochin Cytometry and Immunobiology Facility for the luminex 200 analysis.

Supplementary Table 1. Composition of the experimental diets (g/Kg total) used.

Nutrients	Standard chow (SC)	High-fat diet (HF)
Protein (%)	17.49	23.7
Fat (%)	7.42	34.7
Carbohydrates (%)	75.09	29.6
Sugar (%)	4.05	6.36
Starch – fiber (%)	44.97	5.4
Energy (Kcal/g)	2.85	5.25
Energy (KJ/g)	10.76	22

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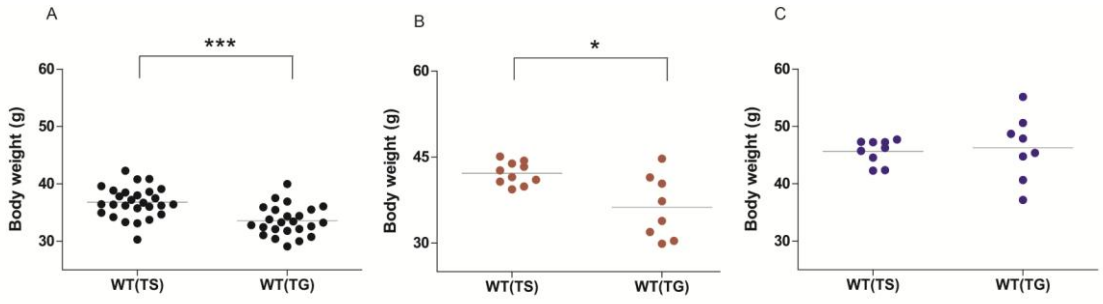
2. Comparative analysis of some obesity and feeding behavioral phenotypes of the wild-type mice from the two Down syndrome mouse models

Accumulated data have confirmed that both trisomic and *Dyrk1A* overexpressing transgenic mice recapitulate several anatomic and functional hallmarks of DS robustly enough regardless the strain. However, since this is the first report of feeding behavior and obesity related phenotypes, in this supplementary result we have compared the wild-type mice that are used as controls of Ts65Dn and mBACTgDyrk1A, to establish to what extent the differences detected between this two DS models could depend on the genetic background: B6EiC3H for Ts65Dn [WT(TS)] and C57Bl6/J for mBACTgDyrk1A [WT(TG)].

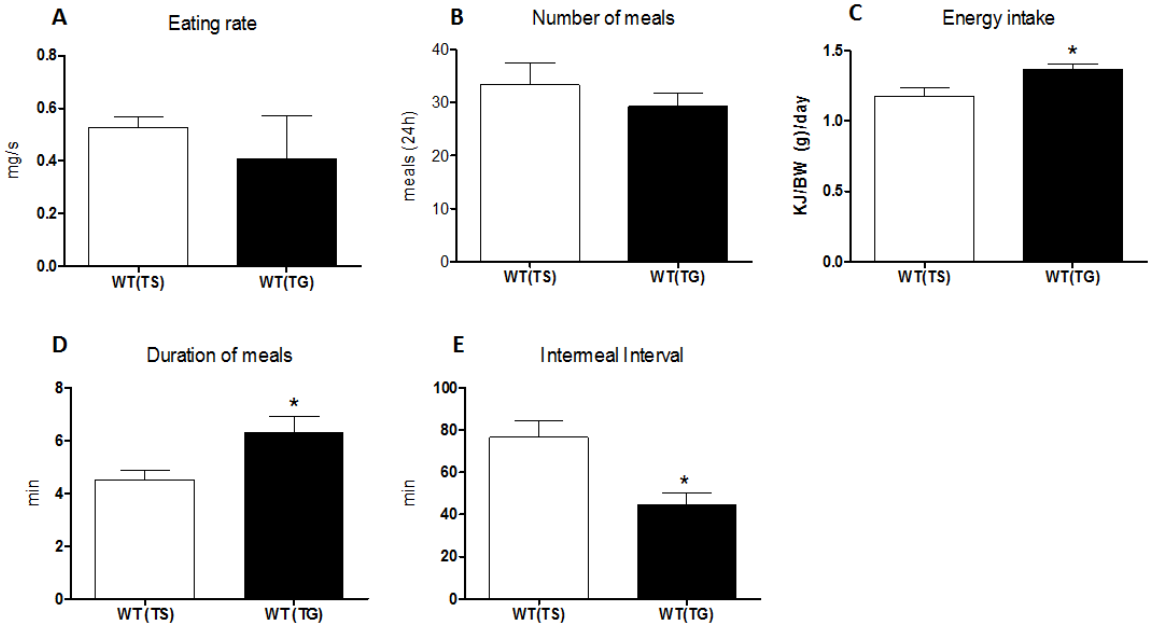
Body weight and feeding behavior

In non-obesogenic conditions, WT(TG) mice presented reduced body weight compared to WT(TS) mice (Student's t test, t (1,49) = 4.40; P<0.01; Supplementary Figure 1A). Both strains gained weight but upon 8 weeks of free choice access to CM, but WT(TG) mice still had lower body weight than WT(TS) (Mann-Whitney-U-test, P<0.05; Supplementary Figure 1B). Instead, WT(TG) body weights were similar to WT(TS) upon 8 weight of HF access (Supplementary Figure 1C), indicating a more important body weight gain in the transgenic strain and certain resistance to CM-driven weight increase.



Supplementary Figure 1. Body weight in non-obesogenic and obesogenic conditions of B6EiC3H [WT(TS)] and C57Bl6/J [WT(TG)] mice. Body weights of WT(TG) and WT(TS) receiving SC **(A),** SC+CM **(B)** or SC+HF **(C)**. SC mice: WT(TS) n = 27; WT(TG) n = 24; SC + CM mice: WT(TS) n = 10; WT(TG) n = 8 SC + HF mice: WT(TS) n = 9; WT(TG) n = 8. The lines represent the mean of the group, individual values are plotted. Student's t test, Mann-Whitney-U-test *P< 0.05, *** P<0.001.

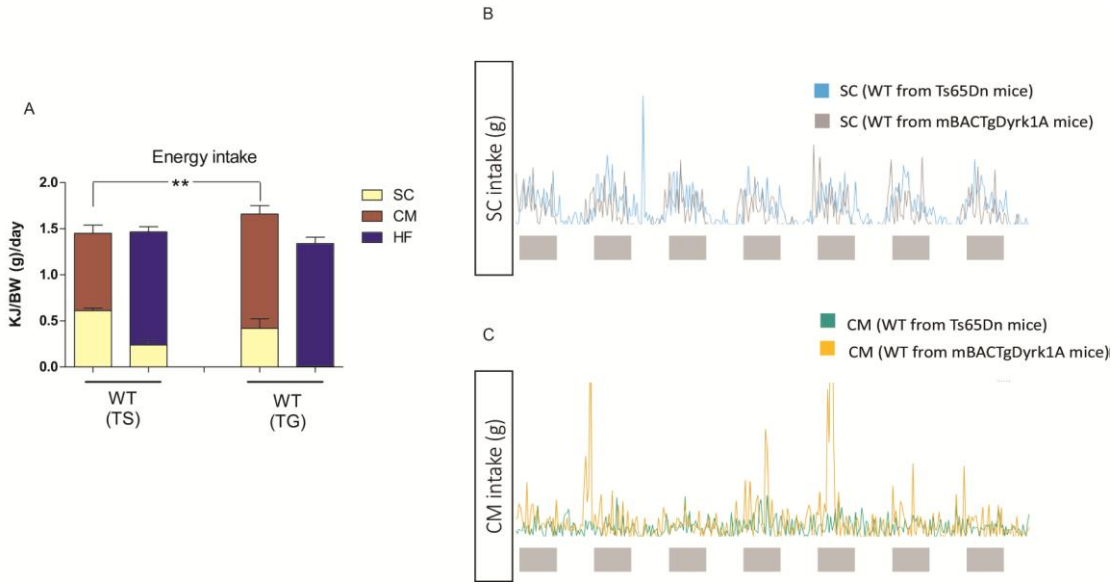
In non-obesogenic conditions, meal pattern analysis revealed no major differences between WT(TS) and WT(TG) (Supplementary Figure 2A-E), with similar eating rate (Supplementary Figure 2A) and number of meals in 24 hours (Supplementary Figure 2B). However, WT(TG) presented higher energy intake (Mann-Whitney-U-test = 3; P = 0.11, Supplementary Figure 2C), and longer meals (Mann-Whitney-U-test = 4; P<0.05, Supplementary Figure 2D) along with reduced intermeal interval (Mann-Whitney-U-test, = 1; P<0.05, Supplementary Figure 2E) compared to WT (TS).



Supplementary Figure 2. Feeding behaviors of B6EiC3H [WT(TS)] and C57Bl6/J [WT(TG)] mice. **(A)** Eating rate (mg/s), **(B)** number of SC meals in 24 hours, **(C)** Energy intake (KJ/g body weight), **(D)** average meal duration (min) and **(E)** time between feeding bouts (average intermeal interval, min) in WT(TS) and WT (TG). Data are expressed as mean ± SEM. SC mice: WT(TS) n = 8; WT(TG) n = 5; Mann-Whitney-U-test * P<0.05.

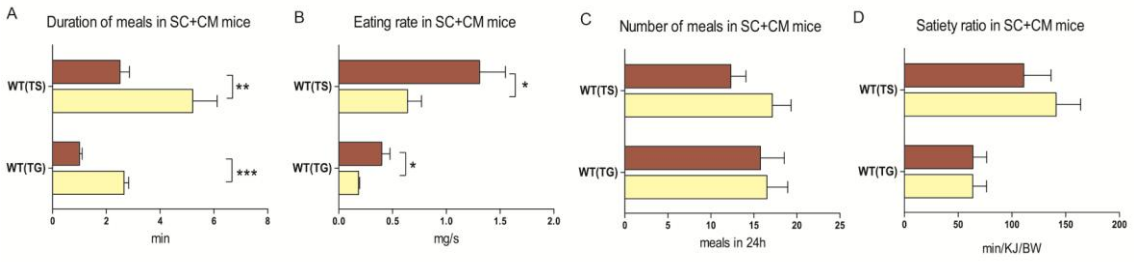
When energy-dense foods were accessible, WT(TG) showed increased CM daily energy intake compared to WT(TS) (Mann-Whitney-U-test P<0.01, Supplementary Figure 3A), suggesting increased preference for CM in C57Bl6/J compared to B6EiC3H. Although no significant, WT(TG) mice showed higher number of CM meals than WT(TG) mice during the active phase (Bonferroni as a post-hoc, P = 0.06). During the inactive phase, the number of CM was similar to the number of SC meals in both C57Bl6/J and B6EiC3H mice (Supplementary Figure 3B-C). This would indicate a preference for CM that is also detected in transgenic mBACTgDyrk1A mice, thus suggesting that the sweet

preference in this model could be partially driven by the genetic background. The energy intake of HF in the SC+HF group was similar (Supplementary Figure 3A).



Supplementary Figure 3. Energy intake in B6EiC3H [WT(TS)] and C57Bl6/J [WT(TG)] mice given free access to energy-dense food. (A) Daily energy intake (KJ/body weight) **(B)** representation of weekly SC and CM intake average in grams of SC and in WT(TS) and WT(TG). The grey squares on the bottom depict the dark (active) periods. Data are expressed as mean ± SEM. SC + CM mice: WT (TS) n = 9; WT (TG) n = 8; SC + HF mice: WT (TS) n = 9; WT (TG) n = 8. Mann-Whitney-U-test ** P<0.01.

Meal pattern analysis in SC+CM group revealed no differences between WT from both mouse models (Supplementary Figure 4A-D). CM diet led to reduced meal duration and increased eating rate compared to SC in both WT (Supplementary Figure 4B-C).



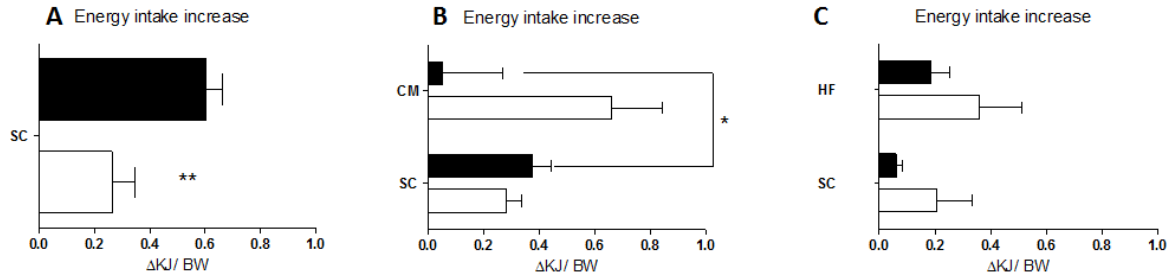
Supplementary Figure 4. Changes induced by chocolate-mixture free access in B6EiC3H [WT(TS)] and C57Bl6/J [WT(TG)] mice. Bar plots represent the meal patterns for each type of food for each experimental group [yellow (SC) and in brown (CM)] upper panel shows WT(TS) mice values, and lower panel results of WT(TG). **(A)** The average duration (min) of meals in 24 hours. **(B)** The eating rate (mg/s). **(C)** The number of meals in 24 hours. **(D)** The satiety ratio after correcting for body weight (BW) of the animal (g) for CM and SC. Data are expressed as mean ± SEM. SC + CM mice: WT (TS) n = 9;

WT (TG) n = 8. Paired Mann-Whitney-U-test for within genotype comparisons (SC vs. CM) * P<0.05, ** P<0.01, *** P<0.001.

We also compared the behaviors of WT mice across the battery of tests addressed to detect compulsive and inflexible behaviors to understand the contribution of the genetic background.

Starvation

After 24 hours of starvation, WT(TG) showed increased SC intake upon refeeding compared to WT(TS) (Mann-Whitney-U-test z <0.01, Supplementary Figure 5A). In SC+CM mice the increase of SC and CM intake upon refeeding was similar in all WT mice (Supplementary Figure 5B), as was also the increase of SC and HF intake upon refeeding in the SC+HF group (Supplementary Figure 5C).

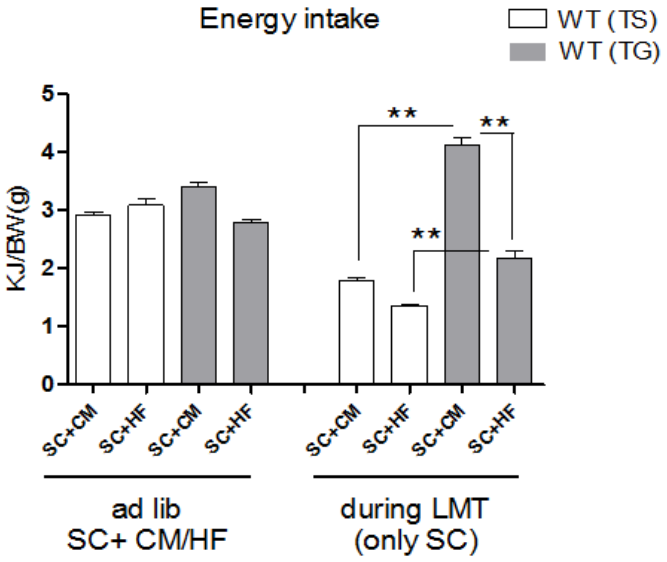


Supplementary Figure 5. Energy intake upon 24 hours of starvation in B6EiC3H [WT(TS)] and C57Bl6/J [WT(TG)] mice. Bar plots show the increase in energy intake calculated as the KJ consumed/BW (grams) 24h after refeeding - KJ consumed/BW (grams) in 24 hours in free feeding conditions. (A) Increase in daily energy upon refeeding in the SC group (B) the SC+CM group, and (C) the SC+HF mice. Data are expressed as mean ± SEM. SC mice: WT (form TS) = 8; WT (from TG) = 8; SC+CM mice: WT (form TS) = 9; WT (from TG) = 8 SC+HF mice: WT (form TS) = 9; WT (from TG) = 8. Only the comparisons between WT are depicted in the figure since those within subjects have been already discussed in the 4.1 and 4.5 of this Thesis). Mann-Whitney-U-test * P<0.05; ** P<0.01.

Limited access to energy-dense food

Mice consumed less energy during the periods of CM/HF restriction than in *ad libitum* conditions (F(1,29) = 19.28; P<0.001). However, ANOVA analysis revealed that WT(TG) mice ate more SC than WT(TS) mice, which reflects energy compensation (Two-way ANOVA, genotype and diet interaction; F(1,32) = 10. 62; P< 0.01). In SC+CM mice, WT (TG) from SC+CM group consumed more energy from SC than WT(TS) (Bonferroni as a post-hoc, P< 0.01) during the days CM was restricted. In SC+HF mice, also WT(TG) consumed more SC than WT(TS) (Bonferroni as a post-

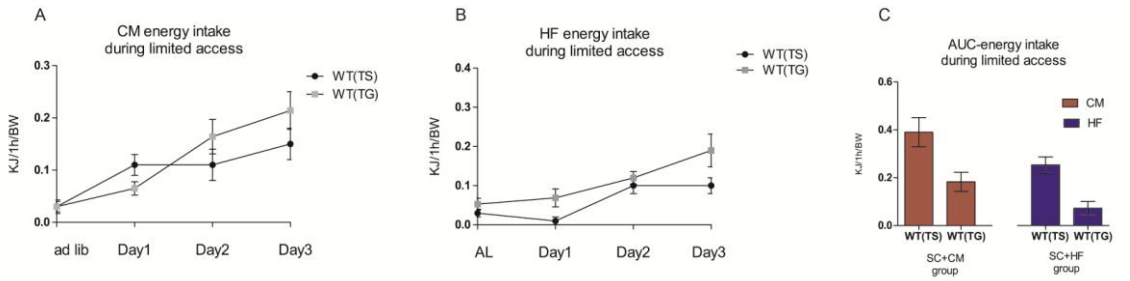
hoc, P< 0.05). WT (TG) from the SC+CM group consumed significantly more SC than those of the SC+HF group (Bonferroni as a post-hoc, P<0.01; Supplementary Figure 6).



Supplementary Figure 6. SC energy intake in B6EiC3H [WT(TS)] and C57Bl6/J [WT(TG)] mice during the periods of absence of energy-dense foods. (A) The bars plots show the mean values of energy intake of SC in *ad libitum f*eeding conditions (when CM and HF are freely available, "*ad lib*") and when energy-dense foods are not available (limited, "LMT"). WT (TS) are depicted in white and WT(TG) in grey. Data are expressed as mean ± SEM. SC+CM mice: WT (form TS) = 9; WT (from TG) = 8 SC+HF mice: WT (form TS) = 9; WT (from TG) = 8. ANOVA, Bonferroni as a post-hoc; **P< 0.01.

When analyzing separately the CM/HF consumption along the three days of the experiment, in the SC+CM group, we found a nonsignificant trend for increasing the consumption of CM in the limited access periods in WT mice from both strains (Supplementary Figure 7A). In SC+HF mice, WT (TG) mice showed higher HF intake across the days ($F(1,14) = 34.18$; $P<0.001$, Supplementary Figure 7B).

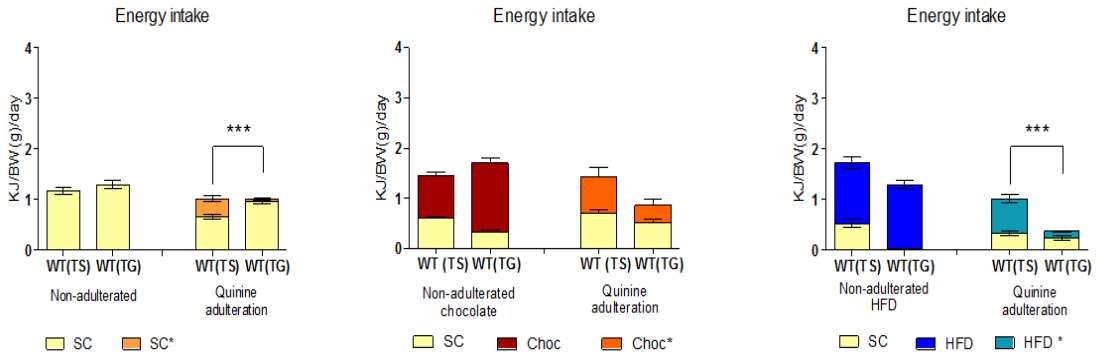
CM/HF energy increase between the *ad libitum* conditions and during the third day of the limited access was similar in wild-type mice from both strains (Supplementary Figure 7C), suggesting similar small "binge-like" events.



Supplementary Figure 7. Energy intake during limited access to energy-dense food in B6EiC3H [WT(TS)] and C57Bl6/J [WT(TG)] mice. (A) Time course of the energy intake of CM (A) and (B) HF during the periods of access. (C) CM/HF energy increase between the *ad libitum* conditions and during the third day of the limited access. *ad lib= ad libitum*; Day 1, Day 2 and Day 3 are the three consecutive days of restricted access (1h) to CM/HF. Data are expressed as mean ± SEM. SC + CM group: WT (form TS) = 10; WT (from TG) = 8; SC + HF mice: WT (form TS) = 9; WT (from TG) = 8.

Food adulteration with quinine hydrochloride

Quinine hydrochloride had stronger inhibitory effect on energy intake in WT from both models than in their TG and TG littermates. However, WT mice from TG model, ate less adulterated food than WT from TG model being significant for both SC and HF diets (bitter SC WT (TS) vs. WT (TG), Mann-Whitney-U-test, z = 0, P<0.001, and bitter HF WT (TS) vs. WT (TG), Mann-Whitney-U-test, z = 0, P<0.001; Supplementary Figure 8).



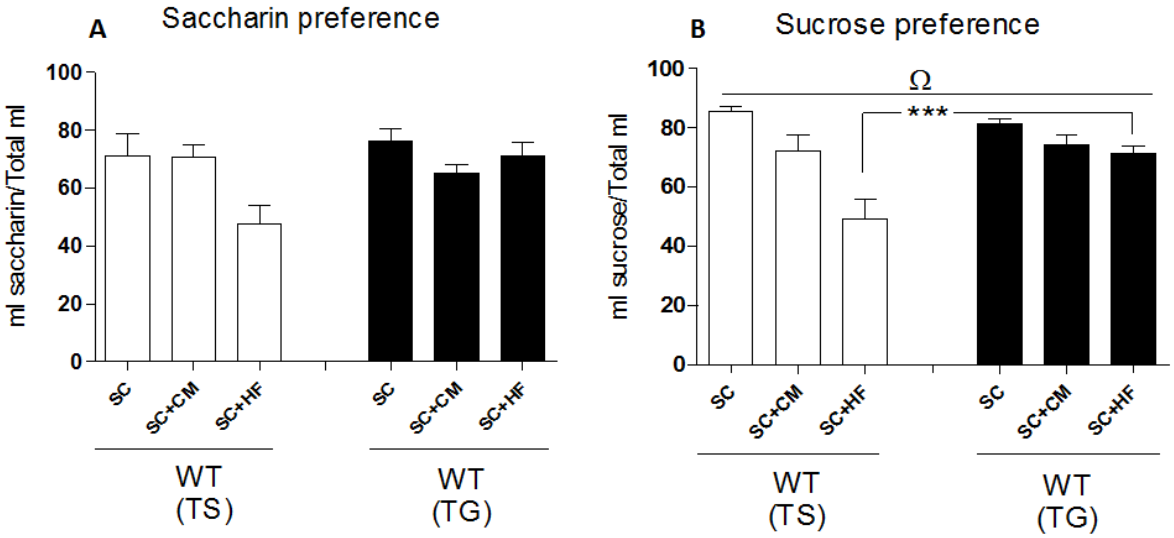
Supplementary Figure 8. Energy intake upon quinine food adulteration in B6EiC3H [WT(TS)] and C57Bl6/J [WT(TG)] mice. Bar plots show food intake (KJ/g of body weight) in 24 hours in non-adulterated conditions (*ad libitum*, "*ad lib*") and the intake after quinine hydrochloride adulteration (Quin) of SC (A), CM (B) and HF (C). Adulterated food is marked by "*". In (A) light orange SC* = bitter SC; in (B) dark orange CM* = bitter CM and in (C) light blue HF* = bitter HF. (A) SC adulteration in SC group. (B) CM adulteration in SC+CM group. (C) HF adulteration in SC+HF group. Data are expressed as mean ± SEM. SC mice: WT (form TS) = 8; WT (from TG) = 8; SC+CM mice: WT (form

TS) = 9; WT (from TG) = 8 SC+HF mice: WT (form TS) = 9; WT (from TG) = 8. Only the comparisons between WT are depicted in the figure since those within subjects have been already discussed in the 4.1 and 4.5 of this Thesis. Mann-Whitney-U-test ** P<0.01.

Preference for sweet solutions

The genetic background had no effect on the preference for sweet solutions in non-obesogenic conditions. Diet had no effect on saccharin preference in WT from both strains (Two-way, diet effect, F(2,51) = 3.01; P = 0.06, Supplementary Figure 9A).

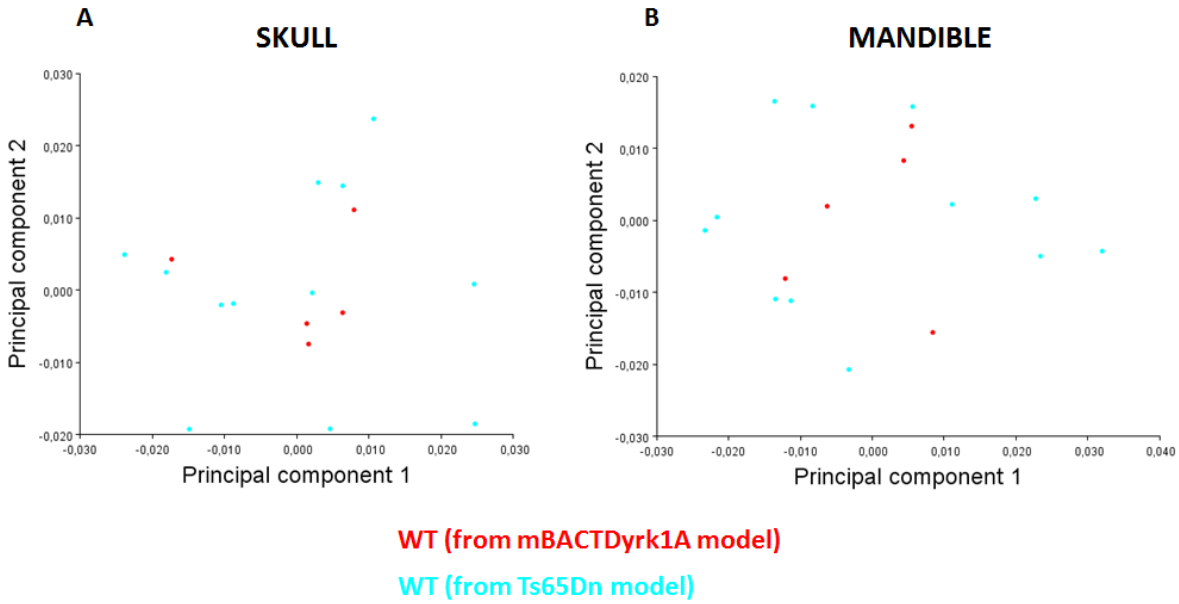
Mice fed with energy-dense diets showed reduced sucrose preference that was only significant in WT(TS), while WT(TG) showed similar preference independently of the diet and preference for sucrose in SC+CM mice was higher compared to WT(TS) (Two-way ANOVA, diet x genotype interaction (F(2,50) = 3.3, P<0.05; Bonferroni as a post-hoc P < 0.01; Bonferroni as a post-hoc P < 0.001; Supplementary Figure 9B). This result suggests a background effect that might be a confounder for the assumptions of a reward sensitivity phenotype between models.



Supplementary Figure 9. Preference for sweets solutions is reduced upon long-term access to energy-dense diets in B6EiC3H [WT(TS)] and C57Bl6/J [WT(TG)] mice. (A) Bar blots show the preference for saccharin against water [ml saccharin/total liquid intake (water + saccharin)] in SC, SC+CM and SC+HF mice. (B) Preference for sucrose against water [ml sucrose /total liquid intake (water + sucrose)] in SC, SC+CM and SC+HF mice. Data are expressed as means ± SEM. Data are expressed as mean ± SEM. SC mice: WT (form TS) = 8; WT (from TG) = 8; SC+CM mice: WT (form TS) = 10; WT (from TG) = 8 SC+HF mice: WT (form TS) = 9; WT (from TG) = 8. Two-way ANOVA, Ω for genotype x diet interaction, Bonferroni as a post-hoc *** P <0.01.

Morphometric analyses of the skull and the mandible

The morphometric analyses revealed that shape of the skull (Procrustes distances among groups for skull: 0.0001; P = ns) and mandible (Procrustes distances among groups for mandible: 0.0002, P = ns) are similar between the wild-type mice from both DS models (Supplementary Figure 10).



Supplementary Figure 10. Results of the Principal Component Analysis (PCA) based on the configuration skull and mandible anatomical landmarks of B6EiC3H [WT(TS)] and C57Bl6/J [WT(TG)] mice. The PCA was performed on the covariance matrix generated from the Procrustes distances coordinates. (A) Morphometric analysis of the skull. (B) Morphometric analysis of the mandible. The three dimensional morphings from the skull and mandible reconstructions have been performed using the software Landmark. Red dots = WT (from TG) = 5; blue dots = The WT (form TS) = 12.

3. Exploring the effect of *Dyrk1A* overexpression on the morphometry of the skull and the mandible: implications for meal pattern

Meal pattern analysis of Ts65Dn mice revealed longer and slower meals as compared to their wild-type (WT) littermates. Orofacial dysmorphologies associated to DS are recapitulated in Ts65Dn mice [1] and have been proposed to contribute to feeding and swallowing difficulties in this population [2]. In a recent paper [3] it has been shown that the modulation of the DYRK1A activity in Ts65Dn mice either through treatment or by genetic deletion of one extra copy, partially restores the altered craniofacial phenotype. However, these anatomical alterations have not been confirmed in monogenic models of DS with only *Dyrk1A* overexpression and in our experiments, mBACTgDyrk1A mice did not show feeding behavior alterations.

Thus, we hypothesized that the craniofacial abnormalities of mBACTgDyrk1A mice might be less severe than those from Ts65Dn mice. To test our hypothesis, we obtained X-ray micro computed tomography (μCT) scans on a subsample of mBACTgDyrk1A (TG) and Ts65Dn (TS) mice and their relative wild-types. We performed a comparative shape analysis of the skull and the mandible of mBACTgDyrk1A mice and their wild-type littermates using Geometric Morphometrics (GM). We also compared TG and TS mice and wild-types of these two DS models to understand the implication of *Dyrk1A* overexpression on the phenotype and the effect of the genetic background.

This work was done in collaboration with Greetje Vande Velde, (KU Leuven – University of Leuven, Belgium), Rubén González Colom and Neus Martínez Abadías (Center for Genomic Regulation, Spain).

Method

The morphometric skull measurements were obtained through X-ray micro computed tomography (μCT) technology using a Skyscan 1278 scanner (https://www.bruker.com), a dedicated high resolution and low dose small-animal μCT scanner. For the analysis, we used mBACTgDyrk1A (TG, n = 4) mice and their wild-type controls (WT (TG), n = 4). The skull reconstructions were made using the NRecon software (V1.6.10.4, Bruker-microCT). The visualization of the reconstructed μCTs and the landmarking were performed using the Amira software (Amira (http://www.fei.com/software/amira-3d-for-life-sciences/). Then we use GM, a

sophisticated body of robust statistical tools developed for measuring and comparing 3D shapes with increased precision and efficiency [4] [5]. The GM analysis started with the recording of 3D landmark coordinates of homologous anatomical points to precisely capture the shape of the skull and the mandible. Here we used a set of landmarks previously used to assess the skull anatomical differences among adults of the DS mouse model Ts65Dn and euploid mice [1]. The skull and mandible of each mouse were manually annotated using the Amira software. Next, to superimpose the 3D coordinate data collected in all specimens and to extract shape information, we applied a General Procrustes analysis [6]. This procedure removes the influence of size and adopts a single orientation for all specimens by shifting the landmark configurations to a common position, scaling them to a standard size and rotating them until achieving a least-squares fit of corresponding landmarks. Centroid size, computed as the square root of the summed squared distances between each landmark coordinate and the centroid of the configuration, was used as a measure of size [6]. We performed Principal Components Analyses (PCA) to analyze shape variation within and among WT, TG and TS mice. PCA is a data exploration technique that performs an orthogonal decomposition of the data and transforms variance covariance matrices into a smaller number of uncorrelated variables called Principal Components (PCs), which successively account for the largest amount of variation in the data [6]. GM analyses were performed separately for the skull and the mandible using MorphoJ (http://www.flywings.org.uk/morphoj_page.htm) [7]. If there are no dysmorphologies associated with DS and overexpression of *Dyrk1A,* the WT, and TG mice will overlap in the PCA scatterplot, showing similar cranial and mandibular phenotypes. If there are significant dysmorphologies, the WT and the TG mice will be separated from each other, showing a differing phenotype.

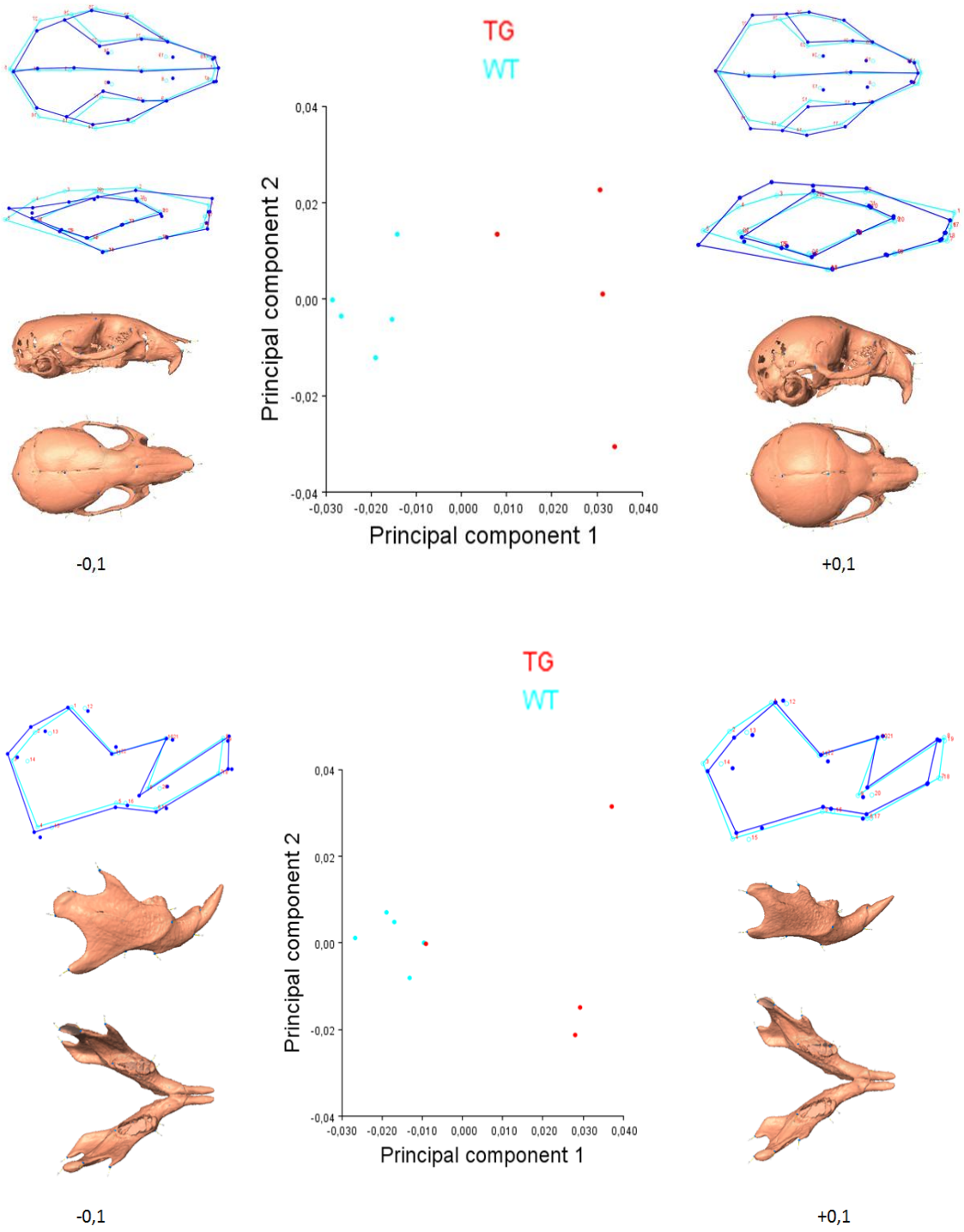
To statistically compare the mean shape differences between the genotypes we used a permutation test under the null hypothesis of no shape differences between the groups. This is a type of statistical test based on the iterative and aleatory redistribution of values between the groups. In each iteration, the distance between the means of each group is calculated in order to generate the distribution for this parameter. Afterwards, the original distance between the means can be tested using a T-test according with the estimated distribution. The resulting P-value is directly correlated with the probability that both groups derive from the same mean. In our study, the groups were resampled 10,000 times using a computer simulation, and the parameter tested was the differences

between the mean shapes of each group (i.e. the Procrustes distances between the groups).

Results

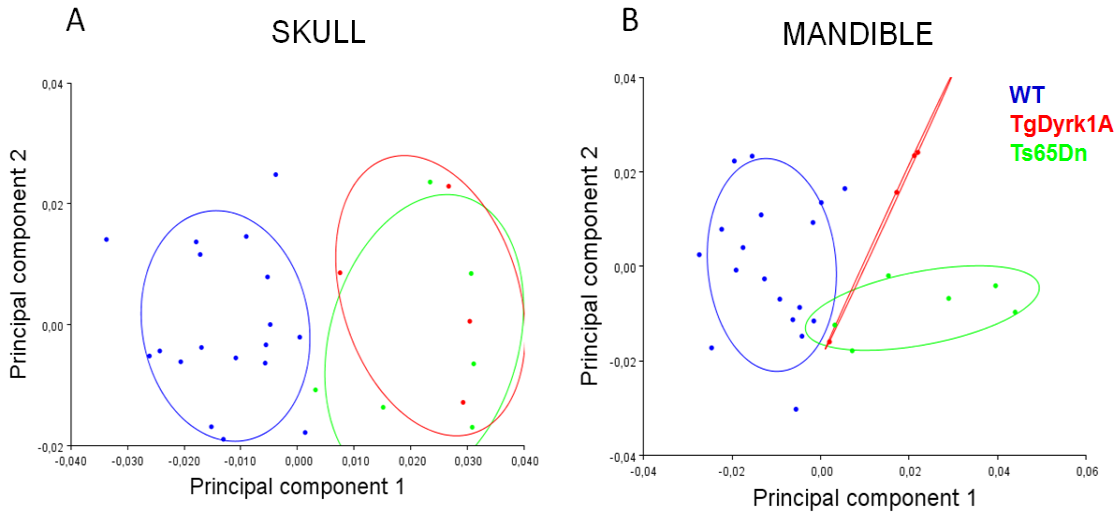
The morphometric analyses revealed significant differences between the mBACTgDyrk1A and the WT mice regarding both the skull shape (Procrustes distances among groups: 0.047; P<0.01, Supplementary Figure 11A) and the mandible shape (Procrustes distances among groups: 0.040, P<0.01, Supplementary Figure 11B). mBACTgDyrk1A and WT mice were separated along the first principal component (PC1), which explains 46% of the total morphological variation. The skull shape changes associated to PC1 were related with changes in the length and width of the skulls. WT mice fell on the positive side of PC1, which is associated with narrower and elongated skulls; whereas mBACTgDyrk1A mice fell on the negative side of PC1, which is associated with wider and shorter skulls (Supplementary Figure 11A). Therefore, in comparison to WT mice, mBACTgDyrk1A mice presented shorter and more globular brachycephalic skulls, which correspond to the most common craniofacial traits in DS individuals [8]. This result suggests that in adult mice the overexpression of *Dyrk1A* is enough to produce the typical craniofacial alterations associated with DS.

Regarding the shape of the mandible, mBACTgDyrk1A and WT mice were also separated along PC1, which explains 40% of the total morphological variation. WT mice were associated with wider, more robust and less curved mandibles, with more pronounced coronoid and condylar processes. In contrast, mBACTgDyrk1A mice showed smaller, thicker and more curved mandibles, with smaller and less pronounced coronoid and condylar processes (Supplementary Figure 11B).



Supplementary Figure 11. Results of the Principal Component Analysis (PCA) based on the configuration of skull anatomical landmarks (top) and mandible anatomical landmarks (bottom) for comparing wild-type (WT) and mBACTgDyrk1A (TG) mice. The PCA was performed on the covariance matrix generated from the Procrustes distances coordinates. (A) Morphometric analysis of the skull. (B) Morphometric analysis of the mandible. The three dimensional morphings from the skull and mandible reconstructions have been performed using the software Landmark.

We took advantage of unpublished skull and mandible measurements in Ts65Dn from our laboratory to compare these phenotypes between the two models of DS. The PCA analysis based on the skull morphology revealed a complete overlap between Ts65Dn and mBACTgDyrk1A mice (Supplementary Figure 12A), indicating that both models of DS have a very similar craniofacial phenotype. However, the PCA analysis based on the mandible morphology showed that mBACTgDyrk1A mice fell between WT and Ts65Dn mice, suggesting an intermediate, less severe phenotype (Supplementary Figure 12B).



Supplementary Figure 12. Results of the Principal Component Analysis (PCA) based on the configuration of the skull and mandible anatomical landmarks for the wild-type (WT), mBACTgDyrk1A (TG) and Ts65Dn mice (TS). (A) TS and TG fell together on the positive side of PC1 whereas WT mice fell in the negative, in that case, associated to narrower and elongated skulls. (B) However, the three genotypes were separated along the PC1 for mandible morphology being the TG in between the WT and the TS mice.

The longer and slower meals of Ts65Dn could be due to different anatomical problems. Among those, the craniofacial contribution to mastication is supported by our results. Our morphometric analysis in skulls of mBACTgDyrk1A mice suggest that the overexpression of *Dyrk1A* is sufficient for the recapitulation of brachycephaly. However, mandible hypoplasia is less pronounced in mBACTgDyrk1A than in Ts65Dn mice, which could explain the more similar feeding behaviors between WT and mBACTgDyrk1A mice than in comparison to WT and Ts65Dn mice.

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