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ORIGINAL ARTICLE

Exploring the link between hedonic overeating and prefrontal cortex dysfunction in the Ts65Dn trisomic mouse model

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Abstract

Individuals with Down syndrome (DS) have a higher prevalence of obesity compared to the general population. Conventionally, this has been attributed to endocrine issues and lack of exercise. However, deficits in neural reward responses and dopaminergic disturbances in DS may be contributing factors. To investigate this, we focused on a mouse model (Ts65Dn) bearing some triplicated genes homologous to trisomy 21. Through detailed meal pattern analysis in male Ts65Dn mice, we observed an increased preference for energy-dense food, pointing towards a potential "hedonic" overeating behavior. Moreover, trisomic mice exhibited higher scores in compulsivity and inflexibility tests when limited access to energy-dense food and quinine hydrochloride adulteration were introduced, compared to euploid controls. Interestingly, when we activated prelimbic-to-nucleus accumbens projections in Ts65Dn male mice using a chemogenetic approach, impulsive and compulsive behaviors significantly decreased, shedding light on a promising intervention avenue. Our findings uncover a novel mechanism behind the vulnerability to overeating and offer potential new pathways for tackling obesity through innovative interventions.

Keywords Down syndrome · Obesity · Overeating · Prefrontal cortex · Dopamine

Introduction

Obesity is highly prevalent among individuals with Down syndrome (DS) compared to the general population [1]. Conventionally, this has been attributed to endocrine alterations, such as leptin resistance or thyroid gland dysfunction [2, 3], as well as reduced physical activity [4]. However, emerging evidence suggests that individuals with DS, particularly adolescents and adults, display a propensity for snacking on energy-dense foods like snacks and sweet beverages [5, 6]. Such behavior depends on the reward system, raising the possibility of reward-induced overeating [7]. The medial

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prefrontal cortex (mPFC), a crucial component of the mesocorticolimbic dopaminergic system, plays a pivotal role in self-control and reward sensitivity [8]. Intriguingly, our previous research demonstrated that chemogenetic inhibition of the prelimbic (PL) mPFC neurons projecting to the nucleus accumbens (NAc) core, known as the PL mPFC-NAc core pathway, led to compulsive food seeking [9]. Furthermore, elevated body mass index (BMI) has been associated with reduced blood flow in the PFC [10], and overeating has been associated with orbitofrontal volume reductions [11, 12]. In individuals with DS, we observed reduced PFC volume [13, 14] decreased functional connectivity [14], and genetic polymorphisms in the catechol-O-methyltransferase (COMTVal158Met) gene resulting in lower DA availability [15]. DS brains also exhibit decreased concentrations of DA [13], along with frequent anhedonia (reduced pleasure responses), associated with psychiatric comorbidities [16] and increased impulsiveness [17]. However, the connection between impaired PFC dopaminergic function, control of eating behavior and obesity in DS remains unexplored.

Hence, we propose that alterations in the brain reward system [18, 19] may contribute to obesity in individuals with DS, potentially heightening their susceptibility to over consume sweet or fatty foods. To investigate this hypothesis, we employed a diet-induced obesity paradigm, offering Ts65Dn mice, a DS mouse model, a free choice access to a highly palatable (chocolate-mixture, CM) diet or a high-fat diet (HFD) over an 8 weeks period. We assessed weight gain, meal pattern, and compulsive and inflexible behaviors both in 2N and Ts65Dn male mice. We focused on male mice since it has been widely reported that male mice have a greater propensity to gain weight and become obese than females when exposed to HFD. We also examined diet-induced changes in monoamine levels in the prefrontal cortex and striatum, regions associated with the reward-driven control of feeding. Additionally, we devised a targeted strategy to manipulate the excitatory projections from PL-mPFC to the NAc core, aiming to reduce the compulsive and inflexible behaviors observed in Ts65Dn mice. By delving into the interplay between the brain reward system, obesity, and eating behavior, we aimed to provide valuable insights that have the potential to pave the way for the development of innovative interventions and therapeutic strategies to combat obesity.

Materials and methods

Animal breeding and diets

Ts(17^{16})65Dn (Ts65Dn; strain #005252) mice were obtained through crossings of a B6EiC3Sn a/A-Ts (17^{16})65Dn (Ts65Dn) female to B6C3F1/J males purchased from The Jackson Laboratory. Genotyping was performed by amplifying genomic DNA obtained from the mouse tail as described in [20]. The colony of Ts65Dn mice was maintained in the Animal Facilities in the Barcelona Biomedical Research Park (PRBB, Barcelona, Spain). Mice received chow and water ad libitum in controlled laboratory conditions with temperature maintained at 22 ± 1 °C and humidity at $55 \pm 10\%$ on a 12 h light/dark cycle (lights off 20:00 h). The control group consisted of wild-type (2N) euploid male littermates (2N = 8, Ts65Dn = 8) receiving a standard chow diet (SC; SDS diets). These mice were utilized to assess genotype-dependent differences in feeding behavior and brain monoamine levels under non-obesogenic conditions. For studying the preference for sweet and fatty foods, obesity development, the emergence of compulsive-like eating, and associated neurochemical changes, separate groups of animals were assigned to two free-choice diet conditions throughout the experiment. In one experimental group (2N = 10, Ts65Dn = 10; SC + CM group), male mice were provided with free access to both standard chow (SC, SDS: 10.76 MJ kg^{-1} digestible energy (17.5% from protein, 75% from carbohydrate and 7.4% from fat) and pellets of a chocolate-mixture (CM) diet, consisting of equal amounts of Bounty®, Snickers®, Mars®, and Milka® chocolate products (20.6 MJ kg^{-1} digestible energy: 17% from protein, 52% from carbohydrate and 24% from fat). The second condition involved free access to SC and a high-fat diet (HFD; commercial pellets: 58G9; Test Diet ®, USA) that contained 22 MJ kg^{-1} digestible energy (24% coming from protein, 30% from carbohydrate and 35% from fat) (2N = 9, Ts65Dn = 9; SC + HFD group). To maintain the organoleptic properties, the pellets were renewed at least twice a week. Mice from both diet groups underwent behavioral experiments and bioamine studies. Additionally, a separate group of male mice (2N = 12, Ts65Dn = 6; SC + HFD group) was utilized to label and manipulate the projections from the prelimbic (PL) to the nucleus accumbens (NAc) core. These mice had free access to both SC and HFD. A control group of 2N animals had their PL-NAc projections labeled with mCherry and served to confirm neuronal activation induced by CNO administration. 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 the use of Laboratory Animals n° A5388-01 (NIH). The experiments were performed by the same experimenter in 5 months-old male Ts65Dn and disomic (2N) 2N littermates, to allow the stabilization of weight gain. Based on the results from previous work, we estimated that for a statistical power of 80%, a sample size of eight/group would detect a 30% difference in the measured variables (alpha < 0.01).

Experiment 1: diet-induced obesity and monoamine levels

For the diet-induced obesity paradigm, we followed the protocol detailed in [21]. Briefly, all mice were individually housed in PheCOMP multi-take metabolic cages (Panlab) and provided with SC and water for two weeks to allow habituation to the housing conditions. Subsequently, the mice were assigned to specific diet conditions, including SC or free access to either the chocolate-mixture (CM) or high-fat diet (HFD). Throughout the experiment, the mice were undisturbed for a duration of eight weeks to ensure stable weight gain. To assess the progress of weight gain, we measured the mice's body weight using a precision scale. Additionally, their meal patterns were automatically recorded and analyzed continuously during the entire experimental period (details of the analysis are provided below). Once the animals reached a state of stable weight, we conducted evaluations of compulsive overeating and behavioral inflexibility through dedicated stand-alone tests. These tests allowed us to gain insights into the presence of these behaviors in the mice. Finally, mice were euthanized with CO_2 and the monoamine content in striatum (ST) and PFC was measured (Fig. 1a).

Feeding behavior analysis

We used the PheCOMP multi-take metabolism cages (Panlab), that provide fine-grain feeding behavior data for detailed meal pattern analysis. Two food dispensers containing SC and CM or HFD were located in the PheCOMP cages, counterbalanced between cages and genotypes (for a complete description, see [22]. The meal pattern was analyzed using the COMPULSE software (Panlab). We analyzed the number and average duration of meals, the total food consumed, and eating rate (food consumed per second). We calculated the food/energy intake, the circadian and ultradian frequency distributions of meals, and the satiety ratio (intermeal intervals divided by the amount (g) of food consumed in the preceding meal). To assess potential pellet loss during the course of the experiment, we developed a custom-made R code specifically designed for this purpose (available upon request). In cases where pellet loss was detected, appropriate corrections were made to the data to ensure accuracy and reliability.

Limited access to energy-dense foods

Access to CM or HFD was restricted to 1 h during the light (resting) phase of the light–dark cycle (from 14:00 to 15:00 h) for 3 consecutive days in overweight mice (SC + CM and SC + HFD) to analyze inflexibility and binge-like eating episodes. Inflexibility refers to the inability to adapt to new or changing events or situations. In our study, we assessed inflexibility by examining the mice's behavioral responses during restricted access to high-caloric food. During periods of restricted access to high-caloric food, animals increase their consumption of standard chow as a compensatory measure for energy intake. The absence of consumption of standard chow in this context would indicate an inflexible response. SC and water were provided unrestrictedly ad libitum (see [21]).

Food adulteration with quinine hydrochloride

After limited access to energy-dense foods, food adulteration with quinine hydrochloride was performed. Foods were adulterated with 3 g of quinine hydrochloride /Kg food (Sigma). Compulsiveness refers to the tendency to engage in repetitive behaviors, even if they are unpleasant or counterproductive, as a means to alleviate anxiety or stress. In the context of our study, we examined compulsive behavior by assessing the mice's persistence in consuming adulterated food despite the presence of an unpleasant bitter taste. This behavior reflects a compulsive and inflexible response, as the mice continue to consume the food despite its negative sensory qualities. To specifically investigate this aspect, we conducted a two-day experiment. During this period, mice from the SC + CM group and SC + HFD group were provided with bitter-tasting CM or HFD, respectively. As a control to ensure the detection of taste, SC mice were given bitter-tasting SC as a control of taste detection. In all experimental groups, unadulterated SC and water were always available (for a complete description, see [21]). We compared the average energy intake of bitter SC/CM/HFD with the daily SC/CM/HFD energy intake in non-adulterated conditions (in SC mice, SC + CM mice, and SC + HFD mice, respectively).

Monoamine levels determination using high-performance liquid chromatography with electrochemical detection (HPLC-ED)

Mice were sacrificed after behavioral testing, when mice were 8 months-old, and the prefrontal cortex and striatum dissected and stored at − 80°C until analysis. For monoamines measurement, frozen tissues were resuspended in 400 µl of ice-cold 0.1 M perchloric acid solution with 0.4% EDTA. Tissues were homogenized with 1.5 mm diameter beads for 2 min using a tissue lyser II (Qiagen). The homogenates were centrifuged at 3000 rpm for 30 min at 4 °C, and the supernatants were collected and centrifuged at 16,400 rpm for 2 min. The HPLC-ED system was composed of Shimadzu apparatus equipped with a LC20AD pump (Shimadzu), and a SiL20AC autosampler (Shimadzu) coupled with an electrochemical detector Waters 2465 with ISAAC/

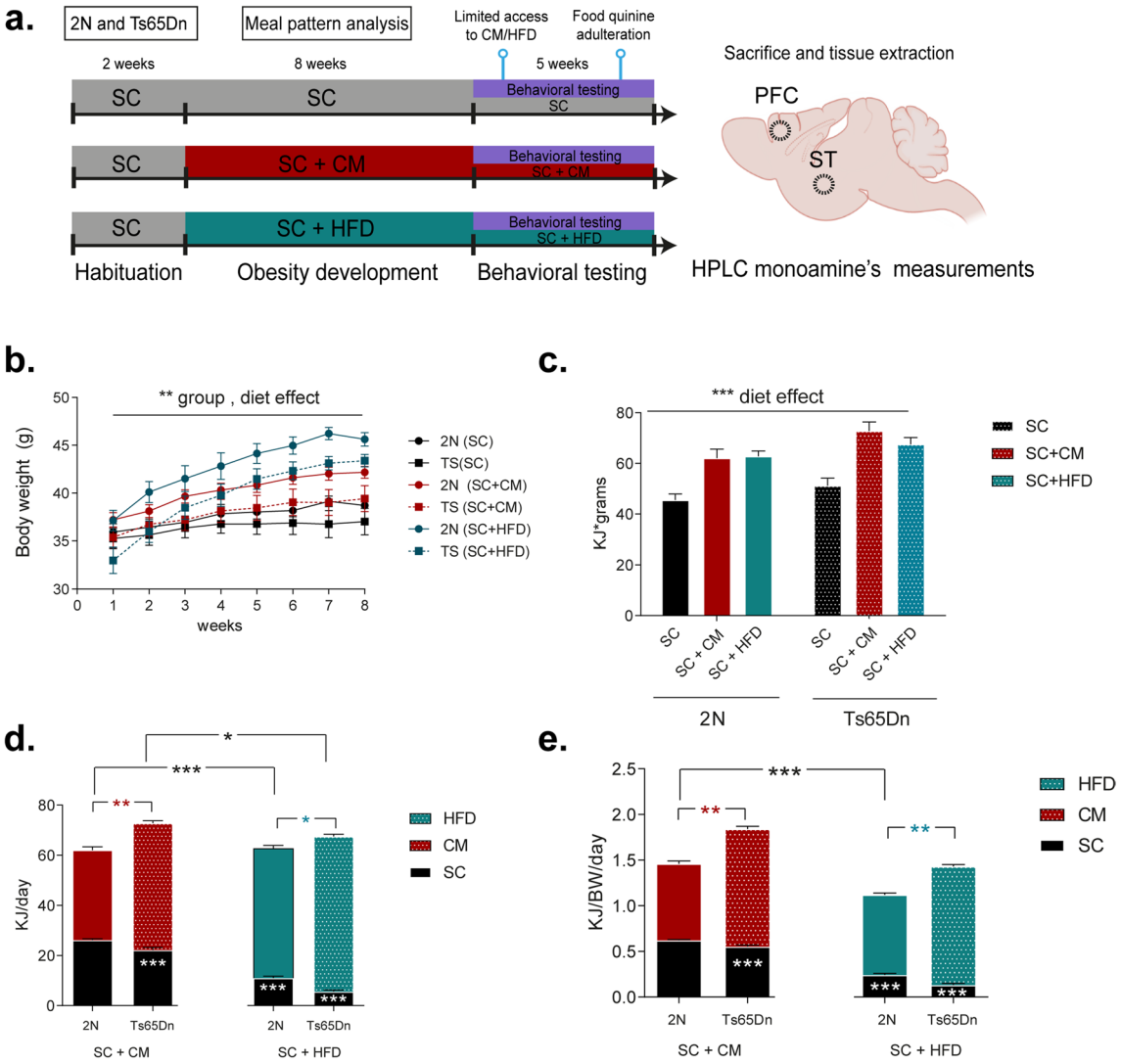


Fig. 1 Body weight increase and energy-intake in euploid controls (2N) and Ts65Dn male mice. **a** Experimental design of Experiment 1. During the habituation period (2 weeks), all mice received standard chow (SC). After two weeks, mice were allocated to receive free access to chocolate-mixture (CM) or high-fat diet (HFD). After eight weeks from the introduction of energy-dense diets, animals gained stable overweight ("Obesity development"), and a battery of behavioral tests was used to characterize compulsive and inflexible behavior. **b** Mean body weight changes along the 8 weeks of the experiment in each experimental group in wild type (2N, circles) and Ts65Dn (TS, squares) mice. **c** Energy intake (KJ consumed per body weight). **d** Daily total energy intake (KJ/day) and **e** daily energy intake corrected by body weight (KJ/BW/day) of SC and CM in mice of each genotype receiving SC + CM (left panel) and intake of SC and HFD in mice receiving SC + HFD. SC group: 2N = 8, Ts65Dn = 8; SC + CM group: 2N = 10, Ts65Dn = 10; SC + HFD group 2N = 9, Ts65Dn = 9. Statistical significance is denoted as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. In white comparisons between SC vs CM/HFD, brown for CM, and green for HFD

GC cell (Waters Corporation). Separation was obtained using a 150 × 4.6 mm C18 5 µM Interchim Ultrasphere column (Interchim) with a security guard system. The separation was done with an isocratic method with 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 0.2 µM membrane filter, degassed before use, and was pumped at a flow rate of 0.8 ml/min. Elutes were detected at an oxidation potential of 700 mV versus a reference electrode. The detection cell was housed within the Faraday cage of the electrochemical detector that was set at 30 °C. Samples with an injection volume of 40 µl were placed in the autosampler and kept at 4 °C. With this method, the retention time allowed by this system was 7 min, 10 min, 13.5 min, 16.5 min, 21 min, 32 min, and 42 min for NA (noradrenaline), AD (adrenaline), DA (dopamine), DOPAC (3–4 dihydroxyphenylacetic acid), 5-HIAA (5-hydroxy-3-indoleacetic acid),

5-HT (serotonin),respectively. The peak areas of the external standards allow the metabolites quantification in the tested samples. All raw measurements were normalized to total tissue weight.

Chemicals

The monoamines AD, DA, DOPAC, 5-HIAA, 5-HT and NA, and the chemicals EDTA (ethylene-diamine-tetraaceticacid), disodium salt, perchloric acid, and hydrogen chloride were purchased from Sigma. Ultrapure water was obtained with a Milli-Q system (Millipore). Standard solutions of each monoamine or metabolite were prepared at 50 ng/ml in ultrapure water for indoleamines and in 0.1 N HCl solution for catecholamines and were stored at − 20 °C.

Experiment 2. Specific tagging and manipulation of PL-NAc projections

Building upon our previous research indicating that the inhibition of PL-NAc core projections induces compulsive-like behavior [9], we conducted further experiments using a separate group of euploid and Ts65Dn male mice. In this study, we employed a tagging method to specifically label the PL-NAc core projections with an excitatory designer receptor exclusively activated by designer drugs (DREADD). This allowed us to selectively activate PL-NAc core projections using an i.p injection of clozapine N-oxide (CNO; Tocris) at 1 mg/Kg dose. The purpose of this manipulation was to investigate whether we could rescue the impulsive and compulsive phenotype observed in the Ts65Dn trisomic mice. Obesity development was induced when mice were 5 months-old, as in the previous experiment, by a free-choice of SC and HFD (see above). Behavioral testing also included limited access to HFD and food quinine adulteration. In this set of experiments, adeno-associated viruses (AAVs) were injected 2 weeks after initiating the obesity development. Behavioral testing started at week 9 (Fig. 6a), when mice reached a stable weight and hM3Dq receptors were expressed in the PL region. After the behavioral testing, when mice were 8 months-old, brains were extracted and processed for immunofluorescence studies.

Viral vectors

We used the following viral vectors: AAV-retrograde-Cre-GFP (AAVr-Syn1-GFP-Cre; 8.2E + 12 gc/ml produced at the Viral Vector Production Unit of Universitat Autònoma de Barcelona). For the detection of AAV8-hSyn-DIO-hM3D(Gq)-mCherry (1.18E + 13 gc/mL), we directly visualized mCherry in the confocal microscope as it was clearly visible without immunofluorescence signal amplification.

Drugs

CNO was dissolved in DMSO and then diluted in 0.9% saline to reach a final DMSO concentration of 0.5%. The saline solution for control injections consisted of 0.5% DMSO in saline 0.9%. CNO (1 mg/Kg) or saline solution was injected 30 min before the behavioral assays. The CNO dose did not induce any seizure activity or behavioral variation.

Stereotaxic injection

Mice were anesthetized using ketamidol (7.5 mg/Kg) and medetomidin (0.2 mg/Kg) and placed in the stereotaxic apparatus (Stoelting 51,730) with a heating pad for adeno-associated virus intracerebral injections. Craniotomies were performed using a 0.45 mm diameter drill. The volume [0.2 µL per site in mPFC and 0.4 µL in NAc core] was injected at a constant rate of 0.05 µL/min (mPFC) or 0.1 µL/min (NAc core) by using a microinfusion pump (Harvard Apparatus) for 4 min. All injections were made through a bilateral injection cannula (33-gauge internal cannula, Plastics One) connected by polyethylene tubing (PE-20; Plastics One) to a microsyringe (Model 1701 N SYR, Cemented NDL, 26ga, 2in, point style 3, Hamilton Company). Cannulas remained 10 min after injection to allow the fluid to diffuse and to prevent reflux, slowly withdrawn for 5 min, and the skin was sutured. Mice were treated with 0.1 mg/Kg meloxicam as analgesic, and anesthesia recovered by Atipemazol (1 mg/Kg) and maintained on a heating pad until fully recovered from anesthesia. Mice were allowed to recover for four days in individual home cages before being introduced again in the PheCOMP cages. We used the following coordinates to target the injections according to Paxinos and Franklin atlas [23]: (PL) AP + 1.98 mm, L ± 0.3 mm, DV -2.3 mm; and (NAc core) AP + 1.34 mm, L ± 1 mm, DV—4.6 mm.

Behavioral testing

To ensure unbiased data collection, mice from the various experimental groups were randomly assigned to cages, and the experimenters remained blind to their group allocation throughout the study. Meal pattern measurements were performed automatically, eliminating the possibility of experimenter biases in data recording and analysis. The first behavioral test performed was the limited access which spanned two consecutive weeks, followed by the HFD quinine adulteration test.

Limited access to HFD

This test was carried out specifically on overweight mice from the SC + HFD group to evaluate inflexibility and

compulsiveness. During the first week, the mice were administered with saline solution 30 min prior to being given free access to the HFD for three consecutive days. In the subsequent week, the mice were administered with CNO at a dose of 1 mg/kg, also 30 min before being given free access to the HFD for three consecutive days.

HFD quinine adulteration test

Food adulteration with quinine hydrochloride was prepared following the above-described method. In this experiment, adulterated HFD and non-adulterated HFD consumption was measured after the activation of PL-NAc projection during a 24 h-period.

Immunohistochemistry

Mice were transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA; Sigma) in PBS (pH 7.4). Brains were extracted and post-fixed in 4% PFA at 4 °C overnight. Brains were then transferred to PBS and 30 µm coronal consecutive brain sections were obtained employing a vibratome (LeicaVT1000S; Leica), collected in PBS and stored in cryoprotective solution (40% PBS, 30%, glycerol (Sigma) and 30% polyethylene glycol (Sigma) for long-term storage. For immunofluorescence studies, 3–5 sections per mice were selected centered on the injection sites and according to stereotaxic coordinates of the PL and NAc regions with the aid of a bright-field microscope (Leica S6D). Brain sections were washed with PBS (3 × 10 min). Then, sections were permeabilized with 0.5% Triton X-100 (Sigma) in PBS (PBS-T 0.5%) (3 × 15 min) and blocked with 10% of Normal Goat Serum (NGS; Thermo Fisher Scientific) for two hours at room temperature (RT). Sections incubated in PBS-T 0.5% and NGS 5% with the primary antibodies overnight at 4ºC washed again (PBS-T 0.5% 3 × 15 min) and incubated with the secondary antibodies (PBS-T 0.5% + NGS 5%) for two hours at room temperature protected from light. Finally, samples were washed with PBS-T 0.5% (3 × 15 min) followed by PBS washing (3 × 10 min) to remove the detergent and sections were mounted and coverslipped into a pre-cleaned glass slide with Fluoromount-G medium with DAPI (Thermo Fisher Scientific). Staining with cFos was performed with rabbit anti-cFos (1:1000, Sc-7202, Santacruz) and visualized with anti-rabbit Alexa-647 (1:500; A-21443, Thermo Fisher Scientific). mCherry and EGFP were directly visualized without fluorescence amplification.

Cell counting

A 20x/0.70 NA objective was used to acquire confocal fluorescence images using a Leica TCS SP5 inverted scanning laser microscope. Using ImageJ software and a z-stack (3 µm step size), cFos + cells were counted both in the PL and NAc region. Within the PL, a region of interest (ROI) was manually delineated according to the mCherry and DAPI signal in each section. In the NAc, a ROI was manually delineated according to the Paxinos and Franklin atlas [23]. The number of cFos + cells was then divided by the volume of the delineated region. For every animal, the cell density of different sections (3–5) was averaged.

RNA extraction from cortex

For RNA extraction, frozen tissues were homogenized in 1 mL of TRIzol. Afterwards, 200 µl of chloroform was added and incubated for 5 min. Then, the samples were centrifuged at 12,000 g for 15 min at 4 °C, and the upper aqueous phase was transferred to a new tube. RNA was precipitated with 2 × volume of molecular-grade isopropanol. The samples were then incubated for 10 min at RT and centrifuged at 12,000 g for 10 min at 4 °C. The pellet was then washed with 75% ethanol and resuspended with nuclease-free water after air drying for 5 min on the benchtop. RNA purity was assessed using a NanoDrop 2000 (Thermo Scientific #ND-2000). RNA concentrations ranged from 30 to 200 ng/µl. All RNA samples were subjected to DNase digestion with DNase I (Sigma #AMPD1- 1KT). Single-stranded cDNA was synthesized from 1000 ng of total RNA using SuperScript™ III Reverse Transcriptase and oligo(dT) primers (Thermo Scientific #18080093) following the manufacturer's instructions.

Quantitative real-time PCR

All experiments were performed with three to five biological and three technical replicates. Briefly, RTqPCR reactions were carried out using LightCycler® 480 SYBR Green I Master Mix (Roche #04-887-352-001) on a LightCycler® Real-Time PCR System (Roche). The final volume for each reaction was 10 µl with 500 nM of corresponding gene specific primers (Ddr1 forward primer: tctggtttacctgatccctca, Ddr1 reverse primer: gcctcctccctcttcaggt, Ddr2 forward primer: tgaacaggcggagaatgg, Ddr2 reverse primer: ctggtgcttgacagcatctc, Gapdh forward primer: ggagattgttgccatcaacga, Gapdh reverse primer: tgaagacaccagtagactccacgac), and 5 µl of total cDNA diluted 1:5. A negative water control was included in each run. The thermal cycling was initiated at 95 °C for 10 min followed by 45 cycles of 10 s at 95 °C and 15 s at 55 °C, the optimal annealing temperature for our target genes. Melting curve analyses were carried out at the end of each run of qPCR to assess the production of single, specific products.

Statistical analysis

Most variables of interest were analyzed using linear mixed models to account for the dependence of the data within the same animal. The time course of body weight gain was analyzed using linear mixed models with the following fixed effects: diet (SC consumption of the SC group, SC and CM separate consumption of the SC + CM group, SC and HFD separate consumption of the SC + HFD group), genotype, and their interaction. A random intercept per mouse was considered. For the analysis of the meal pattern data, linear mixed models were used, in which the diet (SC consumption of the SC group, SC and CM separate consumption of the SC + CM group; and SC and HFD separate consumption of the SC + HFD group), the genotype, and their interaction were included as fixed effects and a random intercept per mouse was considered. Energy compensation during the limited access to energy-dense food test, was analyzed separately for CM and HF diets using linear mixed models with genotype, day, and the interaction of both as fixed effects. A random intercept per mouse was considered. Those sessions in which the PheCOMP cages did not properly record food intake due to technical problems or to HFD destruction by 2N or Ts65Dn mice, were excluded from the analysis. For the pharmacological evaluation of the dopaminergic system on energy-dense foods overeating, the genotype dependency of the effect of the dopaminergic agonist in reducing food intake was assessed using separate nonparametric Wilcoxon tests. All post-hoc pairwise comparisons of interest were carried out in the framework of the corresponding model. The computation of the simultaneous confidence intervals and adjusted p values in order to guarantee family-wise error rates of 0.05 were based on the multivariate t distribution of the vector of test statistics [24].

Results

Ts65Dn mice show increased intake and preference for energy-dense foods

The body weight (BW) changes across time were different between the experimental groups (two way, time and group interaction: ANOVA $F(35,336) = 9.48$, $p < 0.0001$; Fig. 1b). euploid control male mice fed with SC showed a significant increase in body weight during the experiment (BW week 8th vs. week 1st, mean difference: 2.785; 95% CI: [0.7062, 4.864]; $p = 0.009$; Fig. 1b), possibly indicating that their normal growth curve was still progressing, whereas body weight in Ts65Dn male mice receiving SC remained stable (BW week 8th vs. week 1st, mean difference: 1.754; 95% CI: [− 2.574, 6.082]; *N.S.*; Fig. 1b). Conversely, free access to energy-dense food (SC + CM or SC + HFD) led to a progressive body weight gain in both genotypes (for SC + CM group BW mean difference (week 8th vs. week 1st): 2N: 4.969; 95% CI: [1.334, 8.604]; $p = 0.006$; Ts65Dn: 4.065; 95% CI: [1.253, 6.877]; $p = 0.004$. For the SC + HFD group BW mean difference (week 8th vs. week 1st): 2N: 8.509; 95% CI: [5.499, 11.52]; $p < 0.0001$; Ts65Dn: 10.41; 95% CI: [5.130, 15.69]; $p = 0.0005$; Fig. 1b). At the end of the obesity development phase (8th week), 2N mice fed with SC + CM had a significant higher increase in body weight compared to their genotype-matched controls (SC group) (mean difference: 3.466; 95% CI: [0.4101, 6.521]; $p = 0.02$; Fig. 1b). However, for Ts65Dn mice fed with SC + CM, the difference did not reach statistical significance (mean difference: 2.416; 95% CI: [− 4.281, 9.112]; $p > 1$). In both genotypes, mice from the SC + HFD group showed a significantly higher percentage of body weight increase (for 2N: mean difference: 6.924; 95% CI: [3.627, 10.22]; $p < 0.0001$; Ts65Dn: mean difference: 6.380; 95% CI: [0.5205, − 12.24]; $p = 0.03$; Fig. 1b).

Both euploid controls and Ts65Dn mice that received SC + CM and SC + HFD exhibited an increase in energy intake compared to SC mice (diet effect ANOVA $F(1,48) = 13.67$, $p < 0.001$; Fig. 1c). Within each diet group, Ts65Dn mice consumed higher quantities of CM and HFD compared to 2N mice (Fig. 1d–e). Specifically, Ts65Dn mice consumed more CM or HFD than SC, whereas in 2N mice, a clear preference for HFD over SC was only observed in the SC + HFD group. Additionally, in both genotypes, SC intake was nearly neglected in the SC + HFD group (Fig. 1de).

In summary, providing free access to HFD resulted in overweight and increased food consumption in both genotypes. However, Ts65Dn mice exhibited a greater preference for and consumption of energy-dense food compared to 2N mice, even though the extent of body weight gain was less pronounced in Ts65Dn mice.

Energy-dense foods induced similar eating behavior changes in 2N and Ts65Dn mice

Ts65Dn mice exhibited a slower eating rate compared to 2N mice (genotype effect for meal duration, $F(1,83) = 13.63$; $p < 0.001$; genotype effect for eating rate, $F(1,83) = 19.85$; $p < 0.001$). The analysis of meal patterns revealed significant changes in the number of energy-dense food meals compared to SC meals (diet effect, $F(4,83) = 21.76$; $p < 0.001$, Fig. 2a–b). This effect was particularly pronounced in Ts65Dn male mice, where the number of HFD meals, but not CM meals, was significantly increased compared to SC meals (Sidak as a post-hoc; $p < 0.001$). Moreover, diet had a significant effect on the duration of the meals (diet effect, $F(4,83) = 15.14$; $p < 0.001$, Fig. 2cd), and eating rate [diet effect, $F(4,83) = 20$; < 0.001, Fig. 2ef]. Both CM and HFD meals were shorter in duration than SC meals (Sidak

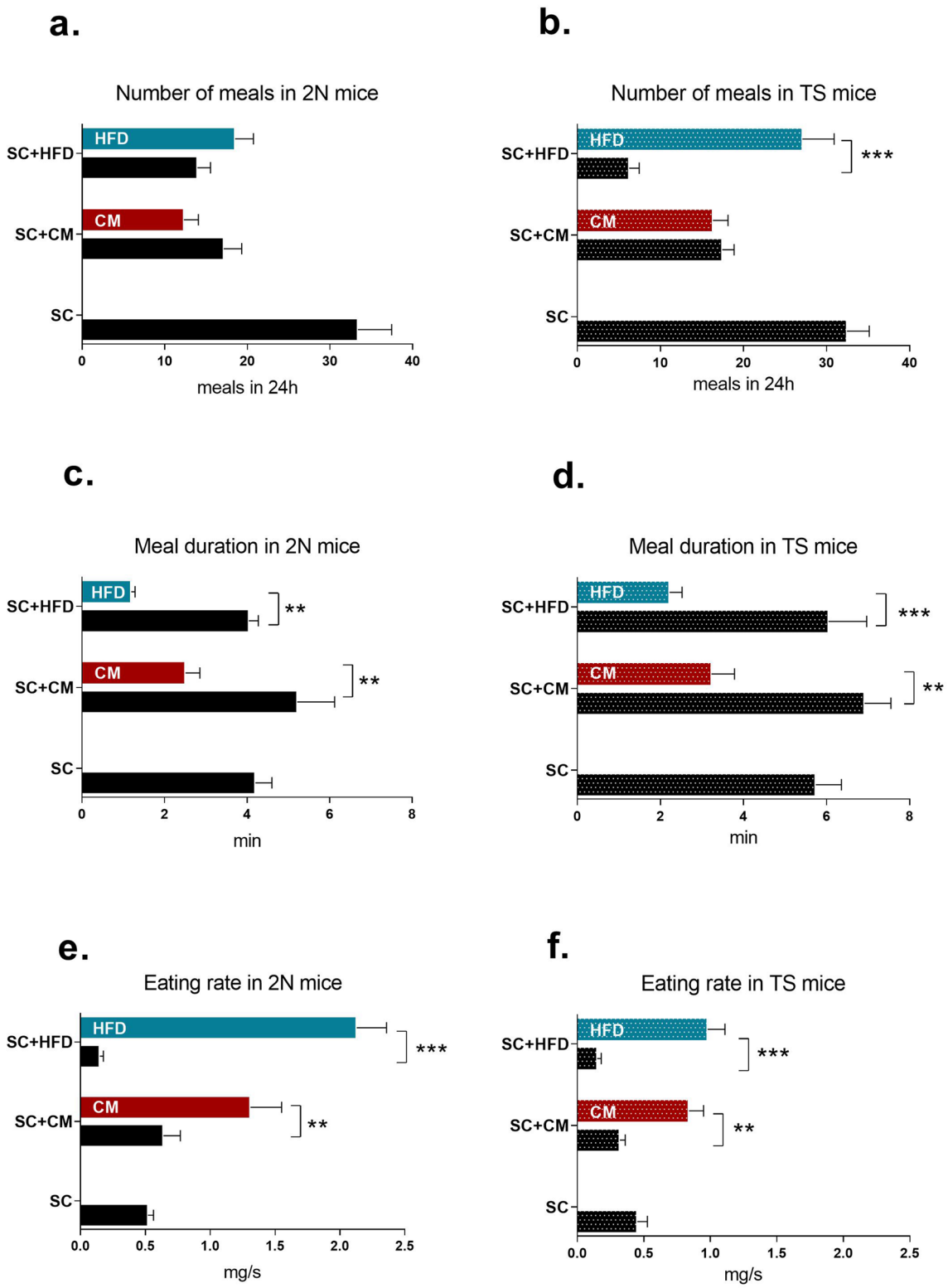


Fig. 2 "Snacking-like" behaviors' in euploid and Ts65Dn (TS) male mice. Bar plots illustrate the meal patterns for each food type in the experimental groups. In all figures, the left panel represents 2N mice, while the right panel represents Ts65Dn (TS) mice. **a**, **b** Number of meals. **c**, **d** Average meal duration (min). **e**, **f** Eating rate (mg/s). Data are presented as mean ± SEM. SC group: 2N = 8, Ts65Dn = 7; SC + CM group: 2N = 9, Ts65Dn = 10; SC + HFD group: 2N = 8, Ts65Dn = 7. Statistical significance is denoted as follows:*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. The figures depict the significance of intra-genotype comparisons, specifically CM vs SC (in SC + CM mice) and HFD vs SC (in SC + HFD mice)

post-hoc test; $p < 0.05$), and CM and HFD were consumed at a faster rate than SC (Sidak post-hoc test; $p < 0.05$) in both genotypes.

Ts65Dn male mice show enhanced compulsive-like food intake compared to euploid controls under limited access to energy-dense food

To assess whether the observed changes in meal patterns could be associated with inflexibility or the development of compulsive-like food intake [as described in [25]], eight weeks after providing free choice access to CM or HFD we evaluated compulsive overeating by limiting the access to CM or HFD to a one-hour period for three days (Fig. 3a). Comparing the restricted to non-restricted (ad libitum) conditions, we found that the consumption of CM was significantly higher during the one hour of limited access for all days in both genotypes (ANOVA repeated measures for CM, $F(3, 54) = 5.16$; $p = 0.003$; Fig. 3b). Instead, mice only exhibited a significantly higher intake of HFD on the 3rd day of limited access in the SC + HFD group (comparing one hour ad libitum to one hour on the 3rd day of limited access: in 2N mice: mean difference: 0.063; 95% CI: [0.009, 0.117]; $p = 0.012$; Fig. 3c; in Ts65Dn mice: mean difference: 0.152; 95% CI: [0.091, 0.213]; $p = 0.001$; Fig. 3c). Post-hoc analysis further revealed that on the 3rd day of limited access, Ts65Dn mice ate more CM than 2N mice (mean difference: 0.117; 95% CI: [0.039, 0.196]; $p = 0.001$; Fig. 3b–d) and HFD (mean difference: 0.084; 95% CI: [0.026, 0.143]; $p = 0.001$; Fig. 3cd).

Furthermore, we investigated the impact of energy restriction on the consumption of SC during the periods when access to calorie-dense CM or HFD was unavailable. It is well-documented that when energy-dense food options

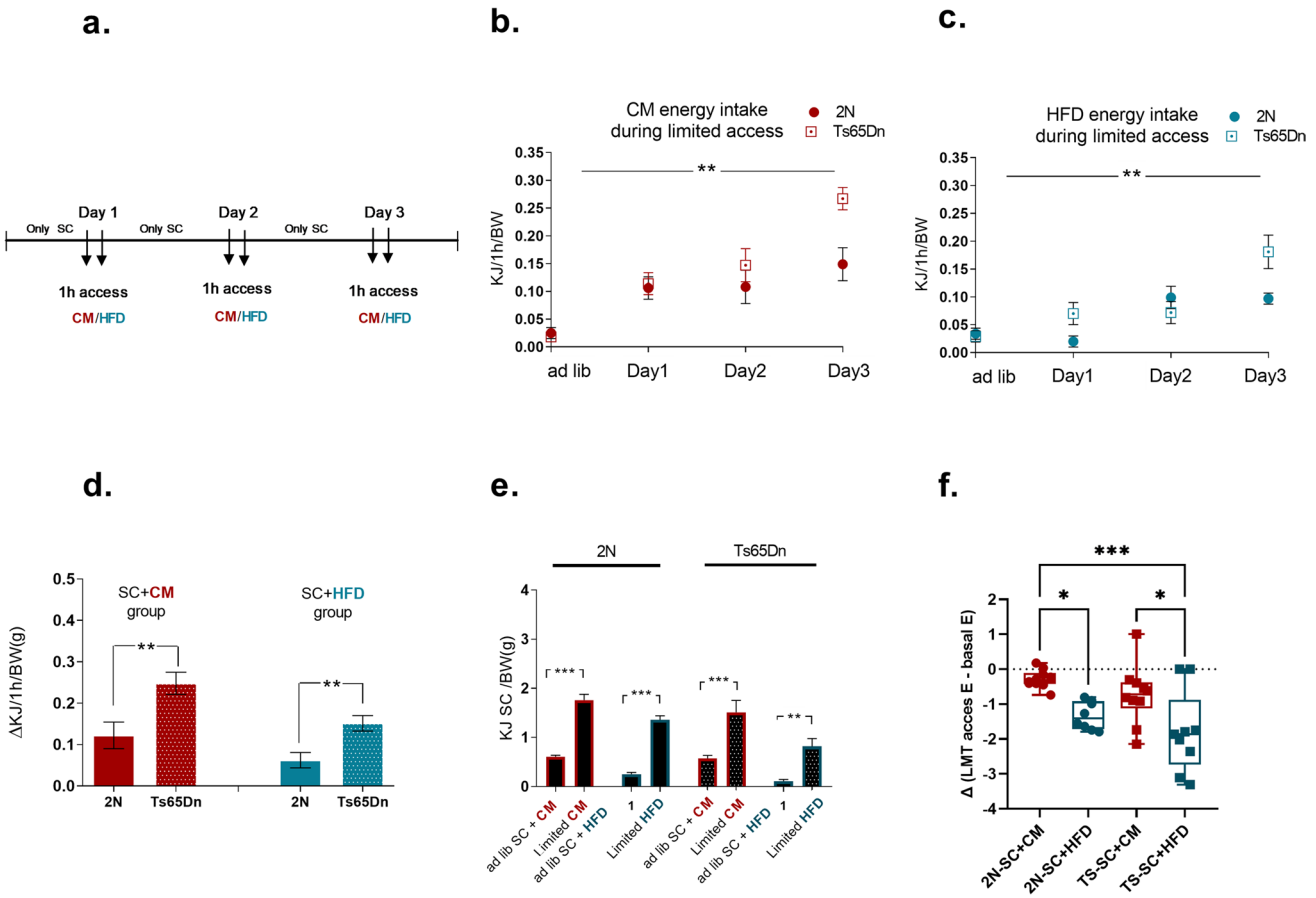


Fig. 3 Limited access to energy dense foods revealed binge-like intake in Ts65Dn male mice. **a** Limited access experiment schedule. **b** CM or **c** HFD energy intake ad libitum and during the three consecutive days of one-hour limited access to CM/HFD. **d** Mean one-hour energy intake of CM and HFD ad libitum conditions compared to the third day of limited access. **e** Mean SC-derived energy intake ad libitum ("ad lib") and in conditions of restricted access to CM or HFD, when only SC was available ("Limited"). Note the significant increases in all groups. **f** Difference between the SC-derived energy intake during restricted access to CM or HFD, when only SC was available and total energy intake (SC + CM/ SC + HFD) in ad libitum conditions. ad lib = ad libitum; LMT = limited access. Data are expressed as mean ± SEM. SC + CM group: 2N = 9, Ts65Dn = 10; SC + HFD group: 2N = 7; Ts65Dn = 9. Statistical significance is denoted as follows: $*p < 0.05$; $**p < 0.01$

are limited, mice exhibit an elevated consumption of the available food source, regardless of their preference, in order to compensate for the reduced energy intake [26]. The inability to adequately compensate for the decreased energy intake is considered an inflexible behavioral response to restriction. In our experiment, all groups showed an increase in energy intake of SC when access to calorie-dense was unavailable, compared to their intake of SC when both SC and CM/HFD were available ad libitum [$F(1,31) = 1115.2$; $p < 0.001$; Fig. 3e]. However, not all groups were able to reach their ad libitum caloric intake (Supplementary Fig. 3). During periods when energy-dense foods were unavailable, there was a significant difference in energy intake from SC between the HFD and CM groups (diet effect, $F(1,31) = 134.9$; $p < 0.001$; Supplementary Fig. 1). In the SC + HFD group, both genotypes exhibited a caloric deficit, with the Ts65Dn group experiencing a more pronounced deficit. Specifically, mice in the SC + HFD group exhibited a greater decrease in energy consumption compared to the SC + CM group, indicating worse compensation in the CM conditions. 2N mice showed similar energy intake between the baseline and the limited access of CM. However, SC + HFD Ts65Dn mice displayed reduced compensation (Fig. 3f). When comparing the difference in energy intake between limited access, when only SC was available, to baseline total energy intake (SC + CM/HFD ad libitum conditions), we observed significantly different degrees of compensation [$F(3,33) = 7.239$; $p < 0.001$; Fig. 3e]. In the SC + CM group, 2N mice were able to maintain their basal energy demand, whereas Ts65Dn mice did not (mean difference Ts65Dn SC + CM ad lib vs. limited access to CM: 0.736; 95% CI: [0.2436, 1.228]; $p = 0.002$; Supplementary Fig. 1). These results might indicate reduced ability to adequately compensate for the decreased energy intake, which suggest an inflexible behavioral response to restriction in Ts65Dn.

Long-term access to HFD induced inflexible behavior in Ts65Dn mice

To investigate the presence of inflexible behavior in Ts65Dn male mice, we conducted additional experiments to assess the influence of quinine hydrochloride adulteration on food preference. The inability to disregard the presence of quinine hydrochloride, either completely or partially is considered an indication of inflexibility [25, 27].

In the SC group, both genotypes exhibited a decrease in consumption of adulterated SC compared to non-adulterated chow under ad libitum conditions (Wilcoxon test; 2N: $p = 0.023$; Ts65Dn: $p = 0.047$; Fig. 4c). Only one mouse per genotype consumed a similar amount of adulterated SC as non-adulterated SC (Fig. 4a-b).

The adulteration of CM resulted in a less consistent anorectic response in the SC + CM group, but the effect was not statistically significant for either genotype (Wilcoxon test; 2N: $p = 0.846$; Ts65Dn: $p = 0.275$; Fig. 4f). However, in terms of behavior, 50% of 2N mice (Fig. 4d) and 30% of Ts65Dn mice (Fig. 4e) consumed adulterated CM despite its bitterness. Additionally, in Ts65Dn mice, 60% of the animals that initially avoided adulterated CM reduced their intake by more than 30% (Fig. 4e).

In the SC + HFD group, quinine adulteration resulted in a reduction in HFD consumption in more than 85% of the mice (8 out of 9 2N and 6 out of 7 Ts65Dn). Only one mouse per genotype consumed a similar or higher amount of adulterated HFD compared to non-adulterated HFD (Fig. 4gh). 2N mice consumed significantly less HFD (50% less) under adulterated conditions compared to non-adulterated conditions (HFD intake vs. bitter HFD intake in 2N, Wilcoxon test; 2N: $p = 0.019$; Fig. 4c). Conversely, the reduction in adulterated HFD consumption in Ts65Dn mice (30%) was not statistically significant compared to non-adulterated conditions ($p = 0.156$; Fig. 4i).

Low dopamine levels in prefrontal cortex of Ts65Dn might facilitate abnormal feeding behavior

Diet-induced obesity is associated with a decrease in dopamine (DA) availability in the prefrontal cortex. Thus, we examined the impact of different diets on the levels of DA in the brains of 2N and Ts65Dn male mice. To this aim, we conducted HPLC analysis in the striatum (ST) and prefrontal cortex (PFC) at the end of the obesity development (Fig. 5, Supplementary Tables 1, 2).

In the PFC, under non-obesogenic conditions Ts65Dn mice displayed lower DA levels compared to 2N mice. Interestingly, the effects of the diet differed between genotypes (Two-way ANOVA, diet x genotype interaction, DA: $F(2,28) = 5.20$, $p = 0.012$; Fig. 5b). As reported in the literature, exposure to energy-dense foods resulted in a reduction of PFC-DA in 2N mice [28, 29]. Similar findings were found in the PFC of rats exposed to CM diet. Instead, Ts65Dn mice exhibited this reduction only in the SC + HFD group. Notably, access to CM increased DA levels in the PFC of Ts65Dn mice compared to SC-fed trisomic mice (Fig. 5b, One-way ANOVA, Supplementary Table 2). To investigate the interplay between neurochemical phenotypes, behavioral changes, overeating, and obesity, we conducted a Spearman's correlation analysis. Our findings revealed significant correlations supporting the association between behavioral inflexibility and weight gain. Specifically, we observed a strong positive correlation (Spearman's rho of 0.6) between the energy intake of quinine-adulterated SC and increase in body weight ($P < 0$ upon z Fisher transformation), suggesting that behavioral

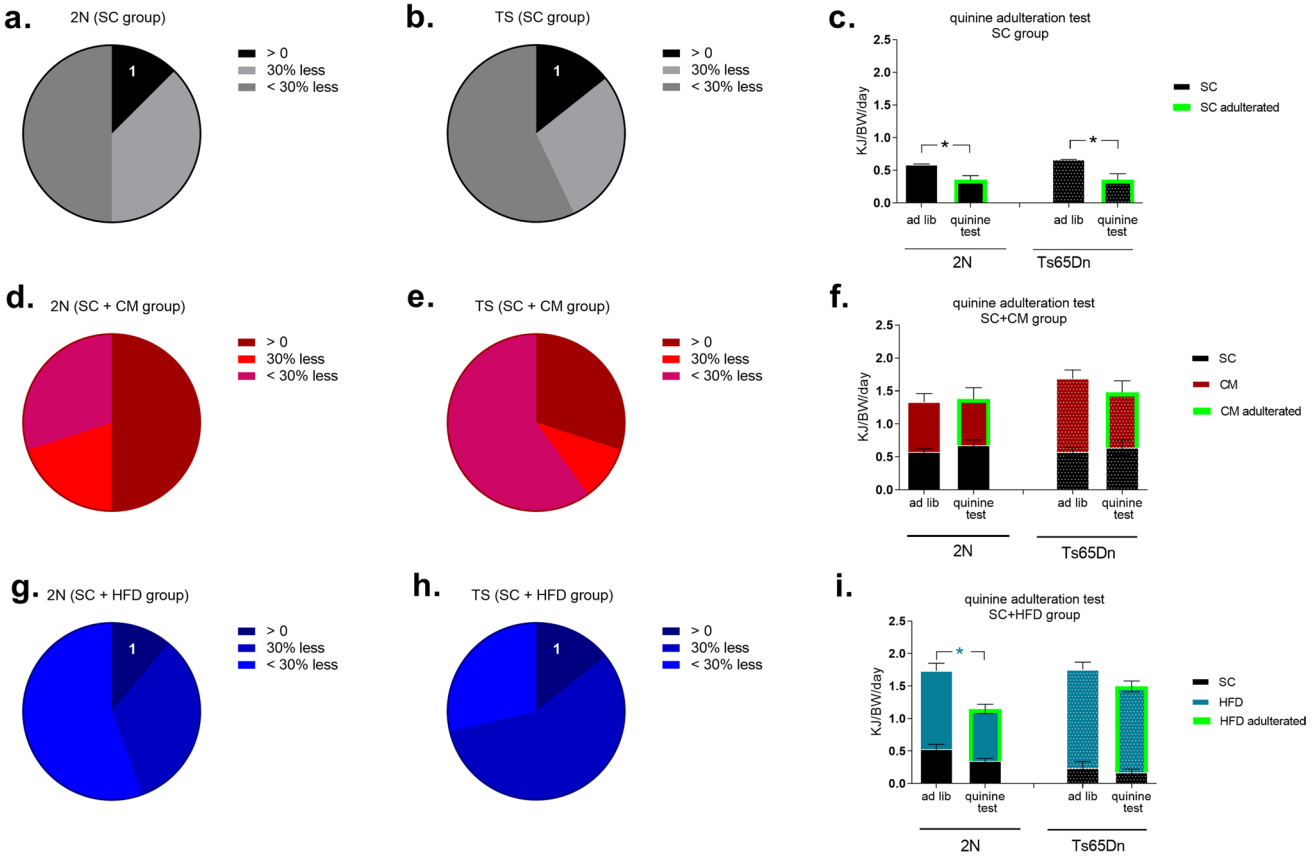


Fig. 4 Energy intake of quinine-adulterated food in euploid and Ts65Dn male mice. The figure depicts the 24 h energy intake (KJ/body weight in g) in non-adulterated feeding conditions (ad libitum, "ad lib") and after food adulteration. Percentage of 2N and Ts65Dn mice consumption of adulterated SC as compared to non-adulterated SC (**a, b**), adulterated CM (**d**, **e**) and adulterated HFD (**g**, **h**). **c** Consumption of adulterated vs. non-adulterated SC in the SC group. **f** Consumption of adulterated vs. non-adulterated CM in the SC + CM group. **i** Consumption of adulterated vs. non-adulterated HFD in the SC + HFD group. SC group: 2N = 8, Ts65Dn = 7; SC + CM group: 2N = 10, Ts65Dn = 10; SC + HFD group: 2N = 9; Ts65Dn = 7; Statistical significance is denoted as $*p < 0.05$

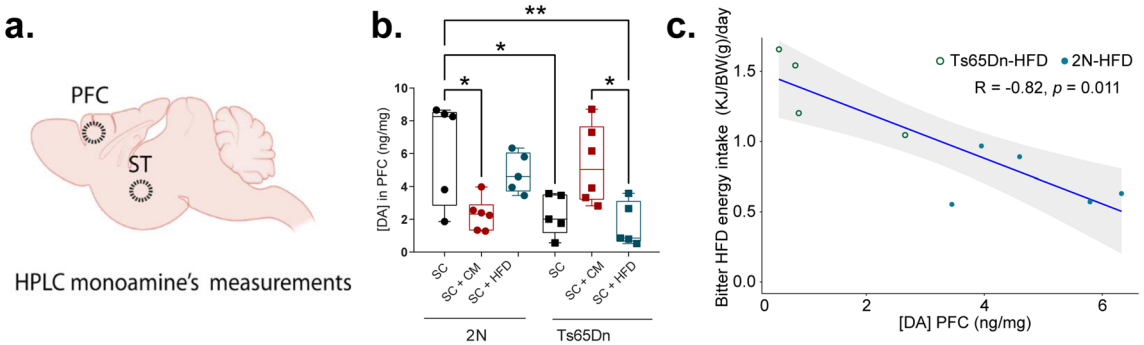
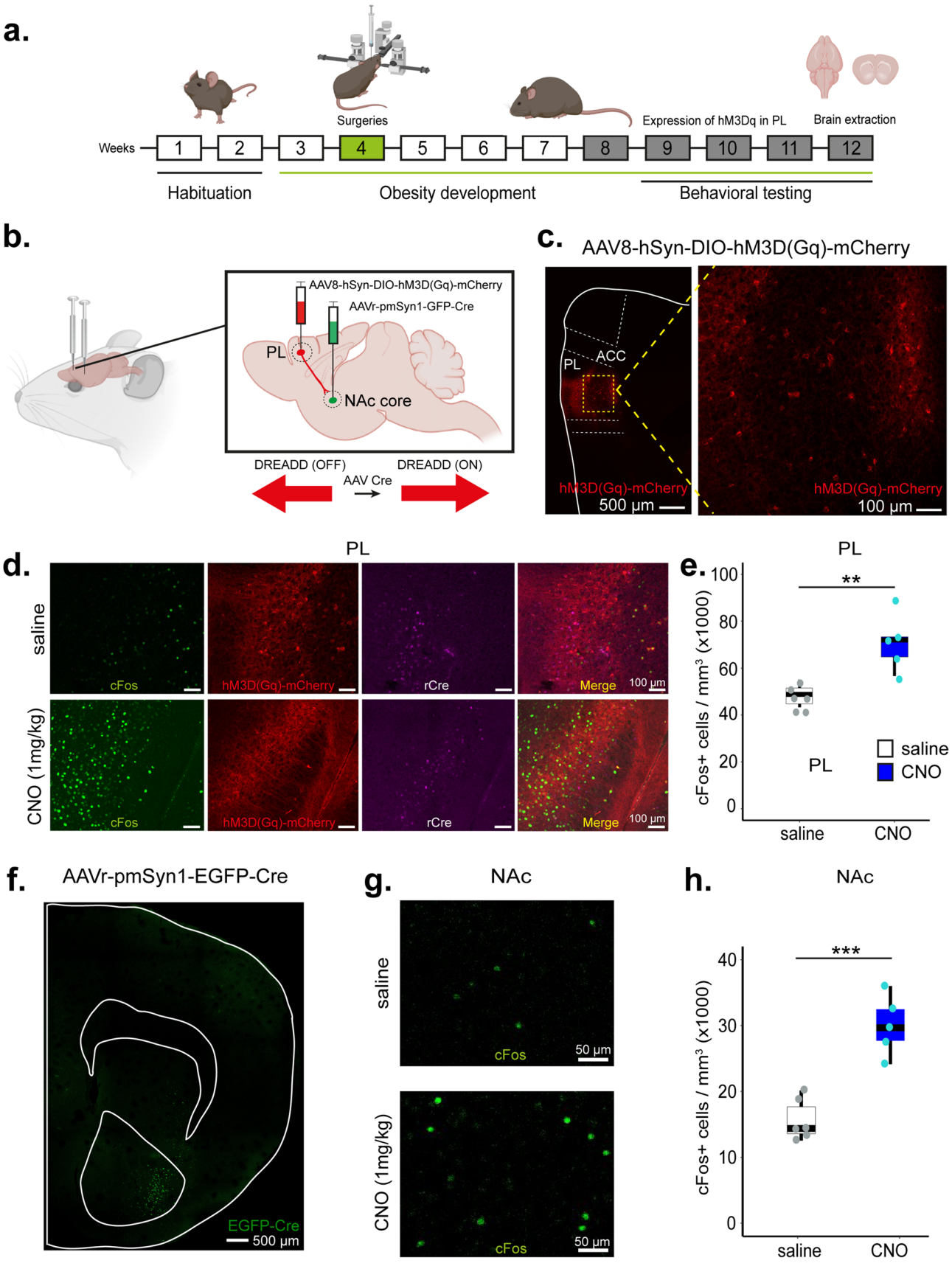


Fig. 5 PFC levels of monoamines and their metabolites in 2N and Ts65Dn male mice. **a** At the end of the behavioral battery, animals were sacrificed, and the PFC and ST were dissected and collected for HPLC monoamine determination. PFC concentration (ng/mg) of **b** dopamine (DA). SC group: 2N = 5, Ts65Dn = 5; SC + CM group: 2N = 6, Ts65Dn = 6; SC + HFD group: 2N = 5; Ts65Dn = 5. Two-way ANOVA, Bonferroni; Statistical significance is denoted as $*p < 0.05$; **a** comparisons showed are One-way ANOVA Bonferroni; $*p < 0.05$. **c** Dot plot showing the relationship between bitter HFD consumed during the quinine adulteration test on the y-axis and dopamine levels in PFC on the x-axis in SC + HFD. 2N mice are depicted as filled dots and Ts65Dn mice as empty dots. SC + HF group: 2N = 5, Ts65Dn = 5


a.
Surgeries
Expression of hM3Dq in PL
Brain extraction
Weeks
1
2
3
4
5
6
7
8
9
10
11
12
Habituation
Obesity development
Behavioral testing
b.
AAV8-hSyn-DIO-hM3D(Gq)-mCherry
AAVr-pmSyn1-GFP-Cre
PL
NAc core
DREADD (OFF)
DREADD (ON)
AAV Cre
c.
AAV8-hSyn-DIO-hM3D(Gq)-mCherry
PL
ACC
hM3D(Gq)-mCherry
500 µm
hM3D(Gq)-mCherry
100 µm
d.
PL
saline
CNO (1mg/kg)
cFos
hM3D(Gq)-mCherry
rCre
Merge
100 µm
e.
PL
**
cFos+ cells / mm³ (x1000)
saline
CNO
f.
AAVr-pmSyn1-EGFP-Cre
EGFP-Cre
500 µm
g.
NAc
saline
CNO (1mg/kg)
cFos
50 µm
h.
NAc
***
cFos+ cells / mm³ (x1000)
saline
CNO

◂**Fig. 6** Activation of PL-NAc projections. **a** Experimental design (Experiment 2). Mice were individually housed in PheCOMP cages to register locomotor activity and meal pattern as described above. During the habituation period, all mice received standard chow (SC). After two weeks, mice were allocated to receive a free access HFD. In the fourth week, surgeries were performed to inject AAV8-hSyn-DIO-hM3D(Gq)-mCherry in PL and AAVr-pmSyn1-GFP-Cre in the NAc core. From week 9 to 12, behavioral tests were performed. At week 12, the brain was extracted and processed for immunofluorescence. **b** Schematic representation of AAV8-hSyn-DIO-hM3D(Gq)-mCherry injection in PL and AAVr-pmSyn1-GFP-Cre injection in the NAc core. Only when both AAVs co-localize in the PL region, the recombination of hMD(Gq)-mCherry is produced. **c** Representative immunofluorescence image of Cre-dependent hM3D(Gq)-mCherry expression restricted at PL injection site (left) and amplified image (right). **d** Representative immunofluorescence images of mice injected either with saline (above) or with CNO (1 mg/Kg) at the PL region. Both hM3D(Gq)-mCherry (red) and rCre (magenta) are detected in the PL region. cFos is shown in green. **e** Quantification of cFos positive cells after saline (white) or CNO (blue) administration in the PL region. (2N saline = 6 male mice, 2N CNO = 5 male mice). **f** Representative immunofluorescence image of Cre recombinase detected at the NAc injection site. **g** Representative immunofluorescence images showing cFos + neurons at the NAc region of mice injected either with saline (above) or with CNO (1 mg/Kg). **h** Quantification of cFos positive cells after saline (white) or CNO (blue) administration in the NAc region. (2N saline = 6 mice, 2N CNO = 5 mice). On the boxplots, the horizontal line indicates the median, the box indicates the first to third quartile of expression and whiskers indicate 1.5 × the interquartile range. Two-tailed t test. ***$p \leq 0.001$

inflexibility may contribute to weight gain (Supplementary Fig. 2a). Within the SC + HFD group, we identified robust correlations between behavioral variables linked to inflexibility and DA levels (Fig. 5c). Notably, low DA levels in the prefrontal cortex (PFC) were correlated with the consumption of the bitter HFD during the quinine adulteration test (Spearman's rho of − 0.8, Fig. 5c).

Diet-induced obesity can also affect the expression and function of dopamine receptors in the prefrontal cortex. Specifically, there is a downregulation of Ddr2 receptors, which play a crucial role in DA signaling. In our experiments, no significant differences were observed in the expression of Ddr1 and Ddr2 in the cortex between 2N and Ts65Dn mice under non-obesogenic conditions nor when exposed to palatable diets. Likewise, the ratios of DA in the striatum to Ddr1 or Ddr2 expression in the cortex showed no significant changes among the different experimental groups. Interestingly, a significant negative correlation was found between the levels of DA in the striatum and the expression of Ddr2 in the cortex (Spearman correlation, $p = 0.032$, Supplementary Fig. 3f) while no correlation was observed with Ddr1 expression (Supplementary Fig. 4e). Additionally, a significant positive correlation was found between the expression of Ddr1 in the cortex and the increase in energy intake from HFD on the third day of limited access, compared to ad libitum conditions, in both 2N and Ts65Dn mice (Spearman correlation, $p = 0.01$, Supplementary Fig. 4a).

Activation of PL-NAc projections rescues compulsive-like behavior in Ts65Dn male mice

Our findings demonstrate the presence of inflexibility and compulsive-like behaviors, particularly in trisomic mice. In a previous study, we established the involvement of D2R-modulated projections from the prelimbic cortex (PL) to the nucleus accumbens (NAc) core in compulsive-like behaviors [9]. Furthermore, our current study reveals a hypodopaminergic phenotype in Ts65Dn mice. We thus investigated whether selectively increasing the activity of PL neurons projecting to the NAc core could alleviate compulsive-like behavior in trisomic mice, we employed a combination of chemogenetics and retrograde adeno-associated virus (AAV) techniques. Specifically, we administered AAV-hM3Dq DREADD (AAV-hSyn-DIO-hM3D(Gq)-mCherry) into the PL and AAV-retrograde-Cre (AAVr-Syn-EGFP-Cre) into the NAc core (Fig. 6ab). This allowed for the selective expression of hM3Dq receptors in PL neurons projecting directly to the NAc core. To validate the injection sites of the AAVs and confirm retrograde transport of Cre, immunofluorescence staining was employed to visualize mCherry and Cre recombinase (Fig. 6cd). Additionally, we confirmed that the administration of CNO (1 mg/Kg) resulted in a substantial increase in neuronal activation in both the PL (Two-tailed t test, $p = 0.009$; Fig. 6de) and in the NAc (Two-tailed t test, $p < 0.001$; Fig. 6gh) compared to control mice that received saline injections. This increase was demonstrated by the higher number of cFos + cells observed in these regions.

We then conducted a study to examine the impact of activating PL-NAc core projections during limited access and quinine adulteration tests (Fig. 7a). Since a HFD consistently induced inflexible and compulsive-like behaviors in Ts65Dn mice, we used this diet to investigate whether activating the PL-NAc projection could prevent compulsive-like behavior in Ts65Dn mice. Neither weight gain nor meal patterns were affected by AAV injection in 2N or Ts65Dn mice (Supplementary Fig. 5a–c).

We examined the effects of activating PL-NAc core projections in the quinine adulteration test. In non-injected mice, we confirmed that adulteration of HFD resulted in reduced energy intake in 2N mice but not in Ts65Dn mice. However, when we activated the PL-NAc core projections, we found a significant decrease in consumption of adulterated HFD compared to non-adulterated conditions in both 2N mice and Ts65Dn mice injected with CNO (Two-way ANOVA $F(1,32) = 8,68$; $p < 0.001$; TukeyHSD multiple comparisons as post-hoc; $p = 0.0005$ for 2N ad libitum vs. 2N quinine test adulteration + CNO; $p = 0.012$ for Ts65Dn ad libitum vs. Ts65Dn quinine test adulteration + CNO; Fig. 7b). These findings suggest that the activation of PL-NAc core projections may contribute to the compulsive feeding behavior observed in Ts65Dn mice.

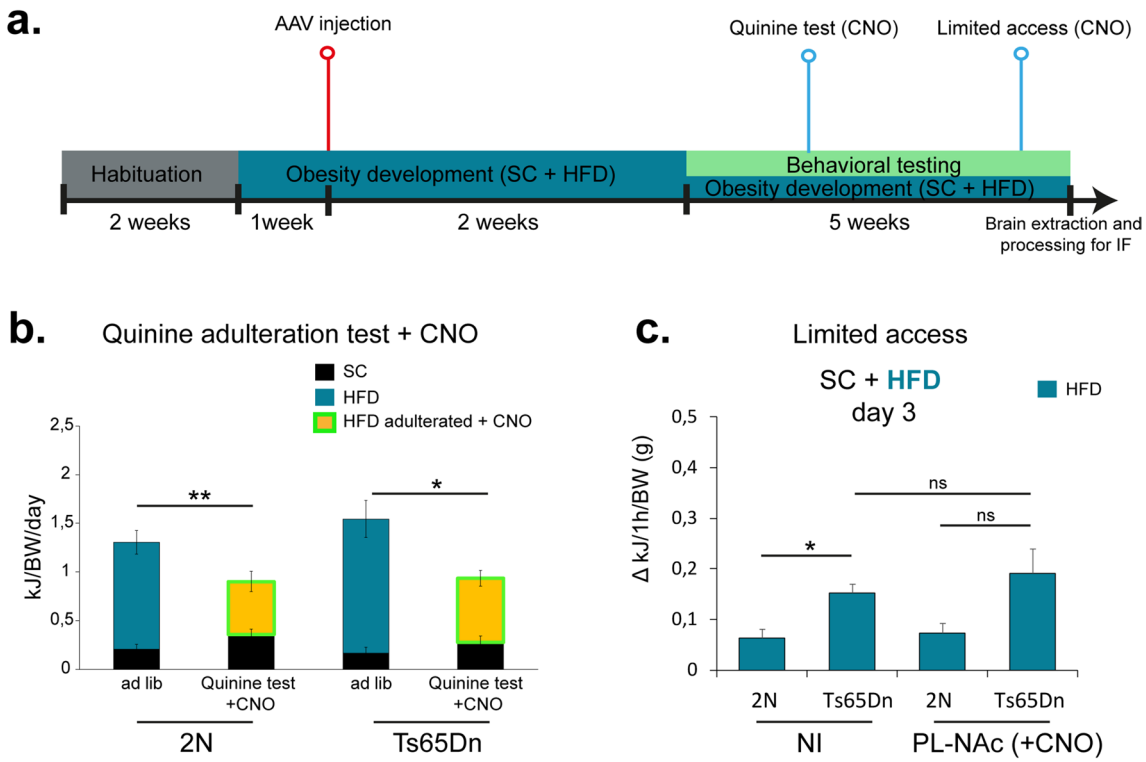


Fig. 7 Activation of PL-NAc projections rescue compulsive-like behavior but not inflexibility in Ts65Dn male mice. **a** Experiment schedule showing the battery of behavioral tests. **b** Energy intake of quinine-adulterated HFD food in 2N and TS mice. 24-h energy intake (KJ/body weight (g) in no adulteration (ad libitum, "ad lib"; left) and with HFD adulteration conditions after activating the PL-NAc projections with CNO (right). Consumption of adulterated HFD in the SC + HFD group. **c** HFD energy increases between ad libitum conditions and day 3 of limited access in non-injected mice (NI) compared to mice in which PL-NAc projections were tagged and CNO was injected i.p. 30 min before the test (2N NI = 9, Ts65Dn NI = 9, 2N PL-NAc = 12, Ts65Dn PL-NAc = 6)0.2N = 12, Ts65Dn = 6. Statistical significance is denoted as follows: $*p \leq 0.05$; $**p \leq 0.01$

Instead, in the limited access to HFD experiment, the activation of PL-NAc projections using CNO did not modify the increase of consumption of HFD during limited access, compared to ad libitum conditions neither in 2N (ANOVA repeated measures for HFD, F (3, 33) = 2.73; p = 0.059; Fig. 7c; post hoc analysis on day 1 in 2N mice: ad libitum vs. day 1; p = 0.019; Fig. 7c) nor in Ts65Dn mice (ANOVA repeated measures for HFD, F (3, 15) = 6.43; p = 0.005; Fig. 7c; Post hoc analysis ad libitum vs. day 1; p = 0.033; ad libitum vs. day 2; p = 0.033; ad libitum vs. day 3; p = 0.033), although this time we did not observe genotype-dependent differences in HFD consumption (Fig. 7c). Comparing the increase in HFD consumption (ad libitum vs. day 3 of limited access) between non-injected mice (NI) and mice whose PL-NAc projections were tagged and activated with CNO, we observed no significant differences in either 2N or Ts65Dn mice (Wilcoxon pairwise comparisons as post-hoc; p = N.S.; Fig. 7c). Taken together, these results suggest that the activation of PL-NAc projections had no effect on inflexible behavior.

Discussion

The findings of our study shed light on the mechanisms underlying the increased preference for energy-dense food and the development of compulsive and inflexible behaviors in the Ts65Dn mouse model. We propose that these behavioral phenotypes are driven by reduced prefrontal dopaminergic tone, which may lead to overeating as an attempt to restore optimal dopamine levels. Moreover, our study demonstrates that activating the PL-NAc core projections can effectively reduce impulsive and compulsive behaviors in Ts65Dn mice, offering potential therapeutic avenues for intervention.

Our analysis of the meal patterns revealed a clear preference for energy-dense food in both Ts65Dn and 2N male mice, as indicated by increased consumption of high-fat diet (HFD) and chocolate mixture (CM) compared to standard chow (SC). This heightened consumption mirrors the overconsumption of energy-dense foods observed in individuals with DS [5, 30]. However, Ts65Dn mice

exhibited significantly higher consumption of both HFD and CM than their wild-type counterparts. Notably, we found that Ts65Dn mice only displayed overweight when exposed to HFD. These results support our previous findings that Ts65Dn mice develop obesity specifically in response to HFD consumption, resulting in increased adiposity [31].

Interestingly, both Ts65Dn and 2N mice showed increased eating rate and reduced meal duration when consuming HFD and CM, indicating a "snacking behavior" associated with impulsivity and overeating [32]. Ts65Dn mice exhibited longer and slower meals, similar to the phenotype observed in individuals with DS [33–35]. Although physical properties of energy-dense foods could potentially influence the feeding rate [36], our results are more suggestive of snacking behavior [37].This suggests a delay of the satiation signals, leading to overconsumption [25].

To further explore the compulsive eating behaviors, we implemented an experiment with limited access to CM and HFD. In our limited access conditions, in contrast with the majority of mouse models for the study of compulsive eating, mice are not submitted to starvation between the periods of energy-dense food access, as they have ad libitum SC [38]. Surprisingly, Ts65Dn mice displayed more pronounced binge-like bouts when refeeding on the energy-dense foods, even without experiencing periods of food restriction or starvation between access periods. This binge-like behavior was primarily attributed to inflexibility rather than a deficient energy state [39]. While 2N mice compensated for the limited access by increasing their consumption of SC, Ts65Dn mice did not fully compensate, particularly in the case of HFD. Additionally, Ts65Dn mice consumed equivalent amounts of non-adulterated HFD and HFD-quinine, despite the latter having an unpleasant taste. This inflexible behavior suggests that Ts65Dn mice struggle with adapting their feeding behavior, particularly when it comes to energy-dense foods, as flexible mice (self-controlled ones) will neglect or significantly reduce the intake of the quinine adulterated [26, 39].

It is worth considering that the taste and/or olfactory deficits reported in adults with DS may contribute to the observed behaviors [40]. However, our findings suggest that inflexibility in Ts65Dn mice is not solely due to sensory deficits, as they showed equivalent avoidance of adulterated SC and CM, but is specific for HFD. Interestingly, it has been well-established that reward circuitries in the brain play a crucial role in regulating the pleasurable aspects of feeding behavior, and diet can influence monoamine levels, which in turn can impact behavioral responses [29, 41–43]. Numerous studies have associated overeating of energy-dense foods with molecular and functional alterations in the reward system, particularly in the PFC and ventral striatum [27, 44, 45]. Specifically, DA is known to modulate PFC function, facilitating flexible behaviors and playing a role in controlling impulsive choices where it mediates flexible behaviors and participates in the control of impulsive choices [46, 47].

Remarkably, our previous research revealed that inhibiting the PL-NAc core pathway induced compulsive-like behaviors [9]. Building upon this finding, we hypothesized that the observed behavioral differences between the genotypes might be linked to alterations in monoamine levels within the PFC and the ventral striatum, particularly the NAc. In our experiments, striatal monoamine concentrations did not differ significantly between genotypes, Ts65Dn mice exhibited lower DA in the PFC under non-obesogenic conditions as compared to 2N. This is in contrast with the results of Dekker and collaborators [48] who did not detect reduced DA levels in brain regions of Ts65Dn mice. One possible explanation for this discrepancy could be attributed to variations in the techniques employed and the age of the mice utilized in both studies. However, our findings align with previous research indicating a deficit in dopamine in human fetuses [13] and in DS adults [49]. Moreover, a recent study utilizing HPLC coupled with electrochemical detection revealed differences in the levels of metabolites associated with dopaminergic, serotonergic, and kynurenine pathways in trisomic mice, and these differences were found to be exacerbated with age [50].

In our experimental findings, we observed that 2N mice, when fed a high-fat diet and exposed to a chocolate mixture meal, displayed lower dopamine levels in the PFC compared to 2N mice on a standard chow diet (2N-SC). These results are consistent with previous studies that have reported similar findings [28, 51]. Interestingly, the effects of HFD and CM on DA-PFC levels in Ts65Dn mice were opposite: CM increased DA levels, while HFD exacerbated the already detected hypodopaminergic state observed under non-obesogenic conditions,.probably driven by a hypodopaminergic state may contribute to a more pronounced phenotype when Ts65Dn mice are exposed to an HFD. Furthermore, we observed a significant negative correlation between PFC-DA levels and the consumption of an adulterated HFD in the quinine test, which serves as an index of inflexibility, for both genotypes. In fact, Ts65Dn mice fed with SC + HFD exhibited the lowest DA-PFC levels and demonstrated the highest signs of inflexibility (Supplementary Fig. 1). In the striatum (ST), following long-term access to energy-dense diets, we observed a non-significant increase in DA levels in both Ts65Dn and 2N mice. Elevated DA levels in the ST have been associated with the severity of binge episodes [52], as the ST is a crucial area involved in seeking behavior [53]. Therefore, it could be speculated that the increase in DA levels observed in mice fed energy-dense diets may represent a neurochemical correlate of the behavioral neuroadaptations that sustain overeating.

We propose that overeating in Ts65Dn mice is, to a certain extent, attributable to a hypodopaminergic endophenotype in the PFC which may contribute to reward deficiency. The observed low DA levels in the PFC could stem from a combination of reduced DA production and/or release, primarily regulated by the activation of D2 receptors [54]. This notion is supported by evidence from studies on obesity-prone rats, which demonstrate pre-existing deficits in dopamine neurotransmission, including diminished DA release [55], decreased D2-receptor availability [56, 57], and heightened sensitivity to dopaminergic agents [51].Multiple lines of evidence suggest that mPFC exhibits hypoactivity in human addicts [58] and in rats displaying compulsive-like behaviors [59]. Notably, we previously demonstrated that reducing PFC activity was sufficient to promote compulsive-like behaviors [9]. Furthermore, PFC hypoactivity has been linked with higher body weight, and the PL region of the PFC has been associated with addiction [60]. In our previous work, inhibiting PL-NAc projections not only increased the percentage of animals classified as food-addicted but also induced compulsive-like behaviors. Given the hypodopaminergic tone in the PFC of Ts65Dn mice, it is possible that decreased activity of mPFC promotes food addiction through reward-seeking behaviors to compensate for the decreased DA levels. Consequently, we specifically decided to increase PFC activity. Through an excitatory chemogenetic approach, we successfully restored compulsive-like behaviors in Ts65Dn mice exposed to highly palatable foods but not inflexibility by selectively activating PL-NAc projections in Ts65Dn mice. As the majority of PL-NAc projections consist of glutamatergic neurons, our results suggest that enhancing glutamatergic transmission in the PL prevents the development of compulsive-like behavior in Ts65Dn mice. In conclusion, our study sheds light on the involvement of reward-related mechanisms in the development of obesity in DS. We have provided evidence for deficits in these mechanisms, which contribute to an increased preference for energy-dense food, as well as the presence of compulsivity and inflexibility. Specifically, our findings demonstrate a reduction in DA levels within the PFC of Ts65Dn mice, suggesting a potential association between diminished dopaminergic tone in the PFC and the occurrence of hedonic overeating and obesity in DS. Significantly, our results not only support the notion of dopaminergic dysregulation in Ts65Dn mice as a facilitator of these behaviors, but also offer novel insights into the underlying mechanisms of food addiction in DS. The identification of this new mechanism provides a fresh perspective on the susceptibility to overeating and unveils potential avenues for addressing obesity through innovative interventions.

It is important to acknowledge that the observed phenotype in Ts65Dn mice may be influenced by the presence of triplicated non-HSA21 genes. This consideration serves as a valuable point for future investigations to explore the impact of additional genetic factors on the observed outcomes.

In this study, we aimed to investigate the mechanisms underlying hedonic overeating in obese mice. For this reason, and since female mice present a reduced propensity to gain weight and become obese when exposed to HFD related to ovarian hormones, since ovariectomy eliminated the protection of female mice to weight gain, we decided to perform the experiments in male mice [61].

The implications of our findings extend beyond DS, as they offer valuable insights into the understanding and potential treatment of other psychiatric disorders characterized by alterations in compulsive behaviors. By unraveling the intricate relationship between reward-related mechanisms, dopaminergic dysregulation, and obesity, our research paves the way for future studies aimed at developing targeted interventions for individuals affected by these conditions.

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Author contributions MD, MF, ÁF, EM and RM designed the experiments and discussed the results. MF, ÁF, AG and MMdL performed the experiments and MF, ÁF and AG analyzed the behavioral studies. MF, JD, and NJ performed and analyzed the bioamine determination by HPLC. CS quantified the expression of *Ddr1* and *Ddr2 by q-PCR*. NL contributed to the acquisition of confocal images. IdT performed the correlations and statistical analysis between the behavioral and the brain bioamine's data. MF, and ÁF did the statistical analysis of the results. MF, ÁF and MD wrote the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All the raw data and supplementary materials are available upon request.

Declarations

Conflict of interest The authors declare no competing financial interests.

Ethical approval and consent to participate 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 the use of Laboratory Animals n° A5388-01 (NIH).

Consent for publication All authors consent for publication.

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