PRIME

Prevention and Remediation of Insulin Multimorbidity in Europe

H2020 – 847879

D2.1. – Report on comorbidity of compulsivity, learning difficulties, and diabetes in TALLYHO mice

Dissemination level	Public
Contractual date of delivery	31.December.2022
Actual date of delivery	27.December.2022
Type	Report
Version	1
Filename	PRIME_Deliverable Report_D2.1.
Workpackage	2
Workpackage leader	Simone Macri (ISS)

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 847879.

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Abbreviations

T2DM	Type 2 Diabetes mellitus
AD	Alzheimer's disease
OCD	obsessive compulsive disorder
ADHD	attention deficit hyperactivity disorder
TH	TALLYHO/JngJ mice
MS	metabolic syndrome
IDF	International Diabetes Federation
WHO	World Health Organization
IR	Insulin Receptor,
IGF-1R	Insulin Growth Factor-1 receptor
MCI	mild cognitive impairment
SW	SWR/J mice, JAX stock #000689
ASST	Attentional set shifting task
PND	post-natal day
IPGTT	intra-peritoneal glucose tolerance test
IST	insulin sensitivity test

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1. Executive Summary

Type 2 Diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by chronic hyperglycaemia, resulting from deficits in insulin secretion, insulin action, or both. Whilst the role of insulin in the peripheral nervous system has been ascertained in countless studies, recent data indicate that it also acts in the central nervous system, wherein its receptors are abundant. Brain insulin has been recently associated with numerous brain disorders like Alzheimer's disease (AD), obsessive compulsive disorder (OCD) and attention deficit hyperactivity disorder (ADHD). Thus, understanding the role of insulin as a common risk factor for the comorbidity of mental and somatic disorders may disclose novel preventative and therapeutic approaches. To further detail this association, while accounting for sex and age differences, we conducted an extended test battery for the evaluation of general metabolism (glucose tolerance, insulin sensitivity, energy expenditure, lipid metabolism, and polydipsia) and cognitive capabilities (attention, cognitive flexibility, and memory), in adolescent, young adult, and adult male and female TALLYHO/JngJ mice (TH). TH mice, selectively bred for baseline hyperglycaemia, have been reported to constitute a valid experimental model of T2DM due to impaired insulin signalling. While TH mice exhibited remarkable deficits in cognitive flexibility, we observed that these alterations emerged either much earlier (males) or independent of (females) full-blown T2DM-like abnormalities. Therefore, the nature of this association suggests that deficits in insulin signalling may represent a common risk factor for both T2DM and CNS-related deficits, and that this association may stem from (partly) independent mechanisms

2. Deliverable report

The association between altered glucose metabolism and mental disturbances is not new. As early as 1919, Kooy[1] reported that several psychiatric patients presented with glycosuria compared to healthy individuals. Following these original observations, the discovery of insulin and of its role as a key regulator of glucose metabolism disclosed relevant avenues to identify potential common risk factors in mental and somatic disturbances. Yet, alterations in insulin signalling have been primarily associated with somatic disturbances like type 1 diabetes, type 2 diabetes mellitus (T2DM), metabolic syndrome (MS), atherosclerosis[2], non-alcoholic fatty liver disease[3], polycystic ovary disease[4], and obesity[5–7], and much less with behavioural and mental disorders. Only recently is experimental and epidemiological evidence supporting the view that altered insulin signalling is also involved in mental disorders like Alzheimer's disease (AD)[8], obsessive compulsive disorder (OCD)[9] and attention deficit hyperactivity disorder (ADHD)[10]. Affecting over 20% of the population, the somatic diseases outlined above account for 1,2 trillion US $ in global healthcare costs annually[11]. Focussing solely on diabetes, according to the International Diabetes Federation (IDF), approximately 537 million adults (20-79 years) were living with this condition in 2021; it is projected that 643 million will face it by 2030 and 783 million by 2045[12]. The societal and economic burden is further amplified when considering the comorbid mental diseases in which insulin is hypothesised to play a role. For example, the World Health Organization (WHO) estimates that around 55 million people have

dementia[13] and, of these, approximately 60-70% have AD[14]. Therefore, understanding the contribution of altered insulin signalling as a common risk factor in the aforementioned comorbidities represents a much-needed knowledge leap in the management and treatment of chronic highly-debilitating diseases.

The role of insulin in T2DM and MS has been ascertained in countless studies[6,15–17] and is directly associated with its primary role in glucose metabolism. Briefly, upon glucose intake, the pancreatic beta-cells release insulin which in turn regulates glucose uptake by liver, gut and muscles, and ultimately suppresses endogenous glucose production[18]. In T2DM, insulin function is defective due to two main anomalies: beta-cell dysfunction and insulin resistance[18]; the latter indicating a reduced sensitivity to insulin of cells in peripheral tissues (primarily muscles, liver and adipose tissue[19]). Decreased insulin sensitivity, at first, triggers a hyperfunction of the beta-cells of the pancreas, resulting in increased production of insulin aimed at maintaining normal blood glucose concentrations (hyperinsulinemia). Subsequently, this process gradually affects beta-cells functionality ultimately causing insulin deficiency and hyperglycaemia[20] (typically set at fasting plasma glucose levels >110 mg/dL), a hallmark of T2DM and MS.

Beside its function in the peripheral nervous system, insulin also exerts a major role in the central nervous system (CNS), wherein its receptors (e.g., Insulin Receptor, IR, and Insulin Growth Factor-1 receptor IGF-1R) are highly expressed[21]. Insulin exerts this role via two primary routes: it can either derive from the periphery crossing the blood brain barrier (accounting for a significant portion of insulin present in brain) or via direct synthesis by neurons[22,23]. There is even evidence showing that, beside regulating glucose metabolism, insulin in the CNS contributes to several non-metabolic functions, such as learning, memory, integration of sensory information, and modulation of synaptic plasticity[24]. Evidence in support of a role of insulin signalling in mental function and disease derives from genetic[25], clinical[26], and preclinical[27,28] investigations. For example, recent studies have shown a correlation between T2DM and AD and have highlighted common pathophysiological characteristics[8,29,30]. The association between T2DM and cognitive decline extends beyond AD, whereby T2DM patients are at increased risk of milder forms of cognitive decline including mild cognitive impairment (MCI) and diabetes-associated cognitive decrements[28,31]. The latter may include cognitive capabilities other than memory, like processing speed and executive functions[32,33]. Importantly, as described by Biessels and Despa[28], these milder forms of cognitive decline may occur during pre-diabetic stages and slowly worsen over time[34]. Mallorquí-Bagué and collaborators recently reported an association between metabolic syndrome and impairments in impulse control in a large cohort of obese patients[35]. Failures in impulse control have also been observed[36] in ADHD[37] and addiction[38,39], both of which have been associated with disturbances in insulin signalling. Genetic and genomics studies also identified a link between insulin signalling and OCD[9]. Specifically, adopting an integrated strategy combining enrichment analyses and review of top-ranked genes derived from genome-wide association studies, van de Vondervoort and collaborators[9] reported that insulin-dependent signalling cascades are associated with OCD. These data have also been supported by Bralten and collaborators[40], who showed that impaired insulin signalling is involved in OCD[40]. Furthermore, clinical investigations

consistently reported that anti-diabetic drugs have beneficial effects on cognitive impairments in both AD, MCI, and other cognitive decrements[41–43].

Within this broad framework, animal models can help deciphering the fundamental mechanisms underlying the comorbidity between T2DM and cognitive decline. Specifically, as cogently summarised by Biessels and Despa, "Animal models that adequately capture the heterogeneity of diabetes seen in humans are essential to uncover a pathological substrate for cognitive dysfunction and dementia in T2DM"[28]. Evidence in support of this proposition is accumulating. For example, Nisticò and collaborators[27] showed that heterozygous mice, haploinsufficient for the beta subunit of the insulin receptor, exhibited impaired memory capabilities and altered synaptic plasticity correlates in the form of long-term potentiation. Complementarily, Ramos-Rodriguez et al.[44] reported that a murine model of AD (APP/PS1), with impaired cognitive capabilities, was also characterised by poor glycaemic control. Furthermore, leveraging an inbred mouse strain (TALLYHO/JngJ, JAX stock #005314, hereafter TH) spontaneously exhibiting diabetes-like abnormalities (late-onset hyperglycaemia and insulin resistance), van de Vondervoort and collaborators[45] reported that alterations in insulin signalling relate to increased compulsivity and anxiety. TH mice are characterised by an elevated degree of construct and face validity in T2DM research, wherein they resemble the human disease in terms of aetiology (construct validity[46,47]) and symptomatology (face validity[46]). Specifically, due to a polygenic inheritance mode, TH male mice develop insulin resistance, hyperglycaemia, hyperinsulinemia, and obesity. These phenotypes, occurring between 10 and 14 weeks of age[48], are associated with increased cholesterol levels and with an enlargement of the islets of Langerhans[49]. TH mice derive from two male mice, belonging to a colony of Theiler mice, exhibiting polyuria and glucosuria. Starting from these deviant mice, a research colony was established by selective breeding of hyperglycaemic mice[50,51].

Based on the association between altered insulin signalling, somatic and mental disturbances, and on the remarkable construct and face validity of TH mice as an experimental model of T2DM, in the present study, we hypothesised that TH mice may also exhibit behavioural abnormalities isomorphic to mental disorders like AD, ADHD, and OCD. Thus, we tested this hypothesis by adopting an ontogenetic perspective. Specifically, we investigated the developmental course of body weight, hyperglycaemia, insulin resistance, energy expenditure, respiratory exchange ratio, and polydipsia in adolescent (5-6-7 weeks), young adult (11-12-13 weeks), and adult (>23 weeks) male and female TH mice. To test whether T2DM-like abnormalities co-occurred with behavioural abnormalities associated with CNS disturbances (AD and OCD), we conducted an extended test battery evaluating behavioural rigidity, attentional set-shifting capabilities, short- and long-term spatial and recognition memory, general locomotion, and anxiety-related behaviour.

MATERIALS AND METHODS

Ethics statement

Experimental protocols were approved by the Italian Ministry of Health (licence n. 216/2020-PR to SM) and performed in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes and Italian law (Legislative Decree 26/2014).

Subjects

Twenty males and 40 females of each strain (TH mice and their controls, SWR/J mice, JAX stock #000689, hereafter SW), 9-13 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and were housed in same-gender and same-genotype pairs per cage. Mice were kept in an air-conditioned room (temperature 24 ± 1°C, relative humidity 40 ± 5%), on a 12-hours reversed light-dark cycle (lights on at 19:30). All cages (polycarbonate cages, 33 cm L × 13 cm W × 14 cm H, equipped with metal tops, – Tecniplast S.p.A. Buguggiate, VA, Italy) were provided with sawdust bedding, replaced weekly, and environmental enrichment in the form of shelter material (Nestlets®). Mice had access to *ad libitum* water and food pellets (Mucedola s.r.l., Settimo Milanese, Italy), except for the attentional set shifting task, which required food restriction (see "Attentional set shifting task (ASST)" for details). After two weeks of acclimatization, males and females were mated to generate the offspring to be used as experimental subjects (the number of animals per group required for behavioural and metabolic tests is detailed below). One week before mating, males were isolated while females were kept in pairs; three days before mating, some sawdust from the cage housing the male was transferred to the cage housing the two females it was planned to be mated with. On the day of mating, the two females were transferred to the cage housing the male. Mating lasted for seven days, after which the male was relocated to individual housing. Females were weighed two days before and two weeks after mating, to ascertain the occurred pregnancy. Pregnant females were housed individually as described above and provided with special food to support gestation and lactation (2019 Teklad global 19%, Envigo, Indianapolis, IN, United States). They were kept in these conditions until weaning, which occurred 21 ± 1 days after delivery (designated as post-natal day, PND, 0). Pup body weight was measured at PND 1, 8, 15 and 22. At weaning, pups were marked through ear-clipping and housed in same-sex pairs. Experimental testing was conducted in an experimental room adjacent to the housing room, minimising gradients in light, temperature, sound and other environmental conditions.

Experimental plan

The study was conducted in three independent experimental cohorts in order to guarantee an adequate number of subjects per test while avoiding pseudo-replications and litter effects, and minimising suffering in each experimental individual. To achieve these aims, each experimental subject was tested on a single test

battery only at a selected life stage: adolescence (5-6-7 weeks), young-adulthood (11-12-13 weeks), or adulthood (>23 weeks). Each test battery was devised in a way that minimised individual suffering by limiting any source of discomfort; additionally, tests were conducted in a sequence wherein the most invasive experimental test was performed at the end of the test battery itself. The experimental plan thus entailed 2 genotypes (TH vs. SW) x 2 sexes (males vs. females) x 3 developmental stages (adolescents vs. young-adults vs. adults) x 3 test batteries (A, B, C). To attain the proposed goals, we conducted the study in three independent cohorts, each consisting of 144 subjects, subdivided into 12 experimental groups of 12 subject each, for 432 total subjects. Each cohort derived from 24 females (12 TH and 12 SW), divided into two batches (12 females mated per batch, six TH and six SW), for a total of 72 females (36 SW and 36 TH). Each litter contributed six subjects (three males and three females), each of which was tested only at one developmental stage: for example, from a single dam, one male and one female were tested in battery A during adolescence, one male and one female were tested in battery B during young-adulthood, and one male and one female were tested in battery C during adulthood. The three independent experimental cohorts were needed in order to complete the test batteries in all subjects and in all developmental stages. Specifically, each cohort was tested in a different order in relation to age (cohort 1: A in adolescence, B in young-adulthood, and C in adulthood; cohort 2: C in adolescence, A in young-adulthood, and B in adulthood; cohort 3: B in adolescence, C in young-adulthood, and A in adulthood). Each test battery lasted three weeks, subdivided as follows: the test battery A included the T-maze test, the open field test, the novel object recognition test and the elevated zero-maze test; the test battery B included the Barnes maze test, the intra-peritoneal glucose tolerance test (IPGTT) and the insulin sensitivity test (IST); the test battery C included the metabolic cages and the attentional set-shifting task (for details see **Figure 1)**. The order of testing of the experimental subjects was randomized within each test.

Metabolic tests

For the evaluation of physiological parameters (intra-peritoneal glucose tolerance test (IPGTT) and insulin sensitivity test (IST)), the number of experimental subjects per group was estimated with a power $1-\beta=0.80$ and a probability of type I error $\alpha=0.05/6=0.00833$ in a Student's t test (for independent samples) at 2 tails, in order to highlight a difference between the 2 genotypes equal to 25% of the mean value observed in the control group, with an expected standard deviation in the same group equal to 15% of the mean value (corresponding to a large effect, Cohen's d=1.66). Based on this analysis, 11 animals per group are required. With respect to the metabolic parameters collected in the metabolic cages (food and water intake, respiratory exchange ratio, energy expenditure, and general locomotion), the number of experimental subject per group was estimated with a power $1-\beta=0.80$ and a probability of type I error $\alpha=0.05/6=0.00833$ in a Student's t test (for independent samples) at 2 tails, in order to highlight a difference between the 2 genotypes equal to 25% of the mean value observed in the control group, with an expected standard deviation in the same

group equal to 12% of the mean value (corresponding to a large effect, Cohen's d=2.08). Based on this analysis, 8 animals per group are required.

Body weight gain

All the animals were weighed, using a precision scale (Kern & Sohn GmbH, Balingen, Germany), on post-natal day 1, 8, 15 and 22. After that, the animals were weighed only during their own battery of tests, based on developmental stages (adolescence, young adulthood and adulthood), early in the morning, once a week (see **figure 2**).

Intra-peritoneal glucose tolerance test (IPGTT)

Mice were tested after 15-h of food deprivation. Each animal was injected (i.p.) with a dose of 2 g/kg (D-glucose 10%; Sigma, St. Louis, MO, USA): blood glucose concentrations were measured at time 0 (baseline concentrations before glucose administration), and then 30, 60, 120, and 180 minutes after glucose administration, with a commercial glucometer (Accu-Chek® Guide, Roche Diabetes care Italy S.p.A., Monza, MB, Italy). Blood samples were obtained using the "tail-nick" technique, which allows collection without compromising animal welfare and minimizing perceived suffering[52].

Insulin sensitivity test (IST)

Mice were tested after a 5-h food deprivation. Experimental subjects were injected (i.p.) human recombinant insulin (0.4 U/kg; Humulin R, Eli-Lilly, 100 U/mL, Indianapolis IN, United States) and then sampled at time 0 (baseline concentrations before insulin administration), 15, 30, 60, 120, and 180 minutes after insulin administration to measure blood glucose concentration with the same glucometer used for IPGTT.

Metabolic cages

To gain insight as to baseline metabolism in terms of energy expenditure, lipid vs. carbohydrate metabolism, general locomotion, and fluid and water intake, mice were individually tested in metabolic cages (*PhenoMaste*r, TSE System GmbH, Berlin, Germany). The latter allow the automated and continuous measurement of the aforementioned parameters using indirect calorimetry (for energy expenditure and lipid vs. carbohydrate metabolism), infrared sensors (for general locomotion), and precision scales (for food and water intake). This automated system has been developed to phenotype mice in undisturbed conditions[53]. Specifically, the PhenoMaster system consists of standard transparent cages (37 cm L x 19 cm W x 12 cm H) equipped with modules monitoring food and water intake (TSE *Food and Drink Measurement*), locomotor activity (TSE *ActiMot3*) and indirect calorimetry (TSE *Indirect Gas Calorimetry*). Food and water intake were continuously

measured through weighing sensors positioned at the top of the cage, to which a food hopper and a water bottle were attached. General locomotion was measured with a grid of two-dimensional infrared sensors, placed around the cage, to monitor horizontal activity, defined as the total number of infrared beam breaks in the X and Y-axes (counts). Indirect calorimetry measures oxygen consumption and carbon dioxide production through an open-circuit system and gas-sensing units, to calculate resting energy expenditure (EE) and respiratory exchange ratio (RER); RER was defined as the volume of carbon dioxide released over the volume of oxygen absorbed during respiration (vCO_2/vO_2)[54]. Oxygen and carbon dioxide sensors were calibrated with calibration gas mixtures (CO_2, O_2, N_2), according to the manufacturer's guidelines. Mice were placed in metabolic cages for 5 days, single housed, with *ad libitum* water and food, on a 12-hours reversed light-dark cycle (lights on at 19:30). During day one, mice were acclimatised to the novel cage and no measurement was collected. Days 2-3 were considered habituation during which data have been collected but not analysed, while days 4-5 were considered proper experimental days during which data have been collected and analysed. All measurements were taken every 21 minutes throughout the entire session.

Behavioural tests

The number of experimental subjects per group was estimated with a power $1-\beta=0.80$ and a probability of type I error $\alpha=0.05/6=0.00833$ in a Student's t test (for independent samples) at 2 tails, in order to highlight a difference between the 2 genotypes equal to 25% of the mean value observed in the control group, with an expected standard deviation in the same group equal to 15% of the mean value (corresponding to a large effect, Cohen's d=1.66). Based on the power analysis performed, 11 animals per group in the behavioural tests are required. The number of experimental groups in the attentional set shifting task, which has consistently been shown to be characterised by a lower variance compared to the other behavioural tasks, was estimated with a power $1-\beta=0.80$ and a probability of type I error $\alpha=0.05/6=0.00833$ in a Student's t test (for independent samples) at 2 tails, in order to highlight a difference between the 2 genotypes equal to 25% of the mean value observed in the control group, with an expected standard deviation in the same group equal to 12% of the mean value (corresponding to a large effect, Cohen's d=2.08). Based on this analysis, 8 animals per group are required. To gain insight of different cognitive domains we conducted the following tests: T-maze (to evaluate the spontaneous alternation behaviour), open field (to assess spontaneous locomotion and anxiety-like behaviour), novel object recognition (to evaluate recognition memory), elevated zero-maze (to evaluate anxiety-like behaviour), Barnes maze (to evaluate spatial memory) and attentional set-shifting task (to assess executive function like attentional and cognitive flexibility). Data of open field, novel object recognition, elevated zero-maze and Barnes maze tests were collected and automatically scored through the ANY-maze software 7.09 (Stoelting Europe, Terenure, Dublin, Ireland), an automated video-recording and video-tracking system which allows an accurate quantification of animal

behaviour, avoiding the experimenter bias. All the tests, except the Barnes maze test, were conducted under the dim light, 12 lux, measured with a lux meter (Giorgio Bormac S.r.l., Carpi, MO, Italy).

T-maze test

The apparatus consists of a T-shaped Plexiglass maze with a metal grid as a floor consisting of three equally sized arms (24 cm L x 10 cm W x 8 cm H). Ten sessions were performed during five consecutive days (two sessions per day, three hours apart), and each session consisted of two consecutive trials. The experimental session started with the mouse positioned in the starting compartment, facing the wall of the apparatus, and allowed to explore the apparatus for two minutes, after which, if it did not make any choice, a cut-off was recorded; if the cut-off occurred during the first of the two trials, the mouse was not subjected to the next trial, the session ended, and it was tested in the next session. When the mouse entered one of the two alternative arms, such choice was recorded as the first choice and the door of the arm was closed. After a few seconds, the animal was gently removed from the selected arm, relocated in the starting compartment, and allowed to perform a second-choice trial. The selected arm (left vs. right) and latency to enter it (with all four paws) were recorded. If the subject re-entered the previously selected arm, a non-alternation trial was scored, while if the subject entered the opposite arm, an alternation trial was scored. The percentage of spontaneous alternations, expressed as the number of alternations divided by the total number of entries in the arms x 100, was calculated. When the animal completed the session, the maze was cleaned with a 30% ethanol/water solution.

Open field (OF) test

Spontaneous locomotion in an unfamiliar environment was evaluated in a square arena (*Open Field 47432*, Ugo Basile® S.r.l., Gemonio, VA, Italy) surrounded by grey PVC walls (40 cm L x 40 cm W x 30 cm H). The experimental subject was located in the centre of the arena and allowed to freely explore it for 10 minutes. The arena was virtually divided by ANY-maze software in two equally-sized (2 x 800 cm^2) differently-shaped zones (centre and periphery). Distance travelled and time spent in the centre and in the periphery of the arena were measured. When the animal completed the test, the arena was cleaned with a 30% ethanol/water solution.

Novel object recognition (NOR) test

The test consists of several phases: a *habituation* phase (corresponding to the open field test) on the first day, an *acquisition* phase on the second day, and two *testing* phases, the first conducted one hour after *acquisition*, and the second conducted 24 hours after *acquisition*. The apparatus was the same described for the Open

field test (see above), and mice were tested individually. During *acquisition* (10 minutes), mice were allowed to explore two identical objects (A) placed next to two adjacent corners symmetrically equidistant (10 cm) from the walls of the arena. At the beginning of each test session/phase, the subject was placed in the quadrant opposite to the objects, facing the walls. In the first *testing* phase (one hour after *acquisition* = short-term recognition memory) one of the two objects was replaced with another (B), identical in size but different in shape and material; in the second *testing* phase (24 hours after *acquisition* = long-term recognition memory) object B was replaced again with another (C), different in shape and material. Both testing phases lasted 10 minutes. Shape and material (glass, plastic or metal) of familiar or novel objects, as well as their relative position, were counterbalanced and randomized for each experimental subject and cleaned between trials with 30 % ethanol solution. The objects were attached to the floor with adhesive Velcro and the total volume of the objects was similar to the volume of the mouse. Time spent to explore objects and novel object percent preference [(time spent exploring novel object/time spent exploring both objects) x 100] represented the primary dependent variables. Furthermore, the latency to enter the object area, the average speed and the frequency of visits to the two objects, were recorded. When the animal completed the test, the arena was cleaned with a 30% ethanol/water solution.

Elevated zero-maze test

The apparatus (*Elevated Zero-Maze 40163*, Ugo Basile® S.r.l., Gemonio, VA, Italy) consists of a circular platform with a 5 cm wide corridor and a diameter of 60 cm, raised 62 cm above the floor. Two opposing sectors were protected by 16 cm high walls (closed sectors), and the two remaining sectors were unprotected (open sectors) and only surrounded by a 0.5 mm slightly raised rim. The subject was placed in one of the two closed sectors, individually, and each session lasted 5 minutes. Time spent and number of entries in both sectors, and percent time spent in open sectors (as an inverse index of anxiety) were analysed. When the animal completed the test, the maze was cleaned with a 30% ethanol/water solution.

Barnes maze test

The maze (*Barnes Maze 40193*, Ugo Basile® S.r.l., Gemonio, VA, Italy) consists of a grey circular platform raised 60 cm above the floor, with a diameter of 100 cm and 20 evenly spaced holes of 5 cm in diameter, along the perimeter. All but one of the holes were blind ending, while one led to an escape box defined "target", which was magnetically attached underneath the target hole. To avoid position bias, the position of the escape box was randomized between subjects but remained constant within subjects. This test was performed with a bright light (310 lux), an aversive stimulus for rodent, providing a motivation to locate the escape box[55]; the escape box contained a handful of sawdust bedding, sampled from the home cage of the experimental subject to increase its rewarding nature. The experimental protocol consisted of one day of

habituation phase, five days of *acquisition phase* and two *probe trials*, conducted respectively 24 hours and seven days after the last day of the *acquisition phase*. At the start of each trial, the mouse was placed under a cylinder (diameter 20 cm), at the centre of the maze for a few seconds. After this, the cylinder was removed, and the animal was free to explore the maze. During the *habituation phase* (day 1), the animal was exposed to two consecutive trials lasting one minute. Habituation trials ended when the mouse entered the target. If the mouse failed to locate the target within the allocated time, after one minute, it was gently directed to the target hole by the operator, and left therein for two minutes. In this phase no parameters were measured. During the *acquisition phase* (days 2-6) mice performed two trials per day, 10 minutes apart, each of them lasting three minutes. Test ended when the mouse entered the target. If the mouse failed to locate the target within the allocated time, after three minutes, it was gently directed to the right hole by the operator, and left therein for three minutes.

The first *probe trial* "probe 24 hours" (day 7), performed 24 hours after the end of the last day of the *acquisition phase*, was used to assess the short-term spatial memory, while the second *probe trial* "probe 1 week" (day 13), performed one week after the end of the last day of the *acquisition phase*, was used to evaluate the long-term spatial memory. Both *probe trials* lasted 90 seconds, during which the escape box was removed, and all the holes were blind ending, so that mice reached the target area (the maze was virtually divided by ANY-maze software in four quadrants, each containing the hole where the escape box was previously placed and the four side holes, two on the right and two on the left) only through the cues outside the platform. In the *acquisition phase*, the latency to enter the escape box and the latency to enter in the target area were recorded, while in the two *probe trials* the time spent in the target area and the latency to enter the target area were recorded. At the end of each trial the platform was cleaned with a 30% ethanol/water solution.

Attentional set-shifting task (ASST)

In this test, mice had to learn a rule associated with a reward, by trials and errors, and subsequently disregarded the acquired rule in favour of a new one. The apparatus consisted of a custom-made ivory coloured PVC U-maze (45 cm L x 30 cm W x 15 cm H) subdivided into three sections: a starting compartment (30 cm L x 30 cm W) connected, through a sliding door, to two identical smaller compartments (15 cm L x 15 cm W), where, in each of them, a metal bowl (4 cm high, 7 cm top diameter, 4 cm bottom diameter) was placed: the metal bowl was positioned inside a 6.5 cm diameter, 3.7 cm deep hole which allowed the bowl to emerge 0.3 cm above the ground (this expedient was adopted to grant mice access to the bowl without the need to climb on it); inside the bowls a small piece of honey cereal (1/4 honey Cheerios®), covered with 2 cm layer of digging medium, was used as a reward. The floor of the maze consisted of a wire-mesh, under which we placed a layer of sawdust bedding from the home-cage of the animal, to reduce the stress of a novel environment. To increase individual motivation to complete the task, three days before testing, mice were allocated to a food restriction schedule aimed at attaining and

maintaining 85-90% of their free feeding bodyweight. To attain the required body weight, we proceeded as follows: individual body weight and food consumption first measured in *ad libitum* feeding conditions starting one week before testing; three days before testing, we removed food from their hopper for twelve hours and then we provided them with approximately 90% of their individualised *ad libitum* food intake; mice were weighed daily in order to ascertain that they attained the required body weight and food provision was calibrated accordingly. Such food restriction schedule was kept until the end of the ASST. At the beginning of the test the mouse was placed in the starting compartment, and the sliding door providing access to the rewarded bowls were closed; as soon as the test began, the operator raised the door, to allow access to the two choice compartments. The test consisted of *habituation and training phase* and *testing phase* (composed of 5 stages). The *habituation and training phase* were performed on the first day: five minutes of free exploration with empty bowls (*habituation*), followed by nine trials (*training phase*) with reward in both bowls (at the bottom of the empty bowls in trials 1-3, on top of the bowls filled with sawdust bedding in trials 4-6, and covered by a 2 cm layer of bedding material in trials 7-9). This phase ended when the mouse ate the reward from both bowls. The *testing phase* consisted of five consecutive stages, in which the animals had to discriminate between two different stimuli belonging to two different dimensions (see **Table 1** for details). During these stages, the experimental subject was free to explore the apparatus and the bowls before making a choice; the choice was registered when the animal dug into a bowl (simple contact or sniffing were not considered as digging). After completing each trial correctly or incorrectly, the mouse returned to the starting compartment and the doors were closed. The first four trials were "exploratory trials": if the mice dug into the unrewarded bowl, the trial was recorded as an error, but the door was not closed, and the mouse was allowed to retrieve the reward from the baited bowl. On subsequent trials, if the mice dug into the unrewarded bowl, the error was recorded the door was closed, and the experimental subject was not allowed to retrieve the reward. A stage was considered complete when the mouse reached the criterion (eight correct responses out of 10 consecutives trials). A session continued until the animal stopped responding (rearing or sniffing without digging): if the mouse did not make a choice after 10 minutes, we registered a cut-off, and the test ended. In the first stage (simple discrimination, SD) only one dimension (olfactory) was introduced, and mice had to choose between two stimuli (odours). In the second stage (compound discrimination, CD) a new dimension, as confounding stimulus, was introduced, but the rewarded stimuli remained the same of SD. In the third stage (compound discrimination reversal, CDR) we introduced the reversal learning paradigm: all stimuli and dimensions were maintained from the CD, but the rewarded stimulus was now the one that had been previously incorrect. In the fourth (intra-dimensional shift, IDS) and in the fifth (extra-dimensional shift, EDS) stages four novel stimuli in both dimensions were introduced: in the IDS, the relevant stimulus was still constituted by the odour, while in the EDS mice had to shift their attention to the previously irrelevant dimension (digging medium). In this test both the number of trials performed to reach the criterion and the number of errors committed in each stage were quantified and analysed. Between sessions the apparatus was cleaned with a 30% ethanol/water solution.

Stage	Relevant dimension	Rewarded stimulus	Discrimination 1	Discrimination 2	Day
SD	olfactory	cinnamon	cinnamon vs. sage (in bedding sawdust)	-	2
CD	olfactory	cinnamon	sawdust + cinnamon vs. cotton + sage	sawdust + sage vs. cotton + cinnamon	2
CRD	olfactory	sage	sawdust + cinnamon vs. cotton + sage	sawdust + sage vs. cotton + cinnamon	3
IDS	olfactory	ginger	crepe-paper + ginger vs. confetti + coriander	crepe-paper + coriander vs. confetti + ginger	4
EDS	tactile-visual	polystyrene	polystyrene + cloves vs. tissue-paper + rosemary	polystyrene + rosemary vs. tissue-paper + cloves	5

Table 1. ASST dimensions and stimuli

Schematic representation of the five stages of the ASST testing phase (first column), with the dimension that was relevant (second column) and the stimulus that was rewarded (third column) in each stage. In each stage mice were presented with either Discrimination 1 (fourth column) or Discrimination 2 (fifth column), according to a pseudo-random sequence. The side where each pair of stimuli was presented (left and right bowls) were counterbalanced through a pseudo-random sequence. In the sixth columns we reported the range of days in which each stage of the task was completed.

Statistical analysis

All statistical analyses were conducted using the softwares StatView 5.0.1 (SAS Institute Inc., Cary, NC, USA), Microsoft Office Excel (Microsoft Corporation, Redmond, Washington, USA), and G*Power 3.1.9.6 (Heinrich Heine University Düsseldorf, Germany)[56]. The experimental design entailed 2 genotype (TH vs. SW) x 2 sex (males vs. females) x 3 developmental stage (adolescence, young-adulthood, adulthood) x *k* repeated measure (variable depending on the specific test) and their interactions. Experimental data have been analysed through the analysis of variance (ANOVA), and when repeated observations were collected on the same animals, these were analysed through repeated measures ANOVA; genotype, sex, and developmental stage constituted between subjects factors while repeated measurements constituted within-subject factors. Post-hoc comparisons were performed using Tukey test. For the evaluation of the subjects who exhibited a severe hypoglycaemic response to insulin administration, during IST, statistical significance was determined by chi-square tests (χ^2). Significance level was set at $p<0.05$

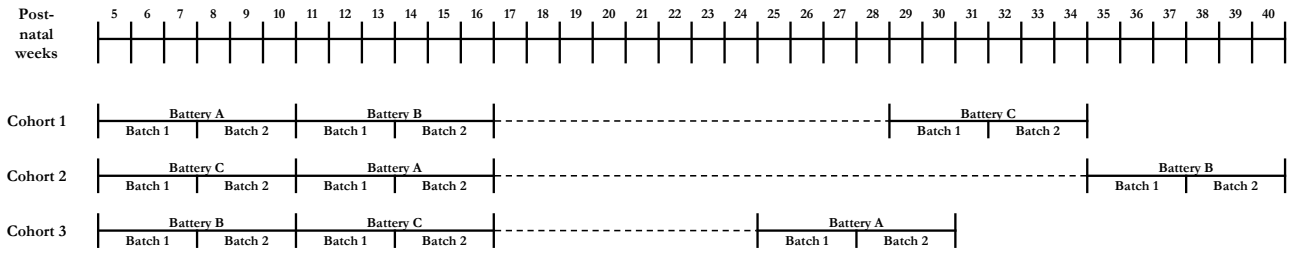

Figure 1. Experimental plan.

Allocation of experimental subjects to behavioural and metabolic testing. Test battery A entailed the T-maze test, the open field test, the novel object recognition test and the elevated zero-maze test; the test battery B included the Barnes maze test, the intra-peritoneal glucose tolerance test (IPGTT) and the insulin sensitivity test (IST); the test battery C included the metabolic cages and the attentional set-shifting task.

RESULTS

Metabolic testing

Body weight gain

As expected, TH male and female mice were heavier than SW controls throughout the entire course of the study (genotype: F(1,26)=159.764, p<0.0001, **figure 2**) with the exception of postnatal day 1. Specifically, significant differences between TH and SW mice emerged as early as postnatal day 8 and remained stable until the end of the experiment. Beside genotype-dependent differences, the sex of experimental subjects played a remarkable role (sex: F(1,26)=129.131, p<0.0001), whereby male subjects of both genotypes were heavier than their respective control females from around puberty onwards.

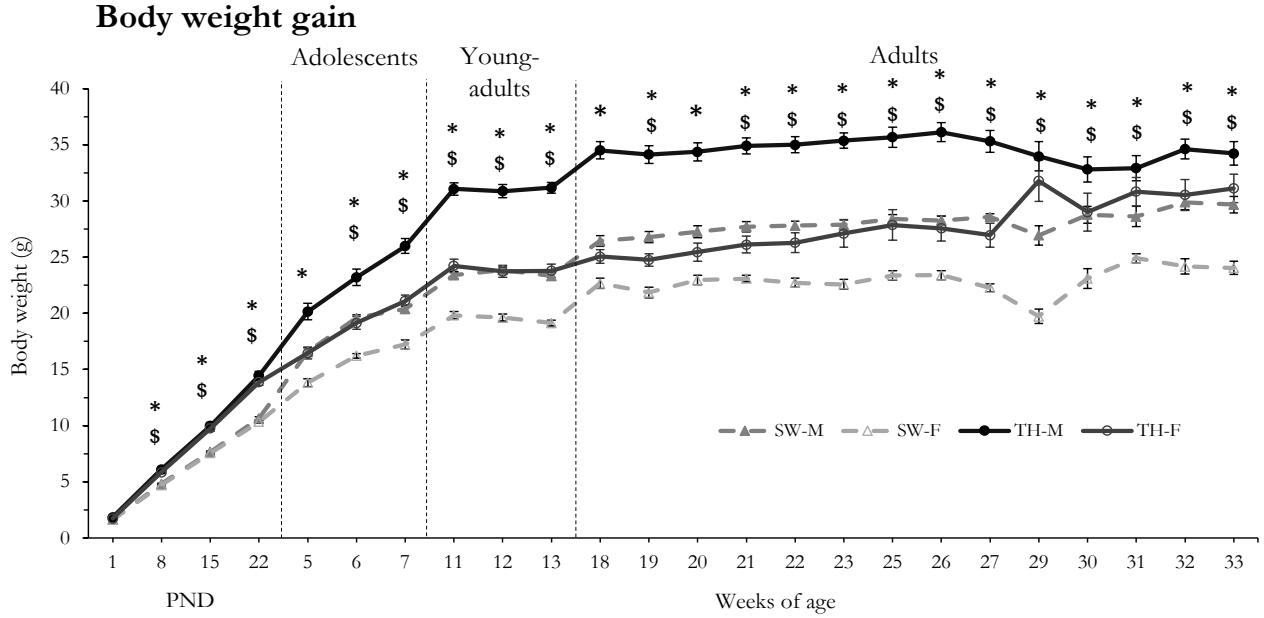

Figure 2. Body weight gain.

Body weight gain (mean ± SEM) in SW male and female and TH males and females at post-natal day 1, 8, 15 and 22 (SW males n=59, SW females n=66, TH males n=43 and TH females n=64), and at adolescence (5-7 weeks of age, SW males and females n=29, TH males n=25 and TH females n=23), young adulthood (11-13 weeks of age, SW males n=34, SW females n=33, TH males n=26 and TH females n=28) and adulthood (18-33 weeks of age, SW males n=13, SW females n=17, TH males n=14 and TH females n=13). * p<0.05 TH-M significantly different from SW-M in post hoc tests. $ p<0.05 TH-F significantly different from SW-F in post hoc tests.

Intra-peritoneal glucose tolerance test (IPGTT)

Experimental data (**figure 3a,b,c**) indicate that glucose administration elicited the expected physiological response in all experimental groups whereby blood glucose concentrations where higher than baseline 30 minutes after glucose administration and then steadily declined to attain baseline values at the end of the experiment (time bins: F(4,484)=315.198, p<0.0001). Additionally, we observed that baseline glucose concentrations (T0) varied as a function of genotype and sex (genotype x sex: F(1,121)=11.817, p=0.0008). Specifically, while TH male mice exhibited baseline hyperglycaemia than SW males at all developmental

stages (p<0.05 in post hoc tests), TH female mice were always indistinguishable from their controls (ns in post hoc tests). Finally, we observed that different developmental stages where characterised by a differential sensitivity to glucose administration (genotype x age: F(2,121)=3.048, p=0.051). Specifically, while TH male mice always exhibited higher reactivity to glucose administration compared to SW males (adolescents T0, T30 and T60, young-adults and adults all time points: p<0.05 in post hoc tests), such genotype-dependent difference was not present in adolescent females (ns in post hoc tests), but emerged in young adulthood (T30 and T60: p<0.05 in post hoc tests) to remain stable later in adulthood (T30, T60 and T120: p<0.05 in post hoc tests).

Insulin sensitivity test (IST)

During the test, many control subjects had to be discarded due to a severe hypoglycaemic response. Specifically, within SW mice, approximately 58% of adolescents, 33% of young adults and 67% of adults exhibited a severe reaction to insulin administration and had to be immediately administered a glucose bolus to guarantee individual survival. Therefore, we preliminarily analysed IST data in terms of survival rates through chi-square test, and observed that TH mice of all developmental stages were insulin resistant compared to controls. Specifically, we observed insulin resistance in adolescent males and females (p<0.05), young-adult males (p<0.05) and adult males and females (p<0.05). Blood glucose concentrations decreased consistently after insulin administration in all experimental groups (time bins: F(5,400)=17.531, p<0.0001, **figure 3d,e,f**). Yet, glucose concentrations in response to insulin administration varied as a function of genotype and sex (genotype x sex: F(1,80)=5.582, p=0.0206) and as a function of genotype and age (genotype x age: F(2,80)=3.836, p=0.0257). Specifically, baseline glucose concentrations were higher in TH females compared to SW controls in adolescence (p<0.05 in post hoc tests), but not in young adulthood and adulthood (ns in post hoc tests). While baseline glucose concentrations were higher in adult TH male mice compared to their respective controls (p<0.05 in post hoc tests), such baseline difference was not observed in adolescence and young adulthood (ns in post hoc tests).

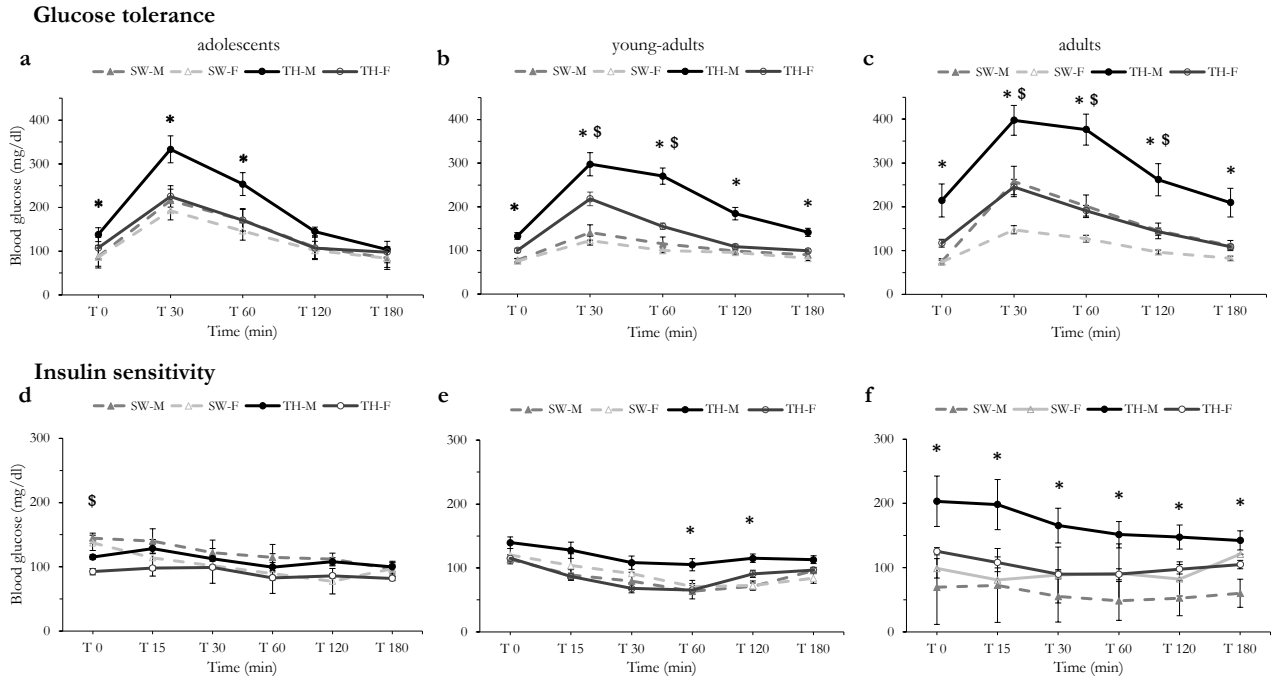

Figure 3. Glucose tolerance and insulin sensitivity tests.

Blood glucose level (mg/dl, mean ± SEM) in glucose tolerance test (a,b,c) at (a) adolescence (SW males and females n=12, TH males and females n=6), (b) young adulthood (n=12) and (c) adulthood (SW males and females and TH females n=12, TH males n=13). Blood glucose level (mg/dl, mean ± SEM) in insulin sensitivity test (d,e,f) at (d) adolescence (SW males and females n=12, TH males and females n=6 but 6 SW males and 8 SW females were excluded from the analysis as they exhibited a severe hypoglycaemic response), (e) young adulthood (n=12, but 3 SW males, 5 SW females and 3 TH females were excluded from the analysis as they exhibited a severe hypoglycaemic response) and (f) adulthood (SW males and females and TH females n=12, TH males n=13, but 6 SW males and 10 SW females were excluded from the analysis as they exhibited a severe hypoglycaemic response). * p<0.05 TH-M significantly different from SW-M in post hoc tests. $ p<0.05 TH-F significantly different from SW-F in post hoc tests.

Metabolic cages

To gauge information about the developmental course of individual metabolic activity, experimental subjects were constantly monitored, for five consecutive days, in metabolic cages, which allowed the continuous and automated assessment of food and water intake, energy expenditure and lipid metabolism (through indirect calorimetry), and general locomotion. In **figure 4** we report the ontogenetic development of the aforementioned parameters displaying data collected over 24 hours. These data suggest that adult TH male mice were characterised by a full-blown diabetes-like phenotype. Specifically, compared to their respective controls, adult TH male mice exhibited marked polydipsia (genotype x sex x age: $F(2,87)=10.682$, $p<0.0001$, **figure 4a,b,c**) and higher food intake (genotype x sex x age: $F(2,87)=2.566$, $p=0.0826$, **figure 4d,e,f**). Furthermore, with increasing age, adult TH showed higher energy expenditure compared to their controls (time x genotype x age: $F(46,2001)=1.783$, $p=0.0010$, **figure 4g,h,i**). Importantly, the quantification of the respiratory exchange ratio (RER) allowed us to analyse the energy substrate (carbohydrates vs. fat) used by experimental subjects to produce heat. This analysis suggested that, while adult SW male mice predominantly used carbohydrates (RER ~ 1), adult TH male subjects also used lipids as an energy source (RER ~ 0.9) (time x genotype x sex x age: $F(46,1978)=2.467$, $p<0.0001$, **figure 4j,k,l**). Finally, we observed

that the aforementioned findings were unlikely to be explained by differences in general activity whereby absolute levels of locomotion were indistinguishable between adult TH and SW mice (time x genotype x sex x age: $F(46,2001)=1.278$, $p=0.1012$, data not shown). Earlier developmental stages were not associated with consistent metabolic differences. Specifically, with respect to TH male subjects, in the absence of differences in adolescence, we observed - in young adulthood - higher food intake (young adulthood genotype: $F(1,14)=23.409$, $p=0.0003$, **figure 4e**), EE (young adulthood genotype: $F(1,14)=13.149$, $p=0.0028$, **figure 4h**), and RER (young adulthood genotype: $F(1,14)=9.980$, $p=0.0070$, **figure 4k**). Notably, the latter difference is opposite to what we observed in adulthood, whereby young adult TH mice used carbohydrates (RER ~ 1) as an energy source. Furthermore, in TH females, of all the parameters considered, we only detected a small increment in EE ($p<0.05$ in post hoc tests, **figure 4h**) in young adulthood, but neither earlier nor later in development.

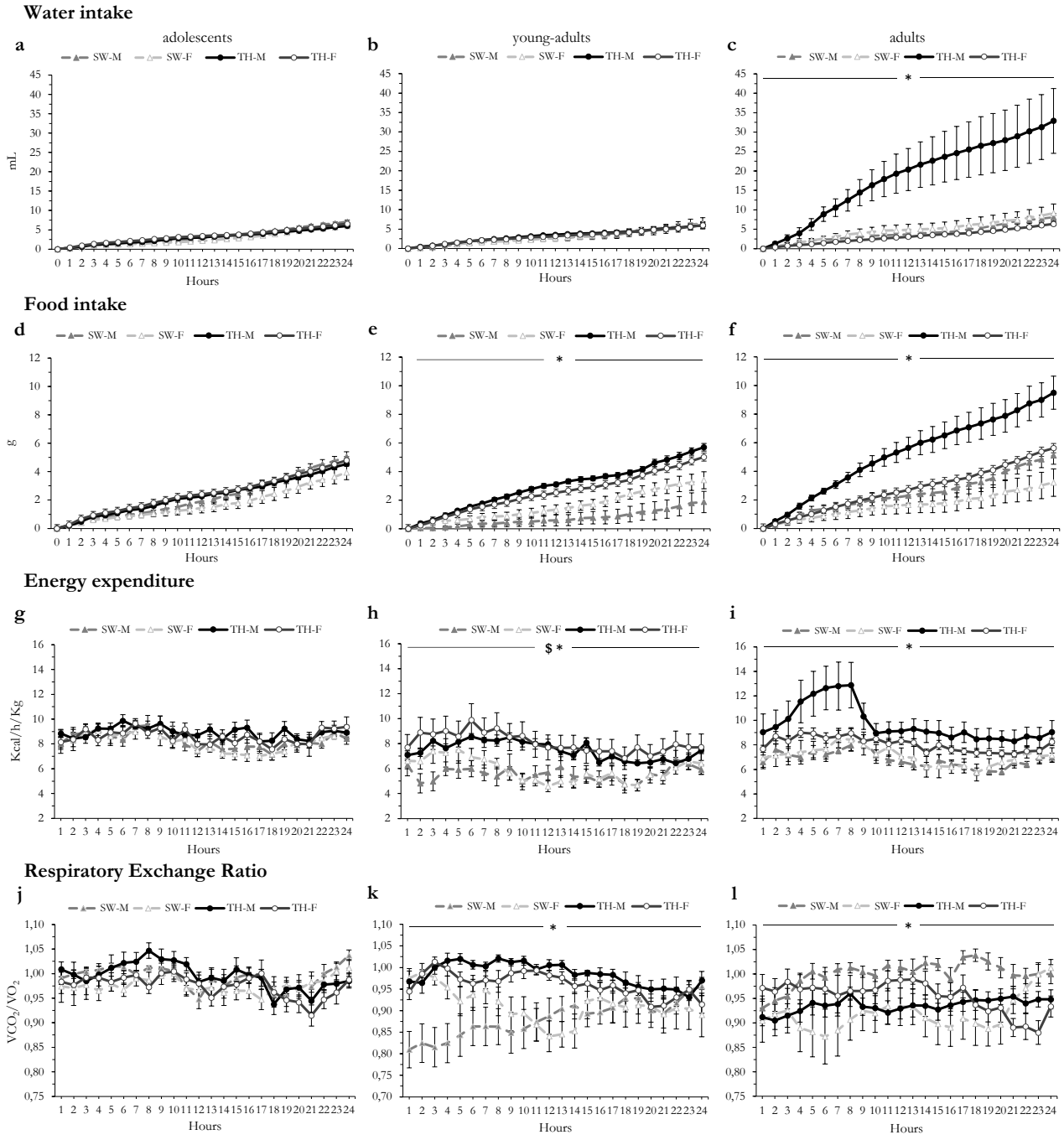

Figure 4. Metabolic cages.

Water intake (mL, mean ± SEM, a,b,c), food intake (g, mean ± SEM, d,e,f), energy expenditure (Kcal/h/Kg, mean ± SEM, g,h,i) and respiratory exchange ratio (VCO_2/ VO_2, mean ± SEM, j,k,l) in metabolic cages at (a,d,g,j) adolescence (SW males and females n=8, TH males and females n=9), (b,e,h,k) young adulthood (SW males, SW females and TH males n=8, TH females n=9) and (c,f,i,l) adulthood (n=8). * $p<0.05$ TH-M significantly different from SW-M in post hoc tests. $ $p<0.05$ TH-F significantly different from SW-F in post hoc tests.

Behavioural testing

T-maze test

In this test, we observed that, compared to SW, TH subjects completed a significantly smaller number of trials during adolescence and young adulthood (strain: $F(1,44)=121.204$, $p<0.0001$ and $F(1,33)=20.117$, $p<0.001$ for adolescents and young-adults respectively, data not shown). Specifically, they performed an average of 3.208 ± 0.381 during adolescence and of 6.833 ± 0.701 during young adulthood. These numbers did not allow us to conduct further analyses during these life stages. In adulthood, all mice performed the required number of trials and allowed further investigations. In agreement with our predictions, we observed that while SW mice exhibited an intact spontaneous alternation, with values significantly higher than 50%, both male and female TH mice showed remarkable impairments in this task. Specifically, neither male nor female TH mice exhibited a significant alternation compared to the 50% chance level (TH males: 95% CI 48.42-70.09, TH females: 95% CI 39.17-65.46). This finding indicates that, once the first choice had been made, TH mice did not significantly prefer the other arm, indicating an impairment in working memory and/or perseveration in behavioural choice.

Attentional set-shifting task (ASST)

The increased perseveration observed in the T-maze test in adult TH subjects was further confirmed in the ASST, since experimental data indicate that TH mice, compared to their SW controls, required a significantly higher number of trials (genotype: $F(1,102)=71.916$, $p<0.0001$) and committed significantly more errors (genotype: $F(1,102)=106.602$, $p<0.0001$) to attain the learning criterion in most stages of the task. This difference varied with age (genotype x age: $F(2,102)=7.600$, $p=0.0008$ and $F(2,102)=8.157$, $p=0.0005$ for trials and errors respectively) and was influenced by sex (genotype x sex x age: $F(2,102)=4.678$, $p=0.0114$ and $F(2,102)=4.244$, $p=0.0170$ for trials and errors respectively). Specifically, as reported in **figure 5**, between group differences were nearly absent during adolescence (with male TH mice exhibiting a selective impairment in the CD and female TH mice in the IDS), were robust, albeit only in TH male mice, in young adulthood, and became fully consolidated in adulthood. During young adulthood, compared to their respective controls, TH male mice required more trials in the CD and the IDS stages (data not shown), and committed more errors in the CD, CDR and the IDS, to attain the learning criterion, while during adulthood, TH mice – regardless of sex – exhibited significant impairments in the CDR, in the IDS, and in the EDS.

Executive functions

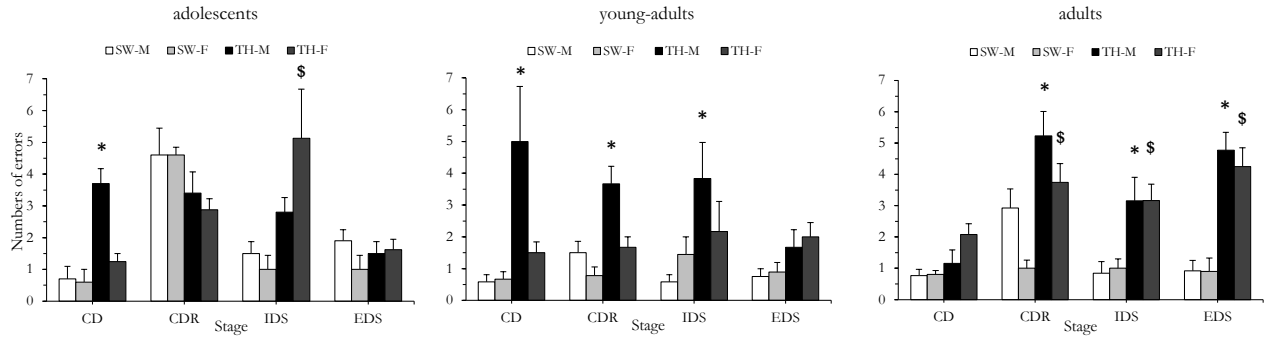

Figure 5. Attentional set-shifting task.

Number of errors (mean ± SEM) committed to complete each stage at adolescence (SW males and TH males n=10, SW females n=9 and TH females n=8, but 4 SW females did not complete the test and were excluded from the analysis), young adulthood (SW males and females n=12, TH males and females n=6, but 3 SW females did not complete the test and were excluded from the analysis) and adulthood (SW males, SW females and TH males n=13, and TH females n=12, but 3 SW females did not complete the test and were excluded from the analysis). * p<0.05 TH-M significantly different from SW-M in post hoc tests. $ p<0.05 TH-F significantly different from SW-F in post hoc tests.

Open field (OF) test

When placed in a novel unfamiliar environment, TH mice consistently exhibited reduced locomotor activity compared to control subjects (genotype: $F(1,94)=118.253$, $p=<0.001$, **figure 6**). Thus, although general locomotion steadily increased throughout development (age: $F(2,94)=30.965$, $p<0.001$), with older subjects moving more than younger ones, TH mice of both sexes always travelled a shorter distance compared to their respective controls ($p<0.05$ in post hoc tests).

General locomotion

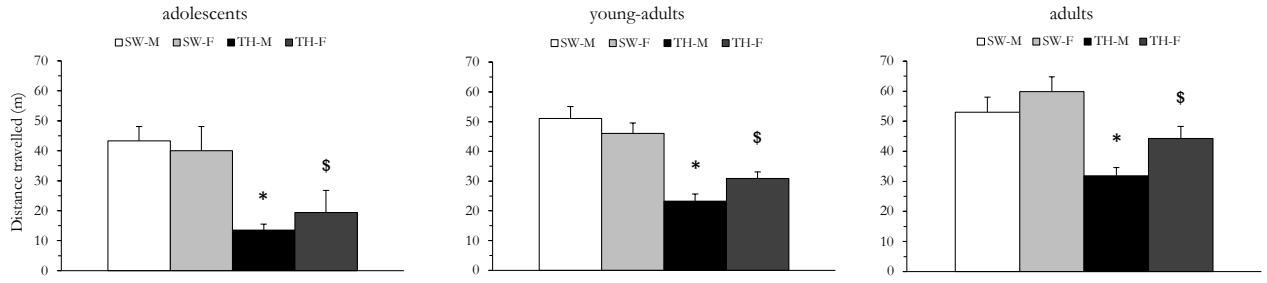

Figure 6. Open field test.

Distance travelled in meters (mean ± SEM) at adolescence (SW males and females n=6 and TH males and females n=12), young adulthood (SW males and TH females n=6, SW females n=7 and TH males n=4) and adulthood (SW females n=11, SW males and TH males and females n=12). * p<0.05 TH-M significantly different from SW-M in post hoc tests. $ p<0.05 TH-F significantly different from SW-F in post hoc tests.

Novel object recognition (NOR) test

In contrast with our predictions, we observed that, while all subjects spent a remarkable portion of time exploring the target objects (subjects explored the objects for approximately 74.7 seconds in the 1 hour test and 75.2 seconds in the 24 hours test), they failed to exhibit the expected preference for the novel object over the familiar one. This statement is supported by the fact that the confidence interval of the novel object preference was never significantly different from the chance level (50% exploration). Such absence of phenotype was exhibited by all experimental groups regardless of sex and age (data not shown).

Elevated zero-maze test

The behavioural phenotype observed in the elevated zero-maze suggests that TH mice exhibited higher levels of anxiety-related states compared to SW subjects (genotype: $F(1,112)=41.001$, $p<0.001$, **figure 7**) and that this difference was partly influenced by sex (sex: $F(1,112)=5.805$, $p=0.0176$) and age (age: $F(2,112)=46.104$, $p<0.001$). Specifically, while in young adulthood and adulthood TH mice spent less time in the open sectors of the elevated zero-maze compared to SW controls ($p<0.05$ in post hoc tests, **figure 7**), but not in adolescence (ns in post hoc tests), such difference within females was significant only during young adulthood ($p<0.05$ in post hoc tests), but not in adolescence and adulthood (ns in post hoc tests). Additionally, the time spent in the open sectors of the elevated zero-maze increased between adolescence and young adulthood, and such increment remained stable throughout adulthood. Finally, it is important to notice that while adolescent subjects predictably preferred the closed sectors over the open ones, such preference was reverted in older SW mice. While this phenotype was predicted in young adult individuals, it was not expected in adult subjects, which have generally been reported to exhibit a preference for the closed over the open sectors of this apparatus.

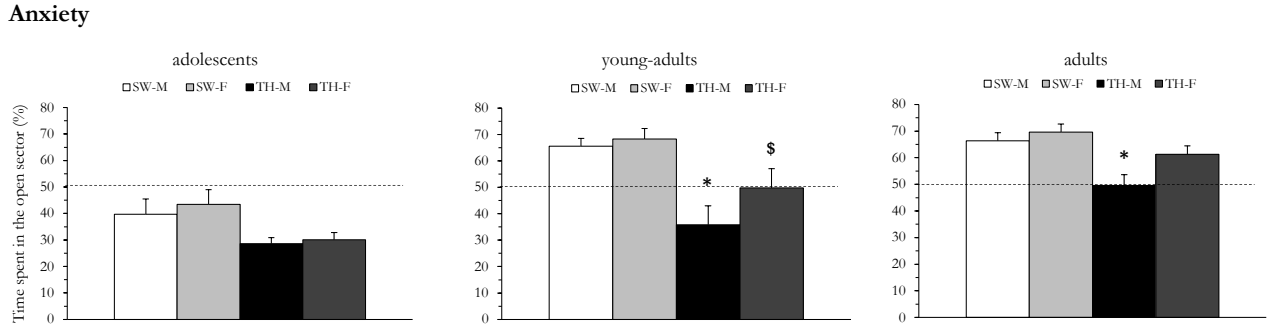

Figure 7. Elevated zero-maze test.

Percent time spent in open sectors (expressed as the time in seconds spent in the open sectors divided by the total duration in seconds of the test x 100; mean ± SEM) at adolescence (SW males and females n=16, TH males and females n=12, but 3 SW males, 4 SW females and 1 TH female fell off the maze and were excluded from the analysis), young adulthood (SW males and TH females n=10, SW females n=9 and TH males n=6, but 2 TH males and 5 TH female fell off the maze and were excluded from the analysis) and adulthood (n=12, but 2 SW males, 1 SW female and 1 TH male fell off the maze and were excluded from the analysis). * $p<0.05$ TH-M significantly different from SW-M in post hoc tests. $ $p<0.05$ TH-F significantly different from SW-F in post hoc tests. The dashed line represents chance level.

Barnes maze test

In this test we observed that TH and SW mice exhibited a differential learning performance and an indistinguishable memory retention of the target location. Predictably, the latency to reach the target location steadily declined between the first and the last trial of acquisition (trial: $F(9,1053)=45.441$, $p<0.0001$). Additionally, we observed that learning impairments were more pronounced during young adulthood and adulthood than in adolescence. Specifically, we observed that, during the early stages of acquisition, TH mice required more time to reach the target location compared to SW subjects in adolescence (males trials 1 and 3, females trials 1 and 5: $p<0.05$ in post hoc tests, **figure 8a**), young adulthood (males and females trials 1, 2 and 3: $p<0.05$ in post hoc tests, **figure 8b**) and adulthood (males trial 1, females trials 1 and 2: $p<0.05$ in post hoc tests, **figure 8c**). This differential profile was no longer present at the end of the acquisition phase, whereby, during the last trials of acquisition, experimental groups were indistinguishable from each other in terms of latency to target. When tested for memory retention (in terms of latency to reach the target zone and time spent in the target quadrant, **figure 8d,e,f,g,h,i**) experimental groups exhibited an indistinguishable phenotype. Noteworthy, the time spent in the target quadrant was always indistinguishable from chance (25% chance level, minimum value: $-0.33 \leq IC \leq 24.01$, maximum value: $24.16 \leq IC \leq 64.74$), thereby indirectly suggesting that all subjects had a remarkable memory impairment regardless of genotype, age, and sex.

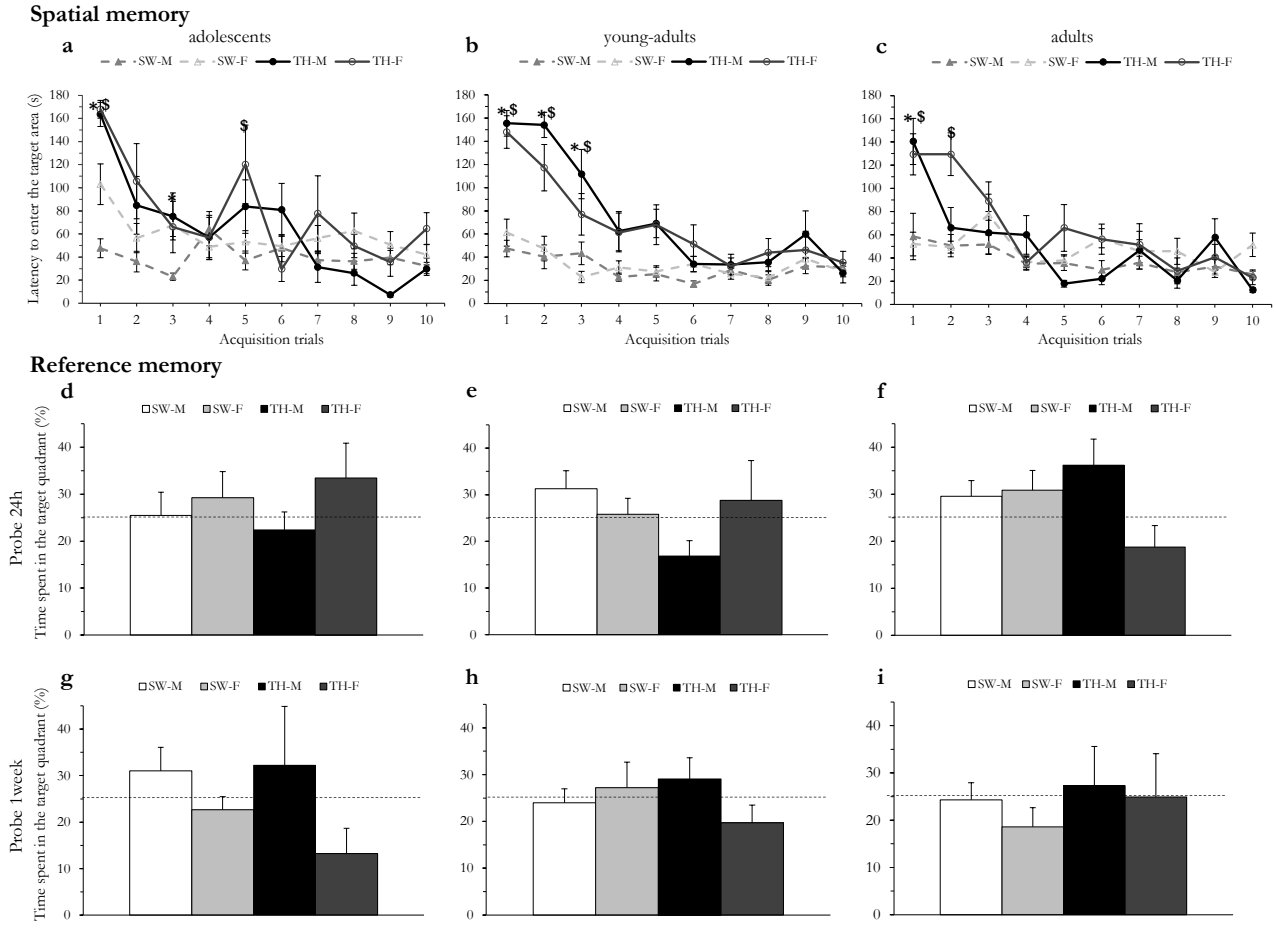

Figure 8. Barnes maze test.

Latency to enter the target area in seconds (mean ± SEM) during the acquisition phase at (a) adolescence (SW males and females n=12, TH males and females n=6), (b) young adulthood (n=12) and (c) adulthood (SW males and females n=10, TH males n=13 and TH females n=12). Percentage of time spent in the target quadrant (expressed as the time in seconds spent in the target quadrant divided by the total duration in seconds of the test x 100; mean ± SEM) in probe 24 hours trial (d,e,f) and in probe 1 week trial (g,h,i) at adolescence (SW males and females n=12, TH males and females n=6, d,g), young adulthood (n=12, e,h) and adulthood (SW males and females n=10, TH males n=13 and TH females n=12, f,i). * $p<0.05$ TH-M significantly different from SW-M in post hoc tests. \$ $p<0.05$ TH-F significantly different from SW-F in post hoc tests. The dashed line represents chance level.

3. Conclusion

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The present data indicate that adult male TH subjects exhibit a comprehensive T2DM-like phenotype (hyperglycaemia, impaired glucose tolerance, insulin resistance, polydipsia, increased food intake and energy expenditure, and altered lipid metabolism) and that this phenotype is associated with overt deficits in attention and perseveration, and memory (albeit with important methodological caveats described below). Furthermore, our data seem to indicate that this comorbid phenotype develops gradually over time, with cognitive deficits preceding full-blown T2DM-like alterations. Similar, but less pronounced, somatic and cognitive alterations are also observed in adult female TH mice. Specifically, whilst both adult male and

female TH mice exhibited altered glucose tolerance and insulin sensitivity, females failed to show any difference in terms of food and water intake, energy expenditure and lipid metabolism. This may suggest the presence of some protective factors in females potentially contrasting the development of the aberrant phenotypes observed in males (see below).

Reduced susceptibility to T2DM-like abnormalities in TH female mice, compared to males, has already been reported in literature[51]. Although the fundamental determinants of this gender difference have not been unequivocally identified, Kim and collaborators[49,50] observed that while male mice exhibited an abnormal structure of Langerhans islets together with degranulation of beta-cells, females did not show this phenotype. Gender differences in vulnerability towards diabetes have also been reported in humans[57,58]. In these studies, the authors reported epidemiological evidence indicating that the prevalence of diabetes is higher in men than in women and that this difference is particularly prominent in middle-aged populations. Sex steroids have been proposed to exert a protective role based on two main lines of evidence: on the one hand, the onset of menopause co-occurs with increased incidence of diabetes[59,60]; on the other hand, rare mutations causing loss-of-function in genes involved in oestrogen function result in impaired metabolism[61]. A protective role of oestrogens in T2DM-like abnormalities has been further substantiated in preclinical studies assessing the role of oestrogen receptors in diet-induced mouse models of obesity, insulin resistance, and glucose tolerance[62,63]. Moreover, clinical studies utilising oestrogen related hormone replacement therapy have reported positive effects on cognition[64] in humans[65].

Beside the reported sex difference, TH mice resemble human diabetes with respect to the ontogenetic progression of the observed abnormalities. Specifically, as already reported elsewhere[51], just as in our species diabetes progressively develops over time[66], so also TH male mice exhibited a full-blown abnormal phenotype no earlier than adulthood. Thus, while male TH mice always exhibited hyperglycaemia compared to controls, only in adulthood did they exhibit insulin resistance, altered lipid metabolism, increased energy expenditure, and polydipsia. The late onset of this phenotype has already been reported in literature[49] and has been related to a progressive degranulation of the β-cells responsible for insulin secretion together with an abnormal structure of Langerhans islets. Whether this relates impairments in cognition and perseveration to altered insulin resistance and lipid metabolism as opposed to glucose management is an intriguing question.

The core aim of our study was to test whether such deficits in insulin signalling were associated with alterations in CNS-dependent behavioural phenotypes. In accordance with our prediction, we observed that TH mice, selected based on their aberrant insulin signalling by selective breeding of hyperglycaemic mice, exhibited consistent impairments in learning and attention. Importantly, we observed that the latter were already present in young adulthood, i.e. long before the onset of a full-blown T2DM-like phenotype in males. Furthermore, similar behavioural impairments were exhibited by adult female mice which, as reported above, did not differ from controls in terms of food and water intake, energy expenditure and lipid

metabolism. Thus, current data support the hypothesis that alterations in insulin signalling may constitute a risk factor for the development of cognitive impairments and that this effect is not necessarily mediated via an earlier onset of diabetes. Rather, this study may suggest an interplay between developmental processes and behaviour in TH mice.

In accordance with previous observations[9,45], present data suggest that the main deficits observed in TH mice may revolve around cognitive flexibility, i.e., the capability to oversee different perspectives and implement appropriate strategies upon changed circumstances[67], and working memory. Thus, TH mice failed to show spontaneous alternation in the T-maze and had difficulties in switching from an acquired rule to a new one upon the attainment of the learning criterion in the attentional set shifting task. Cognitive flexibility is primarily mediated by the prefrontal cortex[68], whereby lesions in this area have been reported to induce severe deficits in executive functions[69]. Additionally, roles for non-prefrontal cortical regions such as thalamus[70] and cerebellum[71] have also been highlighted.

In contrast with our original predictions, memory deficits appeared much less pronounced than originally anticipated. Thus, TH and control mice were indistinguishable in terms of novel object preference (recognition memory) and time spent in the target area of the Barnes maze (reference memory). Before delving into the theoretical aspects potentially explaining these unmet predictions, it is worth considering some fundamental methodological factors that may indicate that both TH and control mice exhibited considerable memory impairments rather than indistinguishable levels of "normal memory". With respect to the novel object recognition memory, by definition[72], the presence of recognition memory shall be claimed upon the observation of a preferential exploration of the novel object compared to the familiar one. This prerequisite has not been met by either control or TH mice, whereby all subjects explored both objects at an indistinguishable rate (confidence interval not exceeding chance level). Analogous considerations may pertain to the Barnes maze, wherein the time spent in the target quadrant did not exceed the 25% chance level in all experimental groups tested. Based on these considerations, we would offer that both TH and SW mice had remarkable memory impairments, especially when compared to literature data in other mouse strains [73,74]. One alternative, and potentially more parsimonious, explanation may point towards the possibility that these tasks cannot be successfully executed in our laboratory. In contrast with this proposition, we repeatedly and successfully conducted these tasks in several different mouse strains[73,74] different from SW. Ultimately, while – based on genetic proximity – SW mice represent the most appropriate control strain for TH mice[48,75,76], we are rather confident that, had we opted for other control strains (e.g. C57BL/6J), the conclusions on memory deficits would have been much stronger. Just as methodological considerations may potentially indicate that memory deficits are more prominent than apparently observed, so also they may devalue the magnitude of the learning deficits exhibited during the acquisition of the Barnes maze. Specifically, while we observed that TH were significantly slower than SW in acquiring the target location, we believe that this deficit was likely dependent on deficits in locomotion. In support of this hypothesis, when tested in the open field, TH mice were much slower than SW. Therefore, we suggest that

– when placed in a novel environment – TH mice may exhibit reduced locomotion and that this may explain the apparent learning deficits. Whether this reduced locomotion represents a natural tendency, a trade off in energy utilisation due to the higher effort required to fuel a heavier body, or an anxiety-related response is unclear. Albeit preliminary, we would favour the anxiety-related explanation whereby, when tested in a non-challenging environment (metabolic cages to which mice were previously acclimatised), TH and SW mice failed to differ in general locomotion.

Regardless of the specificities of memory tests which await further experimental clarification, we observed that deficits in behavioural flexibility emerged either much earlier (males) or independent of (females) full-blown T2DM-like abnormalities. The nature of this association therefore suggests that deficits in insulin signalling may represent a common risk factor for both T2DM and CNS-related deficits, which may act via partly independent biological processes. It also may suggest that altered insulin signalling in TH mice may alter developmental processes to influence the cognitive phenotype independent of metabolic disturbances. While countless studies[5,7,15] detailed the role of insulin signalling in T2DM, a much narrower fraction investigated its role in mental disturbances[8,9]. Among those, Thielen and collaborators[77] reported that altered insulin signalling may alter GABAergic function in the prefrontal cortex and subsequently relate to cognitive deficits. A potential role of insulin in the prefrontal cortex (PFC) is further confirmed by the elevated expression of its receptors (e.g. IR and IGF-1R) in this brain area[78]. Moreover, Martin and collaborators[79] showed that intranasal insulin administration increased neuronal firing by acting upon postsynaptic serotonin 5-HT_{1A} receptors, thus suggesting that insulin may act directly on the serotoninergic tone, and potentially regulate emotional behaviour[79]. Future studies are needed to further detail the role of insulin in the prefrontal cortex as a potential mediator of the observed deficits in executive functions. Finally, the potential role of other brain regions involved in executive functions needs to be clarified. Van de Vondervoort and collaborators[45] hypothesised a potential role for the cerebellum, whereby they reported that deficits in behavioural flexibility and perseveration in TH mice are associated with reductions in IGF1 protein expression in this brain region[45]. While the mechanisms underlying cognitive dysfunction in TH remain to be elucidated, it is interesting to note that insulin appears to interact with other CNS modulators (e.g. oxytocin) which can alter glucose, insulin signalling, body weight balance[80] and dopaminergic transmission[81].

Beside executive functions, insulin signalling has also been shown to directly affect memory and act directly on hippocampal IGF1 receptor populations[82,83]. For example, Nisticò and collaborators[27] observed that mice haploinsufficient for the beta-subunit of the insulin receptor exhibited considerable deficits in synaptic plasticity in the hippocampus and recognition memory. Spinelli and collaborators[84] recently summarized the molecular mechanisms through which insulin may regulate hippocampal neurogenesis and synaptic activity.

Our study provides additional experimental evidence regarding the potential association between insulin signalling and cognitive impairments. The consideration of sex differences, together with the comprehensive

longitudinal phenotypic evaluation, allowed us to suggest that the association between cognitive and metabolic abnormalities is not necessarily causal but potentially correlational in nature. Such correlation may be related to impaired insulin signalling which may, in turn, cause the aforementioned symptoms via partly independent mechanisms. Future studies are needed to further clarify these mechanisms. Another important avenue suggested by the present study concerns the heuristic value of TH mice as a powerful experimental model to further investigate the fundamental determinants of peripheral and central insulinopathies, and to identify innovative therapeutic targets potentially beneficial to patients exhibiting comorbid symptoms. For example, future studies shall investigate whether pharmacological treatments conventionally used in T2DM may be repurposed to mitigate insulin-dependent cognitive disfunctions.

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