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Role of KCNQ1 Gene in Mental and Metabolic Disturbances Associated with Insulin Signalling Dysregulation

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ABSTRACT

Metabolic imbalances (such as diabetes, obesity, and metabolic syndrome) significantly increase the risk of cognitive disturbances, including Alzheimer's disease, dementia, and mild cognitive impairment. Additionally, these metabolic disorders are associated with psychiatric conditions like obsessive compulsive disorders, autism spectrum disorders, and attention deficit hyperactivity disorders. Metabolic disorders can have direct and indirect effects on the brain, contributing to memory impairments, executive dysfunctions, and cognitive inflexibility. They share an altered insulin-signalling that contributes to the cognitive decline, through inflammation and vascular damages. Insulin signalling involves the production and release of insulin and insulin-like growth factors and activates different signalling pathways in the body and in the central nervous system. One of the consequences of insulin signalling dysregulation includes hyperglycaemia: a chronic increase in blood glucose levels that can damage blood vessels in the brain, leading to reduced blood flow and increased risk of vascular dementia and cognitive decline. Moreover, during metabolic dysregulations, together with a peripheral insulin resistance, an altered central insulin resistance occurs, resulting in synaptic dysfunction. Insulin resistance is also linked to the accumulation of beta-amyloid plaques in the brain - a hallmark of Alzheimer's disease. Current clinical research demonstrates that the spectrum of insulin-related syndromes extends well beyond the peripheral nervous system to include the brain. As the first key finding, my doctoral project confirmed the presence of a strong metabolic-cognitive link in animal models (specifically, mice and rats). Through a systematic review of the literature, I showed that hyperglycaemia – as a consequence of insulin signalling dysregulation - has a direct impact on brain function and cognition, especially memory and executive functions (Chapter I). A full understanding of the molecules implicated in this relationship may clarify the interplay between insulin signalling and neural function. In this context, KCNQ1, a voltage-gated potassium channel, is a key connecting metabolism and cognition. Different scholars, including preclinical and clinical scientists, and geneticists, have identified a potential contribution of the *KCNQ1* gene as a key mediator of insulin signalling pathways. Its involvement is well embodied by Romano Word Syndrome patients. Mutations in the *KCNQ1* gene in these patients contribute to cardiac anomalies and insulin resistance. Moreover, mice with a whole-body absence of *kcnq1* show both metabolic and cognitive alterations. Thus, the second objective of my project aimed to identify mechanisms involving peripheral and central dysregulation of insulin signalling, assessing KCNQ1-related effects in the brain. To achieve this aim, I conducted behavioural, physiological, and metabolic tests in a conditional *kcnq1* knockout mouse model. In those experimental subjects, *kcnq1* is downregulated in a time- (during adolescence and adulthood) and spatial- (only in the brain) specific manner (Chapter II). My findings showed that *kcnq1* deletion in the brain significantly impairs cognitive abilities in rodents; specifically, executive functions. Additionally, while cognitive impairments were observed in both ages, the metabolic dysfunction was more pronounced during

adolescence. Specifically, adolescent *kcnq1* knockout mice exhibited distinct lipid metabolism patterns compared to controls. Overall, my PhD results support the view that animal models are characterised by a high experimental validity in the context of insulin multimorbidity. My review indicated that insulin signalling dysregulation is associated to cognitive impairments, even in mice. In addition, I demonstrated that alterations in cognitive domains associated with KCNQ1, especially executive functions, are primarily linked to central dysregulation. Its involvement the metabolism/cognition relationship is also influenced by developmental stage. My experimental findings disclose innovative potential therapeutic avenues revolving around KCNQ1 as a candidate target to mitigate insulin-mediated cognitive decline.

LIST OF ABBREVIATIONS

KCNQ1 - human protein
Kcnq1 - protein symbol for mice
KCNQ1- human gene
Kcnq1- gene symbol for mice
ADHD - attention-deficit/hyperactivity disorders
MetS - metabolic syndrome
T2DM - type 2 diabetes mellitus
AD - Alzheimer disease,
OCD - obsessive compulsive disorder
ASDs - autism spectrum disorders
CNS - central nervous system
RWS - Romano word syndrome
LQTS - long QT syndrome
VSD - voltage sensor domain
PD - pore domain
ICR - imprinting control region
BWS - Beckwith-Wiedemann syndrome
IGF2 - insulin growth factor 2
T1DM - type 1 diabetes
RoB - SYRCLE's Risk of Bias
STZ - streptozotocin
HFD - high fat diet
ZDF - Zucker diabetic fatty rat
GK - Goto-Katazaki
i.p. - intraperitoneal injections
IPGTT - intra-peritoneal glucose tolerance test
IST - insulin sensitivity test
EE - energy expenditure
RER - respiratory exchange ratio
NOR - Novel objects recognition
ASST - Attentional set-shifting task
SD - simple discrimination
CD - compound discrimination
CDR - compound discrimination reversal
IDS - intra-dimensional shift
EDS - extra-dimensional shift
PFC - prefrontal cortex
CSF - cerebrospinal fluid
IGF-1 - insulin-like growth factor 1
TSH - thyroid-stimulating hormone

GENERAL INTRODUCTION

Exploring the link between somatic and mental disturbances

During my three years of PhD studies, I had the opportunity to complete an internship at the Psychoneurobiology of eating and addictive behaviour Unit from the Bellvitge Biomedical Research Institute (IDIBELL), located in Barcelona. The group I worked with specialized in the treatment of eating disorders, dealing with conditions such as obesity, anorexia and other metabolic imbalances. Patients were not only treated from a clinical perspective - addressing their physical health through medical and nutritional care - but were also provided comprehensive psychological support. Albeit limited in time, this experience allowed me to first-hand realizes how deeply interlinked physical and mental health are. This connection extends beyond the emotional realm, such as anxiety and depression, which are often associated with metabolic and eating disorders [1,2]. At a functional level, many of these patients exhibit cognitive impairments, including difficulties with attention [3], memory [4], and executive functions [5].

Research increasingly highlights that metabolic diseases represent a significant risk factor for cognitive decline and brain dysfunctions [6,7]. Those pathologies (such as obesity, diabetes, and metabolic syndrome) are characterized by metabolic imbalances that due to a combination of biological, physiological, and environmental factors may lead to changes in brain structure, function, and performance. Obesity and overweight are defined as *abnormal or excessive fat accumulation that presents a risk to health* [8]; a body mass index (weight (Kg)/[height (m)]^2) over 25 is considered overweight, and over 30 obese [9]. Beyond the peripheral complications (including cardiovascular disease, hypertension, insulin resistance, and type 2 diabetes) [8,10], obesity has also been bi-directionally associated with neuropsychiatric disorders such as depression [11], and cognitive deficits [12]. Children and adolescents with obesity, as well as adults, are at increased risk for deficits in executive function, including inhibitory control and reward processes [13,14]. Moreover, epidemiological studies suggest that obesity may predispose to develop attention-deficit/hyperactivity disorders (ADHD) [15].

Similar to obesity, a broader dysfunction of metabolism can lead to a condition known as metabolic syndrome (MetS); a cluster of disorders including hypertension, dyslipidaemia, and impaired glucose tolerance, which collectively increase the risk of developing diabetes and cardiovascular disease fivefold [16]. MetS is also associated with brain volume loss, particularly in regions like the hippocampus and prefrontal cortex, which are essential for memory and higher-order cognitive functions [17]. Individuals with MetS are at greater risk for cognitive impairments, including reduced verbal fluency, slower processing speed, and deficits in executive function and verbal memory [18]. Moreover, studies have found that people with MetS are 20-30% more likely to develop mild cognitive impairment or dementia compared to those without it [19,20]. Being the most common form of dementia, Alzheimer's disease has been closely linked to MetS [21].

Both obesity and MetS are features of another endocrine disorder of the general metabolism: type 2 diabetes mellitus (T2DM). The World Health Organization defines diabetes as a *metabolic disorder of*

multiple aetiology characterised by chronic hyperglycaemia with disturbance of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both [22]. The "multiple aetiology" of T2DM is due to the environmental, genetic, and metabolic risk factors that contribute to its development [23] . Lifestyle pattern such as high-fat/sugar diet and poor exercise habits (typical of obesity and overweight) can increase the risk of developing T2DM by 90-fold [24]. Nevertheless, even under the same environmental conditions, some individuals are more at risk of developing diabetes due to genetic vulnerability [25]. More than 60 genetic variants related with diabetes risk have now been identified [26]. Age and the biological sex are important moderators of T2DM aetiology. Even if T2DM is considered to be a disease associated with adulthood and the incidence of T2DM increases with age [27], it is becoming more common for children and adolescents to be affected [28]. Finally, globally more men than women are diagnosed with diabetes [29]. T2DM is "characterised by chronic hyperglycaemia with disturbance of carbohydrate, fat, and protein metabolism" [22] that derive from the pathological condition of insulin resistance: cells in insulin-sensitive tissues (muscle, liver and adipose tissue in particular) fail to normally respond to the hormone or downregulate insulin receptors. This reduced sensitivity triggers – at first – a hyper-function of the β-cells, which produce more insulin to maintain normal blood glucose levels (hyperinsulinemia). This compensatory process gradually results in the dysfunction of the β-cells since many islet undergo apoptosis [30]. Peripheral tissues dependent on insulin can no longer uptake nutrients from circulation and adjust to rely on fat and catabolism of intracellular stored macromolecules (such as proteins) [31]. Thus, the ultimate symptoms, "resulting from defects in insulin secretion, insulin action, or both" [22], include dyslipidaemia in skeletal muscle and liver, and lastly a chronic hyperglycaemia. One of the consequences of the hyperglycaemic state is glycosuria. Kidneys are no longer able to filter and reabsorb the glucose and get rid of the excess through the urine [32]. Curiously, the term "diabetes mellitus" was introduced in 1674 by the British physician Thomas Willis referring to the particular sweetness of urine in diabetic patients (from Latin *mellitus* 'containing honey') [33]. Willis defined diabetes mellitus as the "Pissing Evil" [34].

Similar to the other metabolic alterations described above, also diabetes has been associated with cognitive decrements. This association was first proposed in 1922 when Miles and Root *found objective justification for the complaints of loss of memory and of ability to concentrate attention mentioned frequently by persons suffering from diabetes* [35], demonstrating that diabetics performed significantly worse on tasks requiring immediate retention and recall in comparison to non-diabetics. A cognitive decrease has been observed in T2DM, as patients have shown deficits in processing speed, executive and motor function, attention, and learning and memory [7,36–40]. In addition, epidemiological studies indicate an increase in the incidence of dementia in diabetic patients [41]. The most studied diabetes-cognitive association is with the Alzheimer's disease (AD) and dementia [42–44]. There has been a growing interest in the role of β-amyloid and tau protein in the peripheral nervous system, as well as in inducing insulin resistance [45]. Because so many studies showed that AD and T2DM share common

pathophysiological mechanisms [46–48] , the term "type 3 diabetes" has been used to describe the impaired insulin signalling observed in AD [49]. Diabetes is also associated with neurodevelopmental disorders characterized by behavioural and cognitive rigidity [50] (review in which I was directly involved and thus included in the appendix, pag. 105), i.e. obsessive compulsive disorders (OCD) and autism spectrum disorders (ASDs). Increased OCD symptoms have been described in men with diabetes [51]. Moreover, OCD symptoms were found to be positively correlated with blood levels of glycosylated haemoglobin (a diagnostic measure of TD2M) [52]. As for OCD, research in patients [53] and animal models [54] also suggested that altered insulin signalling is involved in the aetiology of ASDs. Finally, those evidences were supported by the interesting association between maternal diabetes and the significantly greater risk of ASDs in the offspring [55].

All these somatic metabolic diseases (T2DM, MetS, and obesity) have in common physiological pathways that can lead to synaptic dysfunction through a dysregulated central metabolism. One key pathway involves chronic inflammation which triggers the release of pro-inflammatory cytokines [56]. These inflammatory molecules can cross the blood-brain barrier, leading to neuro-inflammation that damages brain cells and impairs cognitive function [57,58]. Additionally, the vascular dysfunction reduce the cerebral blood flow and lead to damage in white matter, essential for communication between brain regions, ultimately impairing cognitive function [59]. Lastly, alongside peripheral insulin resistance, these diseases may involve an altered central insulin signalling, which disrupts synaptic function and contributes to cognitive dysregulation [60].

Insulin-signalling: from the periphery to the brain and back again

In 1910, in London, Sir Edward Albert Sharpey-Schafer described the pancreatic islands that were able to secrete a substance controlling glucose metabolism. He termed that substance "insulin", from Latin *insula* with reference to the Langherans islets. A few years later, in 1921, a young surgeon named Frederick Banting and his assistant Beste developed a method to extract insulin from the pancreas of dogs, which proved effective in lowering blood sugar levels in diabetic dogs. In January 1922, Leonard Thompson, a 14-year-old diabetic patient at the Toronto General Hospital, successfully received the first dose of insulin [61]. After that, many other researches have depicted the long story of the discovery of inulin's role.

To cut short this intricate story, insulin is a peptide hormone secreted by the β-cells of the islets of Langerhans of the pancreas. It is the major hormonal regulator of glucose metabolism. Under healthy conditions, following an increase of blood glucose levels, insulin is released via exocytosis from the secretory granules of pancreatic β-cells [62]. Insulin blood concentration is firstly regulated in the liver, where hepatocytes act as the major site of insulin clearance. The concentration of insulin reaching the liver can be 10-fold higher than the concentration in the peripheral circulation. Then, insulin that is not degraded reaches the heart through the hepatic vein. The heart pumps insulin from the arterial circulation to the target tissues (especially skeletal muscle and adipose tissue). The major

function of insulin in muscle and adipose tissues is to increase their uptake of glucose and to store it for the future energetic needs. This is afforded by the insulin dependent-translocation of glucose transporters on the cells surface [31]. Thus, insulin exerts a hypoglycemic role allowing the intra-cellular transport of glucose. Lastly, upon filtration in the glomeruli, insulin proceeds to the kidney wherein it is degraded to end its journey [62]. Paradoxically, only 25% of glucose consumption occurs in insulin-dependent tissues (e.g., muscle and adipose tissue) [63]. Approximately 75% of glucose consumption occurs in insulin-independent tissue and of that, about 50% occurs in the brain [64]. Thus, it not surprising that the brain is an essential actor of the insulin journey.

However, insulin signalling has been primarily associated to peripheral-somatic diseases until the 1978, when Havrankovara et al. showed that *brain insulin, indistinguishable from the pancreas-authentic insulin, was found in extracts of the whole rat brain* [65]. From that moment on, the research on insulin extended from a peripheral treatment for metabolic dysfunctions to an understanding of its broader implications in brain function. Subsequent researches have questioned whether peripheral insulin could cross the blood brain barrier [66–68] or if it was directly produced in the brain [69,70]. Today we know that both mechanisms are involved in the central insulin-signalling regulation.

After being secreted from pancreatic β-cells, insulin crosses into the brain by circumventing the blood-brain barrier through the median eminence, or by transport proteins [71,72]. Transport of insulin into the brain may be altered by conditions such as obesity, diabetes mellitus, fasting, and Alzheimer's disease [72–75]. Additionally, insulin transport varies among brain regions. Although insulin receptors are mainly expressed in the olfactory bulbs [76,77], they are also present in the cerebral cortex, hypothalamus, hippocampus, and cerebellum [78,79]. At the same time, studies in rodents identified insulin mRNA in the central nervous system (CNS), thus hinting at the possibility that insulin may be synthesized, de novo, within the brain [80,81]. Insulin gene expression and insulin synthesis in mammalian neuronal cells localized insulin mRNA to anatomical regions involved with olfaction and in the limbic system [82]. Moreover, C-peptide (a metabolic product in insulin biosynthesis) is also present in some pyramidal cells of the neocortex and hippocampus [83]. Therefore, insulin has an extra pancreatic production. Insulin in the CNS has important non-metabolic functions, whereby it assists neuronal survival [84], synaptic maintenance [85], dendritic development [86], neuronal circuitry formation [87], modulation of both GABA and glutamate release [88] , and learning and memory [89]. Being involved also in the CNS, the insulin signalling encompasses brain disorders.

It is evident that metabolic disorders can lead to various cognitive impairments, but what if the reverse relationship were also true? That is, can cognitive impairments contribute to the onset or worsening of metabolic dysfunctions? The first inverse association dates back to 1919, when Kooy proved that *emotions, especially those of a depressive character, cause an increase of blood-sugar. They therefore have a deteriorating influence on diabetes mellitus. They may cause a temporary hyperglycaemia and even glycosuria in normal persons, not suffering from saccharine diabetes* [90]. Research suggests that mild cognitive impairments may contribute to the development of MetS [91], while an increased risk of MetS was observed in

various psychiatric patient populations [92]. Moreover, patients with AD are more vulnerable to develop T2DM [93], and post-mortem studies have reported alterations in insulin signalling in individuals with AD [94]. These findings may suggest that therapeutic interventions targeting cognitive impairments could also benefit peripheral metabolism. For instance, memantine, a drug approved for the treatment of moderate-to-severe AD, has been shown to improve peripheral metabolic parameters, such as insulin resistance, in mouse models [95].

Just as peripheral insulin imbalances lead to cognitive alterations, so also brain insulin signalling similarly may lead to peripheral metabolic disorders. Insulin action in specific hypothalamic neurons suppresses endogenous glucose production in the liver and leads to lower blood glucose levels [96]. In adipose tissue, brain insulin action regulate lipolysis [97]. Additionally, plasma glucose levels are influenced by serotonergic and dopaminergic system [98]. Lastly, some serotonergic antidepressants reduce hyperglycaemia, normalize glucose homeostasis, and increase insulin sensitivity, whereas some noradrenergic drugs have the opposite effects and may worsen glucose tolerance. [99].

As a consequence, central nervous system impairments can influence peripheral balance, creating a recurrent cycle that links metabolism and cognition. Insulin signalling is implicated in multimorbidity across the lifespan, that beside T2DM, MetS, and obesity, include Romano Ward Syndrome (RWS) [100], and neurological brain disorders like dementia, AD [42–44], OCD [51], and ASDs [101].

The KCNQ1 gene as a link between metabolism and cognition

As anticipated it in the title of my thesis, the *KCNQ1* gene is one of the mediators in this connection. Converging studies, including genomic [102], preclinical [103] and clinical work [100], confirm that the *KCNQ1* gene represents an important actor within the pathway of insulin signalling. Its involvement is well embodied by RWS patients. Mutations in the *KCNQ1* gene in these patients contribute to both the Long QT Syndrome (LQTS) and insulin resistance [100].

KCNQ1 was originally named KvLQT1, reflecting its association with LQTS pathology [104]. LQTS is an inherited (or in many cases drug-induced) cardiac rhythm disorder characterized by a prolonged QT interval on the surface electrocardiogram. It indicates a prolonged time from initiation of ventricular depolarization to the end of ventricular repolarization. This delay, caused by loss-of-function in *KCNQ1* gene, can cause the potentially lethal cardiac arrhythmia and ultimately ventricular fibrillation, which trigger to sudden cardiac death [104]. Moreover, the phenotype of long QT syndrome also includes hyperinsulinemia, the symptomatic reactive hypoglycaemia and, a low potassium level after an oral glucose challenge [100].

The KCNQ1 channel is a voltage-gated potassium channel allowing dynamic cellular repolarization during an action potential; it opens after membrane depolarization to permit K^+ efflux [105]. Similar to other voltage-gated ion channels, the functional unit of KCNQ1 consists of a tetramer, with each monomer containing a short N-terminal helix, followed by six transmembrane helices (S1–S6) and four intracellular helices in the C-terminal domain. The voltage sensor domain (VSD) is shaped by

transmembrane helices S1–S4, while helices S5–S6 form the pore domain (PD)[106]. However, two key structural features of KCNQ1 differ from the other voltage gated channels: a nine amino-acid α-helical insertion in the S2–S3 linker region and the lack of a proline residue in S3 helix which is indeed present in most ion channels [107]. These structural features make KCNQ1's gating properties unique compared to other voltage-gated channels, as it can switch from a voltage-dependent state to a constitutively active state. The voltage sensor domain can lose its voltage dependence upon association with members of the auxiliary subunit family (KCNE), resulting in KCNQ1 becoming constitutively active [108]. Like other ion channels, KCNQ1 undergoes a voltage-dependent three-step activation process, transitioning from an initial resting state to an intermediate state, and finally to a fully activated channel. However, KCNQ1 is unique in that the pore remains open in both the intermediate and activated states [109]. Another interesting feature of KCNQ1 is that, apart from the voltage activation, its function is also mediated by a membrane lipid phosphatidylinositol 4,5-bisphosphate [110].

The best characterized physiological function of KCNQ1 relates to its expression in cardiac tissue, where it co-assembles with KCNE1, a single transmembrane protein, to form a channel complex that mediates the slow delayed rectifier current (a critical electrical component of the cardiac action potential necessary for myocardial repolarization) [110]. Besides its critical function in the physiology of the heart, KCNQ1 associates, in different tissues, with different members of the KCNE auxiliary subunit family (KCNE1–5) that modify KCNQ1 channel properties, creating various phenotypes for different physiological functions [106]. The best-established role for KCNQ1-KCNE2 auxiliary subunits is in the gastrointestinal system, where the channel is involved in acidifying the stomach lumen [111]. Additionally, KCNE2 deletion in mice leads to hypothyroidism, due to reduced activity of another sodium-coupled solute transporter in thyroid cells [112]. Moreover, KCNQ1 has a crucial role in thyroid hormone biosynthesis [113]. KCNQ1 assembles with KCNE3 in the intestine, and it is required for regulation of chloride secretion promoting electrogenic release across the intestinal epithelium [114]. A well characterized role of KCNE1 auxiliary subunit is in the inner ear [115] where the bond with KCNQ1 facilitates the recycle of the endolymph. Autosomal mutations of KCNQ1 can cause deafness [111]. Finally, KCNE4 [116] and KCNE5 [117] might appear to exert similar effects on KCNQ1 – each resulting in robust inhibition of activity – even if the mechanisms and functional outcomes are different.

A lot of observations indicate that *KCNQ1* is expressed in pancreas revealing a role of that gene in insulin-signalling [118,119]. The specific KCNE(s) co-assembling with KCNQ1 in pancreatic β-cells remain unclear. KCNE2 is a potential candidate, as KCNE2 deletion in mouse models has been shown to result in altered insulin secretion [120]. Additionally, the absence of KCNE2 induces extensive changes in the pancreatic transcriptome that are consistent with various aspects of T2DM [120]. *Kcnq1*$^{-/-}$ mice demonstrate enhanced insulin sensitivity, which in turn inhibits insulin release, leading to decreased plasma insulin concentrations [121].

Further evidence supporting kcnq1's role in glucose control comes from in vitro studies. In the MIN6 β-cell line (cells from pancreatic tumor in transgenic mice) the overexpression of *kcnq1* increased K+ currents and significantly reduced insulin secretion, leading to hyperglycaemia [122]. Conversely, inhibiting *KCNQ1* with siRNA in human pancreatic β-cells increased insulin secretion [123].

A lot of clinical studies have shown that single nucleotide polymorphisms within *KCNQ1* are strongly associated with diabetes mellitus across diverse populations, including those from Africa [124], America [125,126], East Asia [127–129], Europe [130–132], and Latin America [130]. Furthermore, a common genetic variation in *KCNQ1* is associated with dysregulation of β-cell function, incretin, and insulin secretion [133], highlighting a major risk factor for T2DM. Additionally, a case study of a patient with a gain-of-function mutation in *KCNQ1* revealed hyperinsulinemia following an oral glucose load, further confirming the role of KCNQ1 in insulin secretion [134].

Another interesting but complex aspect of *KCNQ1* gene is the presence of imprinting [135]. In humans, *KCNQ1* is part of a larger cluster of neighbouring genes, all on chromosome 11p15 that are imprinted (their expression depends on the parental origin of the allele). These genes are normally expressed on the maternal allele and are silent on the paternal allele [136]. The cluster is regulated through an imprinting control region (ICR) that exhibits parental-specific epigenetic modifications, such as DNA methylation and histone modifications, which govern their activity. The ICR serves as a promoter for a regulatory non-coding RNA (*KCNQ1ot1*). The paternal ICR allele is un-methylated and associated with *KCNQ1ot1* expression which leads to the repression of adjacent genes. In contrast, methylation of the maternal ICR allele represses *KCNQ1ot1* and activates the adjacent imprinted genes. Mutation on the paternal allele results in a biallelic expression of *KCNQ1* and in a failure to express *KCNQ1ot1* that have been implicated in several genetic disorders [137]. For instance, Beckwith-Wiedemann syndrome (BWS) is associated with the loss of maternal imprinting information leading to developmental overgrowth, and neonatal, and sometimes persistent, hyperinsulinemia [138]. The aetiology of the hyperinsulinemia in BWS has been suggested to involve insulin-like growth factor 2 (IGF2) and KCNQ1 in the pancreatic β-cell [139]. As a matter of fact, another gene involved in the cluster of imprinted genes on 11p15 is IGF2 [136]. Furthermore, some T2DM-associated single nucleotide polymorphisms in *KCNQ1* indicate an effect of parental imprinting and revealed a maternal allele-specific association of the T2DM's risk [140].

In mice, the imprinted cluster at the distal end of chromosome 7 is the orthologous human chromosome 11p15. *Kcnq1* expression is firstly from the maternal allele, but by the late embryogenesis, it becomes dependent from the paternal allele, while in adult mice there is no obvious difference in expression between the two alleles [141]. Similar to mice, in humans, *KCNQ1* seems to exhibits a time-specific imprinting. Travers et al., demonstrate that *KCNQ1* expression patterns differ by developmental stage in human adult islet and fetal's pancreas samples with

preferential expression from the maternal allele in fetal tissues, and biallelic expression in adults [142]. This could suggest that diabetes risk effects may be mediated in early development.

Despite its low regional specificity, *KCNQ1* is expressed in various tissues of the human and mouse brain (*figure 1*) with the highest nTPM (number of transcripts per million) in the choroid plexus and in the pituitary gland [143]. A role for KCNQ1 in the choroid plexus is in regulating cerebrospinal fluid composition and thus, contributing to neuronal excitability disorders [144,145]. Goldman et al. confirmed the presence of *kcnq1* subunit mRNA in multiple regions of the developing and adult wildtype mouse. Expression of *kcnq1* was detected at different ages in the hippocampus, the cortex, the dentate gyrus, and the pyramidal cells of the CA1-3 regions [146]. Moreover, knock-in mice with a *kcnq1* variant associated with LQTS were found to have epilepsy and cardiac arrhythmic episodes, suggesting a role for kcnq1 in regulating neuronal excitability [146]. KCNQ1 is also involved in dopaminergic system. D_2 dopamine receptors and KCNQ1 channel co-localise postsynaptically in several brain regions modulating the neuronal excitability by dopamine release [147].

The influence of KCNQ1 in the CNS is further supported by genomic studies. Variants in *KCNQ1* may alter white matter electrical signal transmission and thus contribute to cognitive deficits such as slower processing speed in schizophrenia [148]. Additionally, a single nucleotide polymorphism of *KCNQ1* gene showed a strong association with AD [149] and with OCD [102].

All these aspects make *KCNQ1* a pivotal candidate for understanding the interplay between metabolic and cardiac disorders.

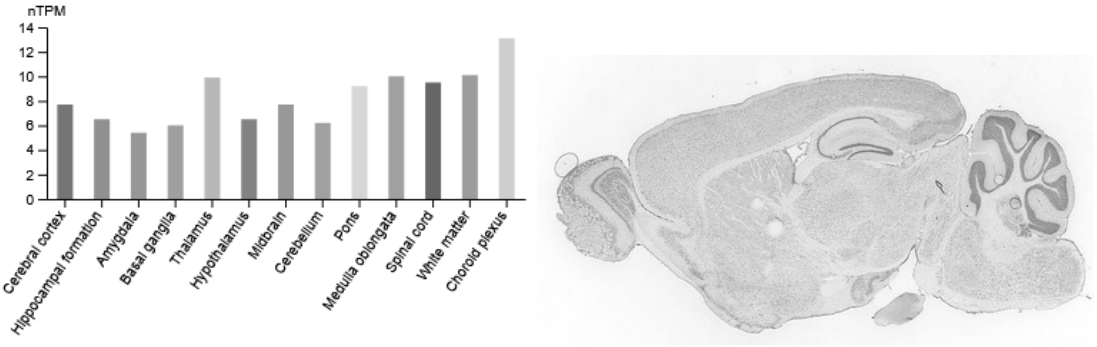

Figure 1. Normalized RNA expression level (nTPM) showed for 13 brain regions in human brain; the bar shows the highest expression among the subregions included [143]; the expression of *KCNQ1* based on in situ hybridation in mouse brain [150].

Main preclinical assumptions behind my hypothesis and aim of the work

Before starting my experimental plan, I confirmed the relationship between metabolism and cognition in the animal model. To achieve this aim, I systematically reviewed the association between hyperglycaemia – as a proxy of insulin-related metabolic dysfunctions – and impairments in working and spatial memory, and attention in laboratory rodents (see Chapter I, pag. 16). My review

confirmed that hyperglycaemia in experimental models of metabolic dysfunctions is associated with cognitive impairments [151].

Once assessed how insulin-signalling extends beyond metabolism to encompass the brain and why KCNQ1 is a key molecule in this interplay, I focused on the preclinical assumptions that allowed me to shape the experimental hypothesis of my PhD project: to evaluate whether the site of action of KCNQ1-related effects on multimorbidity depends on insulin in the periphery and/or in the CNS. Specifically, I conducted behavioural, physiological, and metabolic tests in a conditional *kcnq1* knockout mouse model. In this experimental model, *kcnq1* expression is decreased in a time (during adolescence and adulthood) and tissue (in the brain) specific manner.

The use of animal models in research raises important ethical considerations, but their role in advancing science and medicine is undeniable. They provide invaluable insights into disease mechanisms, enable the testing of new treatments, and contribute to numerous medical breakthroughs. Given that, many preclinical studies preceded my conditional mouse model and suggested a KCNQ1's role in the comorbidity between insulin-related metabolic and mental disorders (*figure 2*). My work fits within the broader framework of the PRIME project [152] which aim is to unravel the insulin-dependent mechanisms underlying both somatic conditions and brain disorders. My experimental hypothesis gains added significance through collaboration with other PRIME research groups showing that: (*i*) TALLYHO/JngJ mice (mouse model of TD2M) had a metabolic phenotype which is associated with deficits in attention and perseveration, and memory [153] (work in which I was directly involved and thus included in the appendix, pag. 142); (*ii*) *kcnq1*$^{-/-}$ whole body mice demonstrate both altered insulin sensitivity, altered insulin release [121], and cognitive alterations (i.e. repetitive behaviour, compulsive object checking, and cognitive and behavioural rigidity) (Glennon J., Sullivan M., not yet published). Thus, the conditional brain *kcnq1* knockout model allowed me to distinguish the KCNQ1 central role from the peripheral one in the insulin multimorbidity.

Another important aspect that I considered is the value of variability for biological research. In behavioural neuroscience, standardized procedures are promoted in methodology to homogenize environmental variables and to ensure reproducibility within and between laboratories [154]. While these standardized approaches have led to significant scientific advancements, they also have notable limitations, underscoring the need to balance standardization with an understanding of natural variability's role in research. Previous analyses demonstrated that heterogeneous experiments yielded more stable results and were characterised by a reduced number of false positive results compared to homogenised experiments [155]. Metabolic dysregulations are highly heterogeneous disorders having gender and age-specific differences [27]. For that reason, a vital tool in my project has been to use animal models carrying significant heterogeneity of that pathology, along with the spectrum of phenotypes seen in cognitive dysregulation.

I adopted a heterogeneous strategy and an ontogenetic perspective. This allowed me to study the developmental nature and time course of insulin multimorbidity and to emphasize the importance of systematically studying gender differences across the lifespan. For that reason, I firstly pooled the experimental subjects on the basis of certain characteristics (age, sex, and treatment) so that the animals are as homogeneous as possible within blocks, but different between blocks. As between block variation can then be eliminated by comparing treatments within blocks only, statistical power and precision is high [155]. Then, I analysed the specific variables (sex and treatment) separately in the two developmental stages (adolescence and adulthood).

Finally, I care a lot about the 3Rs principle (Replacement, Reduction and Refinement) in animal research to reduce the number of animals used, to minimise individual suffering and at the same time to guarantee an adequate number of subjects per test. All the tools I adopted to achieve this aim have been: (i) an execution of a detailed preliminary power analysis, and (ii) a reduction of the number of experimental groups used as controls (well clarified at pag. 59; Chapter II, Materials and methods).

		PRECLINICAL STUDIES				CLINICAL STUDIES			
		TALLYHO *model of obesity*	**App/Ps1** *model of AD/dementia*	**Conventional KO** *whole body kcnq1 -/-*	**Conditional KO** *brain kcnq1-/-*	**Diabetics**	**Romano Word Syndrome**	**Obsessive Compulsive Disorder**	**Alzheimer**
TYPE OF ALTERATION	**Metabolism**	x	x	x	?	x	x	x	x
	Cognition	x	x	x	?	x		x	x
	KCNQ1			x	x	x	x	x	x

Figure 2. Clinical and preclinical assumptions supporting my hypothesis; the 'x' indicates the kind of genotype alteration (*KCNQ1* gene) and/or phenotype variation (metabolic and/or cognitive) observed.

CHAPTER I

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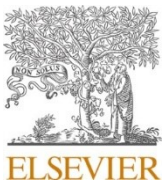

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A systematic review of preclinical studies exploring the role of insulin signalling in executive function and memory

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ABSTRACT

Beside its involvement in somatic dysfunctions, altered insulin signalling constitutes a risk factor for the development of mental disorders like Alzheimer's disease and obsessive-compulsive disorder. While insulin-related somatic and mental disorders are often comorbid, the fundamental mechanisms underlying this association are still elusive. Studies conducted in rodent models appear well suited to help decipher these mechanisms. Specifically, these models are apt to prospective studies in which causative mechanisms can be manipulated via multiple tools (e.g., genetically engineered models and environmental interventions), and experimentally dissociated to control for potential confounding factors. Here, we provide a narrative synthesis of preclinical studies investigating the association between hyperglycaemia – as a proxy of insulin-related metabolic dysfunctions – and impairments in working and spatial memory, and attention. Ultimately, this review will advance our knowledge on the role of glucose metabolism in the comorbidity between somatic and mental illnesses

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Introduction

Whilst impaired insulin signalling has been traditionally associated with metabolic dysfunctions like type 1 (T1DM) and type 2 diabetes mellitus (T2DM), metabolic syndrome (MS), and obesity (Klimova et al., 2018; Landau and Pinhas-Hamiel, 2019; van de Vondervoort et al., 2016), recent evidence indicates that it is also associated with impairments in cognitive capabilities (Moheet et al., 2015). Both T1DM and T2DM are associated with mental and motor slowing and decrements in attention and executive functioning (McCrimmon et al., 2012). A deficit related to memory is frequently reported in patients with T2DM (Zhang et al., 2015); moreover, anhedonia and impulse control disorders (eating disorders and addiction) are often associated with diabetes (De Jonge et al., 2014). These symptoms constitute hallmarks of specific mental disorders in which, accordingly, alterations in insulin signalling have been observed: attention deficit hyperactivity disorder (ADHD) (Landau and Pinhas-Hamiel, 2019), Alzheimer's disease (AD) (Burillo et al., 2021), obsessive-compulsive disorder (OCD) (Grassi et al., 2022), depression (Lyra e Silva et al., 2019), and drug addiction (Brambilla et al., 1976). The socioeconomic costs of these diseases are huge and continue to rise. For example, the International Diabetes Federation estimated that while 537 million adults (20-79 years) are currently diabetic, these numbers are projected to increase steadily by 2045, when 783 million adults will live with this condition. The yearly associated healthcare economic burden of diabetes *per se* accounts for approximately 1.2 trillion US dollars (da Saúde, 2017). Should altered insulin signalling also represent a risk factor for mental disorders, these costs would further increase. For example, although the contribution of insulin signalling to e.g. AD still needs to be elucidated, approximately 35 million people worldwide currently have this form of dementia (World Health Organization, 2022). Predictably, these numbers are projected to increase at an unsettling rate (approximately 85 million AD

patients by 2050) (World Health Organization, 2022). The aforementioned estimations are only the tip of the iceberg whereby they do not account for other insulin-related mental comorbidities like ADHD and OCD. Thus, exploring the role of insulin signalling in executive functions and memory may beget remarkable advantages in terms of public health and its associated costs.

How insulin regulates somatic functions has been the key question of countless scientific studies. Most early investigations focussed on its role in mediating general metabolism (Samson and Garber, 2014). These studies contributed to understanding how insulin regulates glucose homeostasis and energy balance (Boden, 2001; Brown and Walker, 2016; Huang, 2009) and how derailments in these processes result in metabolic disorders like T1DM and T2DM (DeFronzo, 2004), and MS (Banday et al., 2020). The primary deficit in T2DM is insulin resistance (Brunton, 2016), characterised by a reduced insulin sensitivity of cells in peripheral tissues (e.g., muscles, liver, and adipose tissue). This generates hyperfunction of the β-cells of the pancreas which ultimately elicit hyperinsulinemia: an increased production of insulin aimed at maintaining normal blood glucose concentrations. This process gradually impairs β-cells functionality and causes insulin deficiency and hyperglycaemia, with fasting plasma glucose concentrations >110 mg/dL (Banday et al., 2020) representing the symptomatic threshold for T2DM.

Recently, the interest for the role of insulin has started to extend beyond energy metabolism to encompass the central nervous system (CNS) (Banks et al., 2012; Clarke et al., 1986). Evidence for a role for insulin signalling in the CNS is related to its widely-expressed (Chiu et al., 2008) receptors (e.g. Insulin Receptor, IR, and Insulin Growth Factor-1 receptor IGF-1R) in the brain. The presence of insulin in the brain derives from two main paths: from the periphery as it can cross the blood brain barrier or via direct synthesis by neurons (Creo et al., 2021; Fanelli et al., 2022). Accordingly, beside its role in glucose metabolism, recent evidence indicates that insulin contributes to several cognitive functions, such as learning, memory, integration of sensory information, and modulation of synaptic plasticity (Nisticò et al., 2012).

Genetic, clinical and preclinical (Biessels and Despa, 2018; Blázquez et al., 2014; Koekkoek et al., 2015) studies support the evidence that altered insulin signalling is involved in mental function and disease. For example, several studies reported a correlation between T2DM and AD and observed that insulin signalling may represent a common pathophysiological risk factor (Burillo et al., 2021; Pardeshi et al., 2017). Beyond AD, T2DM patients are at increased risk of milder forms of cognitive decline other than memory, such as processing speed and executive functions (Monette et al., 2014; Palta et al., 2014). These may occur during pre-diabetic stages and slowly worsen over time (Biessels et al., 2014). Importantly, impairments in impulse control, as a characteristic of ADHD, have also been observed in a large cohort of obese patients (Sinclair et al., 2000) further strengthening the potential association between metabolic syndrome and cognitive impairments. Furthermore, genetic and genomic studies reported that dysregulated insulin-dependent signalling cascades are associated with OCD (Bralten et al., 2020; van de Vondervoort et al., 2016). Accordingly, clinical investigations consistently reported that anti-diabetic drugs have beneficial effects on cognitive impairments in both AD, OCD, and other forms of cognitive decline (Fink et al., 2018; Munõz-Jiménez et al., 2020).

Although the historical analysis of this literature suggests that the interest in the somatic function of glucose preceded the interest in its role in the brain, a very early account looked at this relationship from the opposite side. Thus, even before the discovery of insulin, Kooy (Kooy, 1919) hypothesised that mental disorders triggered the emergence of hyperglycaemia. Ultimately, the view that insulin signalling may be involved in both somatic and mental disturbances is now consolidated. Yet, it is unclear whether mental disturbances are secondary to somatic alterations, whether the latter are consequence of the former, or whether they are due to diverse insulin-related mechanisms acting independently on the peripheral and the CNS.

The fundamental mechanisms underlying the comorbidity between T2DM and cognitive decline have been investigated in preclinical studies by means of animal models. For example, several authors reported that a consolidated experimental model of AD (transgenic mice expressing human amyloid precursor protein and presenilin 1, APP/PS1) exhibited impaired cognitive capabilities associated with poor glycaemic control (Denver et al., 2018). Similarly, insulin receptor β-subunit deficient mice exhibit impaired memory capabilities associated with altered long-term potentiation, the latter representing a form of synaptic plasticity (Nisticò et al., 2012). Finally, van de Vondervoort and collaborators (van de Vondervoort et al., 2019) reported increased compulsivity and anxiety in an experimental model (TALLYHO/JngJ mice) recapitulating most of the metabolic abnormalities observed in T2DM patients: insulin resistance, hyperglycaemia, hyperinsulinemia, and obesity.

Based on these considerations, we aimed to further detail the role of insulin signalling in the comorbidity between mental and somatic disturbances by systematically analysing available literature in rodents. To this aim, we propose a qualitative description of available preclinical studies – conducted in adult mice and rats exhibiting hyperglycaemia – investigating the role of impaired glucose metabolism in the comorbidity between somatic and mental impairments (limited to working memory, spatial memory and/or attention).

Methods

Review protocol

The systematic search was conducted in accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Moher et al., 2015). The protocol (SYRCLE's protocol; Supplementary item 1) for animal intervention studies (de Vries et al., 2015) was submitted to the PROSPERO registry on May 16th, 2022 and registered on June 12th, 2022 (registration number: CRD42022331458).

Literature search and study identification

A comprehensive systematic literature search was conducted – on April 7th, 2022 – in three online databases (PubMed, Scopus, Web of Science). The search strategy involved, as issues of interest, altered insulin signalling (with hyperglycaemia representing its proxy) and the investigation of the behavioural phenotypes isomorphic to specific symptoms of mental disturbances (i.e., executive function and memory); the search was limited to studies conducted in rats and mice. The complete search strategies used in each database were:

- TITLE-ABS-KEY (hyperglycaemia OR hyperglycemia) AND TITLE-ABS-KEY ((spatial AND memory) OR attention) AND TITLE-ABS-KEY (mouse OR mice OR rat OR rats)) for Scopus database;
- "hyperglycaemia"[Title/Abstract] OR "hyperglycemia" [Title/Abstract] AND "spatial"[Title/Abstract] AND "memory"[Title/Abstract] OR "attention"[Title/Abstract] AND "mouse"[Title/Abstract] OR "mice"[Title/Abstract] OR "rat"[Title/Abstract] OR "rats"[Title/Abstract] for Pubmed;
- TS=((hyperglycaemia OR hyperglycemia) AND ((spatial AND memory) OR attention) AND (mouse OR mice OR rat OR rats) for Web of Science.

During the first phase of examination (i.e., screening of titles and abstracts), the following prioritization of exclusion criteria was used: (1) language other than English; (2) non-original researches (e.g., reviews, commentaries, editorials, book chapters); (3) no full-text articles (e.g., meeting abstracts); (4) studies in vitro, studies in humans, studies in non-human animals other than rats and mice; (5) outcome measures other than working memory, spatial memory and/or attention. Two observers (MP and AMO) independently screened the articles of the first phase. The additional exclusion criteria in the second phase of full-text screening of the eligible articles were: (5) outcome measures other than working memory, spatial memory and/or attention; (6) experimental manipulations not resulting in hyperglycaemia; (7) other control conditions (e.g., low-fat diet used as control instead of standard diet, etc.). The data were independently extracted by two reviewers per site (MP and AMO; AO and DS; MS and JCG, respectively) and discrepancies were resolved by principal investigators of each site (SM, JCG, and DS).

Data extraction and synthesis

The study characteristics extracted from the full-text articles eligible for qualitative data included the following categories: (i) bibliographic details (DOI, title, authors, publication year, journal); (ii) study design characteristics (number of experimental groups, number of subjects per group, type of study design); (iii) animal model characteristics (species, strain, sex, age and/or weight at the beginning of the study, type of test used to evaluate spatial memory, working memory and/or attention); (iv) intervention characteristics (type of experimental manipulation adopted to induce hyperglycaemia, details regarding the experimental manipulation, type of non-hyperglycaemic control, details on the assessment of hyperglycaemia, e.g. higher blood glucose concentrations compared to controls, and or to a predefined threshold); (v) outcome measure (direction of the variation of the behavioural phenotypes isomorphic to working memory, spatial memory and/or attention in experimental subjects exhibiting hyperglycaemia and in non-hyperglycaemic controls). If available, data on the variation of glucose metabolism-/insulin signalling related parameters (obtained after the original induction of hyperglycaemia, for example through glucose tolerance, insulin resistance, etc.) were collected.

Assessment of the risk of bias

To evaluate the methodological quality and validity of the included studies, we used the SYRCLE's Risk of Bias (RoB) tool for animal studies, developed by Hooijmans and co-authors (Hooijmans et al., 2014) by adjusting the Cochrane's RoB tool (Higgins et al., 2011). The RoB tool for animal studies is divided into 10 items (for Selection bias: sequence generation, baseline characteristics, allocation concealment; for Performance bias: random housing, blinding; for Detection bias: random outcome assessment, blinding; for Attrition bias: incomplete outcome data; for Reporting bias: selective outcome reporting; for Other: other sources of bias). With specific reference to the Reporting bias, we note that adequate tools to pre-register the experiments (and thus allow a systematic evaluation of the consistency between the planned and the reported studies) are available only since 2021 (Olevska et al., 2021). Thus, for studies preceding this date, we deemed the Reporting bias as not applicable. Two reviewers per affiliation site (MP and EP; AOL and DS; MS and JCG) performed the quality assessment of each article by independently assessing the aforementioned criteria.

Results

Study selection

The search strategy described above resulted in 851 bibliographic records. The process of selection is summarized in **Figure 1** by using the PRISMA flow diagram. References were exported to Microsoft Excel and, after duplicates were removed, 447 studies remained. The first selection phase (for language other than English, non-original research, no full text article) resulted in 347 studies; the second selection phase (i.e., full-text articles screening) resulted in 91 studies eligible for inclusion in the systematic review, while a total of 256 articles were excluded for: assessment of phenotypes other than attention, spatial memory, and working memory (n=188), species other than mouse/rat (n=44), experimental manipulations not resulting in hyperglycaemia (n=19), and lack of an appropriate control group (n=6).

Study characteristics

The characteristics of the 91 studies included in the present review are summarized in **Figure 2**.

Whilst the behavioural phenotypes in association with hyperglycaemia included working and/or spatial memory, and/or attention, only one article (Moreira et al., 2007) simultaneously evaluated all of them using the same lever-press test to discriminate the three different domains (the position of the levers served as a spatial and operant stimulus at the same time and the ability to change a discrimination, once learned, was considered an attentional task). The most evaluated phenotype was spatial memory: of all the studies considered, only in 11 of them was memory not investigated.

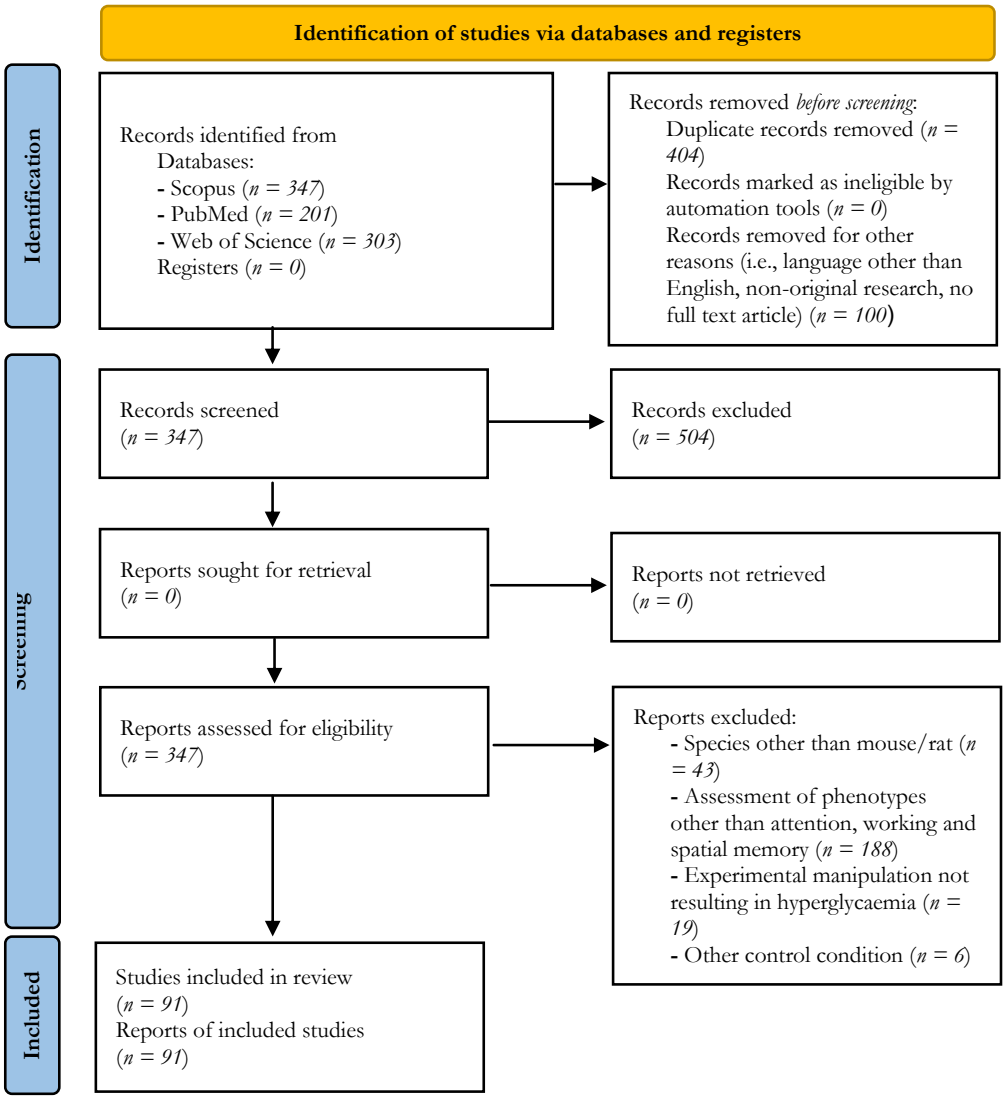

Fig.1 PRISMA flow diagram for preclinical studies (Moher et al., 2009). Diagram of the identification, screening, eligibility, and inclusion of the literature search.

The most widely adopted procedure to investigate this domain was the Morris water maze, which has been applied in 68 studies (n: 1,4,5,7,8,14-16,18,20-24,26,29,30,33-46,50-62,64-66,68-70,72-77,79,80,82,83,85-91). Some of these studies also used the Elevated plus maze learning task (Bhutada et al., 2010), the Novel object recognition test (Taylor et al., 2015) and/or the Barnes maze test (Jin et al., 2018; Momeni et al., 2021) to retest the same spatial memory phenotype. The Barnes maze test was used exclusively (and not as a restest) by three other authors (Li et al., 2012; Madhavadas et al., 2016; Madhavadas and Subramanian, 2015) while three articles used a variation of the Novel object recognition test: Object location (Braga et al., 2021; Van Der Kooij et al., 2018a) and Place recognition test (de Senna et al., 2017). Moreover, the Radial arm water maze test (Malone et al., 2008; Rababa'h et al., 2019), the T-maze (Joshi et al., 2021; Tanokashira et al., 2021; W. Wang et al., 2019), and the Y-maze (n: 15,25,47,49) were employed as spatial memory tasks.

Although the Morris water maze test is currently used for spatial memory, it has been originally devised to dissociate "spatial mapping" and "working-memory" theories of hippocampal function; accordingly, five articles of the present review (n: 15,34,64,73,74) used this test to assess working memory. The latter was also examined in 17 studies, which assessed spontaneous alternation as a proxy for working memory: of these, five articles used a T-maze test (n: 2,11,12,63,84), either in its original dry version or in a water incarnation (Tanokashira et al., 2018; W. Wang et al., 2019), nine used the Y-maze test (n: 13,19,27,31,49,67,78,81,89), while only one (Choeiri et al., 2005) employed the Four-arm maze test. The remaining two articles used the Passive avoidance test (Georgy et al., 2013) and the Step-down inhibitory avoidance task (Remor et al., 2019) to assess aversive associative memory. With respect to these tasks, we note that, as also described by the authors themselves (Georgy et al., 2013; Remor et al., 2019), they are predominantly used to investigate emotional memory rather than working memory. Yet, the same authors also specified that they considered these cortical-dependent tasks related to working memory, whereby the latter affects the impact of aversive stimuli.

Regarding attention, only two articles analysed it through a rewarded lever press task (Moreira et al., 2007) (the ability to change the discrimination of the rewarded lever, once it had been learned, was considered an attentional task) and the nest construction (Yeh et al., 2015), that according to the authors, is a task involving a broad network of brain regions and has previously been used to evaluate attention in mice (Filali and Lalonde, 2009).

Herein, we evaluated the different experimental manipulations adopted to induce hyperglycaemia, the details on the assessment of hyperglycaemia, and the type of non-hyperglycaemic control (**Table 1**). All articles with no appropriate control condition (e.g., low-fat diet used as control instead of standard diet, control group not exposed to vehicle, etc.) were excluded. Therefore, all control conditions belong to the following categories: (1) control strains, be them inbred or outbred, specific for those models that spontaneously exhibited variations in glucose metabolism/insulin signalling; (2) a corresponding control condition for the environmental manipulation (e.g. subjects exposed to standard diet for models based on dietary interventions); (3) wild-type or hemizygous mice as controls for genetically-engineered mice; (4) vehicle-

Fig. 2 The radial graph summarizes the studies assessed in the present review as a function of experimental subjects, experimental manipulations, methodologies adopted to confirm the presence of hyperglycaemia, and behavioural outcomes. Accordingly, each 90-degree quadrant describes all of the 91 studies contemplated in the review as a function of a different aspect. Moving from the centre to the periphery of the radial graph, each category is further subdivided into granular details. STZ: streptozotocin, HFD: high fat diet, MSG: monosodium glutamate, NIC: nicotinamide.

treated animals for the pharmacological modulation. In nine manuscripts (n: 18,21,44,58,60,64,67,68,70), the information on the type of non-hyperglycaemic control was unclear; we took this limitation into consideration during the assessment of the risk of bias.

While detailing the methodologies adopted to induce hyperglycaemia, we observed that 48 studies (n: 1,3,4,8,14-24,28,30-34,36-40,43,44,47,50,52,53,55,57,64,66,73,74,77,79,83,84,86-91) capitalized upon the administration of streptozotocin (STZ), a natural toxic agent capable of affecting the functionality of pancreatic β-cells. In some of them, the injection of STZ was associated with insulin to modulate the level of hyperglycaemia (Biessels et al., 1996) or to induce temporal fluctuations in blood glucose concentrations (H. Wang et al., 2019). The remaining studies that induced hyperglycaemia via a pharmacological approach used different drugs such as isoflurane/sevoflurane (Wu et al., 2014), monosodium glutamate (Jin et al., 2018; Madhavadas et al., 2016; Madhavadas and Subramanian, 2015), nicotinamide plus STZ (Kumar and Maqbool, 2020; Zhang et al., 2016), dexamethasone (Patel and Udayabanu, 2014) and alloxan (Diegues et al., 2014). With respect to environmental manipulations, the majority of studies fed mice with high fat diet (HFD) (n: 2,11,26,29,41,48,51,85) and/or moderate HFD (Arnold et al., 2014); a minority of studies exploited other approaches like psychological stress (Gu et al., 2017; Van Der Kooij et al., 2018a), drinking water supplemented with arsenic, aspartame or fructose (Collison et al., 2012; Rodríguez et al., 2016; Dharavath et al., 2019), administration of cookie pellets with clozapine (Babic et al., 2018), and glucose injection (Rejdak et al., 2001; H. Wang et al., 2019). Pharmacological and environmental manipulations to obtain hyperglycaemia were combined in nine studies (n: 12,42,54,58,60,67,68,69,80) considered in this review (STZ + HFD). One of them (W. Wang et al., 2019) adopted these two approaches in separated experimental groups (a group treated with STZ and a different one subjected to HFD). A genetic approach, as the independent variable responsible for hyperglycaemia, was exploited in eight articles: in particular, one study has been conducted on Irs2-deficient mice (Tanokashira et al., 2021), six on the Lepr $^{db/db}$ mouse (n: 25,56,65,71,75,81) and one on the Ins2C96Y Akita (Choeiri et al., 2005). Finally, three studies were conducted on strains that spontaneously exhibit diabetes-like abnormalities: Goto-Kakizaki and Zucker rats (Moreira et al., 2007; Skapare et al., 2012) and KKAy mice (Li et al., 2018).

Since the presence of hyperglycaemia was an inclusion criterion, we systematically evaluated how this has been assessed. Hyperglycaemia was ascertained in the vast majority of the studies, in comparison either with the control group (37 studies) or with a predefined threshold (50 articles). While most of the articles assessed glucose concentrations in the blood, one measured it in serum (Mirshekar et al., 2011) and another in urine (Baranowska et al., 2020). In two instances (Liu et al., 2022; Moreira et al., 2007) hyperglycaemia was not directly measured, and its presence was assumed based on the known characteristics of the experimental model (inbred strains). Two articles confirmed hyperglycaemia by assessing blood glucose concentrations but did not report the data (Joshi et al., 2021; H. Wang et al., 2019). Those articles that confirmed the presence of hyperglycaemia resting upon a predefined threshold, adopted a similar approach with a threshold ranging between 250 and 280 mg/dL in nine studies (n: 19,50,66,70,83,84,86,90,91); between 300 or 360 mg/dL in eight studies (n: 16,17,18,32,33,77,87,89); between 200 and 210 mg/dL in 12 studies (n: 1,4,21,28,34,38,39,40,45,47,58,79); and >7nmol/L (corresponding to 126 mg/dL) in 20 studies (n: 15,22-24,30,36,37,42,43,52,53,55,57,60,69,73,74,76,82,88); a single study (Hardigan et al., 2017) measured, as threshold, the glycated haemoglobin (HbA1c >8.0%). A variation of glucose/insulin metabolism, after the original validation of the hyperglycaemic state, was also detected during or at the end of the experimental schedule to confirm hyperglycaemia in most of the manuscripts (68 of the 91 reviewed articles).

Since blood glucose concentrations fluctuate as a function of the time elapsed between the last meal and its measurement, we deemed relevant evaluating whether and how fasting has been considered in the relevant articles. While in 39 articles glucose concentrations have been measured following a fasting period, in seven studies (n: 44,70,78,79,87,88,90) it was assessed without fasting. The remaining articles did not provide details regarding this parameter.

All the studies reported were conducted in mice (40 articles) or rats (51 articles). Mouse strains included: ICR mice (n: 14,15,33,36,37,55,67), Swiss mice (Patel and Udayabanu, 2014; Rejdak et al., 2001), C57BL/6J strain (n: 2,7,11,12,22,24,31,35,38,39,42,43,48,49,66,68,89), BALB/c (Noor and Zahid, 2017; Rajab et al., 2017), transgenic mice with mutations linked to Alzheimer's disease such as APP/PS1 (Yeh et al., 2015), 3×Tg-AD (Huang et al., 2019) and PS19 (Nakaoku et al., 2019), spontaneous diabetes-associated dysfunctions animal models such as KKAy mice (Li et al., 2018), Ins2^{C96Y} Akita mice (Choeiri et al., 2005), *Irs2*$^{-/-}$/6J mice (Tanokashira et al., 2021) and C57BLKS/J-lepr^{db}/lepr^{db} (n: 25,56,65,71,75,81). Studies conducted in rats used a transgenic line of non-obese model of T2DM like Goto-Kakizaki (Moreira et al., 2007; Skapare et al., 2012; H. Wang et al., 2019) and a rat model of genetic obesity, the Zucker $^{fa/fa}$ (Gu et al., 2017; Skapare et al., 2012), while the most common strain was Wistar (n: 1,2,14,16,17,18,20,24,27,28,32,42,43,49,56,57,69,75-77,82,85,87-91) followed by Sprague–Dawley (n: 1,5,9,10,23,30,34,44,47,51,53,60,61,63,73,74,80,88) and Long-Evans (Wirt et al., 2021).

Only eight articles used female subjects (n: 16,26,35,48,54,59,62,63), three of which (Collison et al., 2012; Patel and Udayabanu, 2014; Yeh et al., 2015) entailed a pool of males and females. All experimental subjects were adult at the time of behavioural and metabolic phenotyping. Only in three articles did the treatment to induce hyperglycaemia begin at a neonatal stage (Jin et al., 2018; Madhavadas et al., 2016; Madhavadas and Subramanian, 2015). Given that our aim was the comparison between the hyperglycaemic group and the relative controls, all the studies had a between-subjects study design; in one of them (Rodríguez et al., 2016), the behavioural and metabolic tests were performed in two independent groups with the same experimental treatment.

Hyperglycaemia and behavioural outcomes

The effects of hyperglycaemia on the behavioural parameters of interest (within each test) are illustrated in **Table 2**. The tables include, under separate headings, the direction of the variation of the behavioural phenotypes isomorphic to working memory, spatial memory, and attention, respectively. Most of the studies observed an impairment in the behavioural phenotype in the comparison between hyperglycaemic subjects and their relative controls; only one study (Dharavath et al., 2019a), conducted in female rats, reported an improvement in the spatial memory domain after 16 and 20 weeks of a HFD treatment lasting 24 weeks (although, at the end of treatment, they reported an impairment), and 11 papers described no significant difference between the two groups of interest for spatial (n: 7,8,12,17,22,39,42,43,46) and working memory (n: 13,46,63).

N	Ref.	OUTCOME and TEST to evaluate working memory	OUTCOME and TEST to evaluate spatial memory	OUTCOME and TEST to evaluate attention
1	(Georgy et al., 2013)	Passive avoidance test	Morris water maze test	n/a
2	(Arnold et al., 2014)	T-maze test	n/a	n/a
3	(Joshi et al., 2021)	n/a	T-maze test	n/a
4	(Remor et al., 2019)	Step-down inhibitory avoidance task	Morris water maze test	n/a
5	(Wu et al., 2014)	n/a	Morris water maze test	n/a
6	(Moreira et al., 2007)	lever press task (total presses)	lever press task (left versus right lever discrimination)	lever press task (active presses in FR2, FR3, FR5 and PR)
7	(Rodríguez et al., 2016)	n/a	*Morris water maze test*	n/a
8	(Biessels et al., 1996)	n/a	*Morris water maze test*	n/a
9	(Madhavadas et al., 2016)	n/a	Barnes maze test	n/a
10	(Madhavadas and Subramanian, 2015)	n/a	Barnes maze test	n/a
11	(Tanokashira et al., 2018)	Water T-maze	n/a	n/a
12	(W. Wang et al., 2019)	Water T-maze	*Water T-maze test*	n/a
13	(Tanokashira et al., 2021)	*Y-maze test*	Water T-maze test	n/a
14	(Du et al., 2014)	n/a	Morris water maze test	n/a
15	(Fang et al., 2017)	Morris water maze test	Y-maze test	n/a
16	(Tabatabaei et al., 2016)	n/a	Morris water maze test	n/a
17	(Rababa'h et al., 2019)	n/a	*Radial arm water maze test*	n/a
18	(Babri et al., 2013)	n/a	Morris water maze test	n/a
19	(Mirshekar et al., 2011)	Y-maze	n/a	n/a
20	(Baranowska et al., 2020)	n/a	Morris water maze test	n/a
21	(Utkan et al., 2015)	n/a	Morris water maze test	n/a
22	(Taylor et al., 2015)	n/a	Morris water maze test and *novel object recognition test*	n/a
23	(Yang et al., 2014)	n/a	Morris water maze test	n/a
24	(Momeni et al., 2021)	n/a	Barnes maze test and Morris water maze test	n/a
25	(de Cossío et al., 2017)	n/a	Y-maze test	n/a
26	(Dharavath et al., 2019a)	n/a	**Morris water maze test**	n/a
27	(Skapare et al., 2012)	Y-maze test	n/a	n/a
28	(Malone et al., 2008)	n/a	Radial water maze test	n/a
29	(Treviño et al., 2015)	n/a	Morris water maze test	n/a
30	(Nurdiana et al., 2017)	n/a	Morris water maze test	n/a
31	(Hardigan et al., 2017)	Y-maze test	n/a	n/a
32	(de Senna et al., 2017)	n/a	Place recognition test	n/a
33	(Wu et al., 2012)	n/a	Morris water maze test	n/a
34	(Lin et al., 2018)	Morris water maze test	Morris water maze test	n/a
35	(Collison et al., 2012)	n/a	Morris water maze test	n/a
36	(Zhou et al., 2015b)	n/a	Morris water maze test	n/a

(Zhou et al., 2017)	n/a	Morris water maze test	n/a
(Huang et al., 2012)	n/a	Morris water maze test	n/a
(Huang et al., 2007)	n/a	*Morris water maze test*	n/a
(Huang et al., 2019)	n/a	Morris water maze test	n/a
(Lin et al., 2017)	n/a	Morris water maze test	n/a
(He et al., 2020)	n/a	*Morris water maze test*	n/a
(Zhou et al., 2018)	n/a	*Morris water maze test*	n/a
(Sibiya and Mabandla, 2017)	n/a	Morris water maze test	n/a
(Kumar and Maqbool, 2020)	n/a	Morris water maze test	n/a
(Choeiri et al., 2005)	*Four-arm maze test*	*Morris water maze test*	n/a
(Marissal-Arvy et al., 2018)	n/a	Y-maze test	n/a
(Braga et al., 2021)	n/a	Object location test	n/a
(Van Der Kooij et al., 2018a)	n/a	Y-maze test and Object location task	n/a
(Bhutada et al., 2010)	n/a	Morris water maze test and Elevated plus maze learning task	n/a
(Pathan et al., 2008)	n/a	Morris water maze test	n/a
(Esmaeili et al., 2017)	n/a	Morris water maze test	n/a
(Ren et al., 2013)	n/a	Morris water maze test	n/a
(Yeh et al., 2015)	n/a	Morris water maze test	Nest construction
(Pei and Sun, 2018)	n/a	Morris water maze test	n/a
(Ye et al., 2018)	n/a	Morris water maze test	n/a
(Mao et al., 2008)	n/a	Morris water maze test	n/a
(T. H. Liu et al., 2020)	n/a	Morris water maze test	n/a
(Patel and Udayabanu, 2014)	n/a	Morris water maze test	
(J. Liu et al., 2020)	n/a	Morris water maze test	n/a
(Jin et al., 2018)	n/a	Barnes maze test and Morris water maze test	n/a
(H. Wang et al., 2019)	n/a	Morris water maze test	n/a
(Babic et al., 2018)	*T-Maze alternation test*	n/a	n/a
(Lee et al., 2014)	Morris water maze test	n/a	n/a
(Wu et al., 2020)	n/a	Morris water maze test	n/a
(Wang et al., 2015)	n/a	Morris water maze test	n/a
(Lee and Yang, 2019)	Y-maze test	n/a	n/a
(Ren et al., 2019)	n/a	Morris water maze test	n/a
(Noor and Zahid, 2017)	n/a	Morris water maze test	n/a
(Zhang et al., 2016)	n/a	Morris water maze test	n/a
(Li et al., 2012)	n/a	Barnes maze test	n/a
(Gu et al., 2017)	n/a	Morris water maze test	n/a
(Ahmed et al., 2020)	Morris water maze test	Morris water maze test	n/a
(Ahmed et al., 2019)	Morris water maze test	Morris water maze test	n/a
(Liu et al., 2022)	n/a	Morris water maze test	n/a
(Diegues et al., 2014)	n/a	Morris water maze test	n/a
(Kamsrijai et al., 2020)	n/a	Morris water maze test	n/a
(Rejdak et al., 2001)	Y-maze test	n/a	n/s
(Lazcano et al., 2014)	n/a	Morris water maze test	n/a
(Cai et al., 2020)	n/a	Morris water maze test	n/a
(Wen et al., 2020)	Y-maze test	n/a	n/a
(Li et al., 2018)	n/a	Morris water maze test	n/a
(Ahmadi et al., 2017)	n/a	Morris water maze test	n/a
(Wirt et al., 2021)	T-maze test	n/a	n/a
(Nakaoku et al., 2019)	n/a	Morris water maze test	n/a

86	(Delkhosh-Kasmaie et al., 2018)	n/a	Morris water maze test	n/a
87	(Lupien et al., 2003)	n/a	Morris water maze test	n/a
88	(Heng et al., 2011)	n/a	Morris water maze test	n/a
89	(Jash et al., 2020)	Y-maze test	Morris water maze test	n/a
90	(Rajab et al., 2017)	n/a	Morris water maze test	n/a
91	(Sharifzadeh et al., 2017)	n/a	Morris water maze test	n/a

Table 2. Behavioural test for each cognitive domain of interest and relative outcomes; bold text refers to an improvement and italic text refers to a no change in that phenotype, otherwise there was an impairment of the domain investigated.

Other considerations

Although the treatment therapies of hyperglycaemia were not the purpose of our review, some of the presented studies have also analysed the effects of several treatments. Therefore, while we are not able to provide a systematic reading of this aspect, we believe that these considerations may help analysing the predictive validity of animal models of diabetes (i.e., whether treatments used in our species are also effective in experimental models), in terms of changes in blood glucose concentrations and cognition. Although metformin is a first-line therapy for the treatment of diabetes, only in three articles (Delkhosh-Kasmaie et al., 2018; Li et al., 2012; Tanokashira et al., 2018) has it been used as a treatment for diabetes-associated dysregulation. In all of them, metformin ameliorates diabetes-associated decline in hippocampal neurogenesis, learning and memory. Several authors used alternative approaches such as herbal medicine (Mao et al., 2008; Mirshekar et al., 2011; Tabatabaei et al., 2016; Wu et al., 2012), dark chocolate (Madhavadas et al., 2016), prebiotic and probiotic (de Cossío et al., 2017; T. H. Liu et al., 2020) and physical exercise or a backward switch from HFD to a regular diet (Braga et al., 2021; de Senna et al., 2017). All these treatments ameliorated metabolic and cognitive dysfunctions related to the hyperglycaemic condition in the animal model. While metformin is a validated anti-diabetic drug, these alternative treatments clearly deserve special consideration on whether results can be translated to humans or not.

Sex differences constitute an additional important aspect that would warrant a systematic approach. While this aspect was not among the primary scope of our study, we can nonetheless provide some preliminary considerations. Of all the 91 studies considered, only eight included female subjects. In two of them, the authors pooled subjects of both sexes thus limiting the possibility to discern between males and females (see Table 1, n: 54,59). One study (see Table 1, n: 35) reported a direct comparison between males and females and observed gender-specific effects, with males more affected by hyperglycaemia than females. The remaining five articles (see Table 1, n: 16,26,48,62,63) presented data on female subjects only. One of them (see Table 2, n: 26) constitutes the only instance in which hyperglycaemia resulted in a temporary cognitive improvement: i.e. hyperglycaemia resulted in improved spatial memory 16 and 20 weeks after the beginning of a high fat diet, and in impaired spatial memory four weeks later. The other four studies either reported a general impairment (see Table 2, n: 16,48,62) or lack of differences (see Table 2, n: 63) as a function of hyperglycaemia. While these articles partly reverberate the findings observed in the studies conducted in males, their scant numerosity poses some caveats as to whether the findings reported in the majority of studies (male-biased) may translate to females.

Risk of bias (RoB)

The risk of bias assessment of all included studies is shown in **Figure 3**. The assessment of RoB included 91 articles, for which often the experimental details were only partly reported (Avey et al., 2016). This resulted in an overall unclear risk of bias (52.09%). Yet, when data were correctly reported, there was a generally low risk of bias based on SYRCLE's RoB tool (33.19%), with a limited percentage of high risk of bias (5.27%). The judgement "not applicable" resulted in a 9.45% overall risk of bias but it was only influenced by the "Reporting bias" (Hooijmans et al., 2014).

General discussion

The primary purpose of the present systematic review was to identify whether experimental rats and mice characterized by hyperglycaemia also exhibit behavioural abnormalities in the domains of working and spatial memory, and attention. The studies of interest are characterized by hyperglycaemia, which has been induced via different methodologies, be them pharmacological interventions, environmental modulations, transgenic approaches, naturally occurring mutations based on strain differences, or a combination of them. All of them allow the analysis of mechanisms related to diabetes and are important to understand the pathogenesis and progression of the disease as well as to evaluate potential therapeutic strategies with an elevated translational value. Accordingly, an animal model relevant for the study of diabetes, shall mirror the pathophysiology and natural course of diabetes, and/or it should develop complications of the disease with an aetiology similar to the human condition (Varga et al., 2015). These considerations are particularly relevant to diabetes, which is characterised by multiple facets and different main diagnoses: T1DM is an autoimmune disease in which pancreatic β-cells are targeted to be destroyed by antibodies produced by immune cells (Gillespie, 2006). In contrast, the pancreatic β-cells are active in T2DM and synthesize insulin but at dysregulated level and/or not sufficiently efficiently (Brunton, 2016). Chronically elevated blood glucose concentrations represent a commonality in T1DM or T2DM. Since T1DM is characterized by the deficiency of insulin production, the deficit is achieved in experimental animals through chemical destruction of pancreatic β-cells or through breeding of rodents that spontaneously develop diabetes.

However, although the endpoint of β-cell destruction is similar to T1DM in humans, the mechanism for the β-cell destruction is not autoimmune; therefore the aetiology differs from the human condition. On the other hand, T2DM animal models should

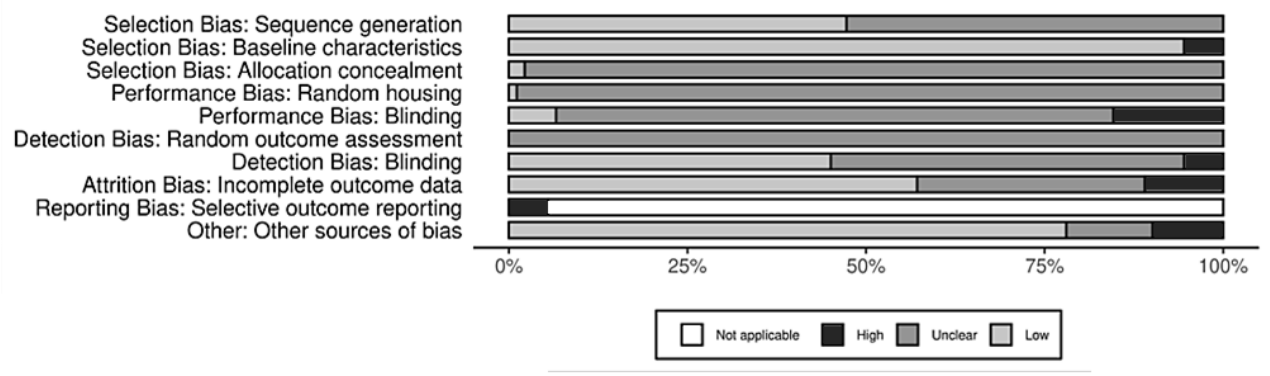

Fig.3 Risk of bias assessment, score (%) per risk of bias item. The RoB tool for animal studies is divided into 10 items. "Random outcome assessment" bias was considered "Unclear" because the details on the sequence of animal testing were never reported. "Reporting bias" was judged as "not applicable" for all the studies published before 2021. In this respect it should be noted that the "Reporting bias" item was prospectively included in the SYRCLE's tool (in agreement with the Cochrane's tool) although at present it is difficult to assess, as protocols for animal studies are not yet mandatorily registered in central, publicly accessible databases (Hooijmans et al., 2014; Olevska et al., 2021).

recapitulate insulin resistance, a certain degree of β-cell failure, and obesity. It appears that no single animal model involves all of these characteristics, but some of them could provide very similar traits in one or more aspects of diabetes in humans.

Although we selected the relevant studies based on hyperglycaemia, our primary interest was the role of insulin signalling. Therefore, an important prerequisite is that hyperglycaemia constitutes a valid proxy of altered insulin signalling. To assess this prerequisite, we first discuss the extent to which the experimental models considered in this review adequately mimic diabetes. Following this examination, we proceed with the evaluation of the association between diabetes and cognitive alterations. Finally, we interpret the observed results as a function of their risk of bias.

Pharmacological induction of hyperglycaemia

Some experimental models utilise a chemical approach to induce diabetes, particularly in the form of diabetogenic agents such as streptozotocin and alloxan. Alloxan is a toxic glucose analogue whose accumulation in pancreatic β-cells inhibits insulin secretion and induces reactive oxygen species formation that are ultimately responsible for the death of the cells (Cefalu, 2006). Streptozotocin is a highly selective pancreatic islet β-cell-cytotoxic agent and inhibits insulin secretion causing a state of insulin-dependent diabetes mellitus (Lenzen, 2008). It is often administered at a single high dose to produce an immediate blood glucose concentration >500 mg/dL and to cause β-cell total necrosis (Cefalu, 2006). However, lower doses of STZ administered multiple times, are capable to delay the onset of hyperglycaemia with a partial damage of pancreatic islets. This slower process triggers an inflammatory process that causes an additional loss of β-cells, which, in turn, results in insulin deficiency, hyperglycaemia, polydipsia, and polyuria (Radenković et al., 2016). This response more closely resembles T1DM in pathogenesis and morphologic changes than the single, high-dose of STZ (Furman, 2015). Another protocol of diabetes induction entails the concurrent administration of STZ and nicotinamide, wherein the latter partially protects the β-cells damage induced by the former (Fukaya et al., 2013; Szkudelski, 2012). This combination produces a model of insulin-deficient, but not insulin-resistant T2DM that is a major feature of most human cases. It is characterized by stable, moderate hyperglycaemia associated with an approximately 60% loss of β-cell function.

Although most of the reviewed articles used models of diabetes (see **Table 1**), our interest was directed towards any type of manipulation capable of inducing hyperglycaemia. Some scholars used different drugs to achieve this goal or to study the behavioural effects of glucose dysregulation. One of these studies reported that isoflurane and sevoflurane (Wu et al., 2014) may impair glucose tolerance by decreasing insulin secretion and glucose utilization. An additional pharmacological approach was constituted by the use of monosodium glutamate (MSG), that is frequently used as a flavour enhancer in the food industry. Some evidence indicate that MSG treatment in animals, during the first day of life, leads to the development of obesity and hyperglycaemia in adulthood (Bahadoran et al., 2019; Dolnikoff et al., 2001). Although these observations suggest that MSG may constitute a useful agent for the induction of T2DM-like abnormalities in animals, the underlying mechanisms are not completely understood. As one of the reviewed articles showed, chronic dexamethasone administration may induce diabetes-related metabolic dysfunctions in animal models (Patel and Udayabanu, 2014). Dexamethasone represents a first-line anti-inflammatory drug. Unfortunately, long-term therapy is associated with metabolic side effects, including hyperglycaemia, hypertension, and hepatic steatosis that contribute to insulin resistance and diabetes (Cefalu, 2006). Additionally, one manuscript used an antipsychotic drug, clozapine. While it generally improves the cognitive symptoms of schizophrenia, it can cause serious metabolic side-effects (Siskind et al., 2016), likely mediated by its impact on glucagon-like peptide (Siskind

et al., 2019). Usually, all these pharmacological models are invaluable when studying the mechanisms by which hyperglycaemia may contribute to microvascular complications such as neuropathy, nephropathy, and retinopathy. However, because they may be toxic to organs and tissues other than the pancreatic islet β-cells, these models do not precisely mimic the human condition (Zhang et al., 2008). Chemically-induced models present a phenotype that closely resembles that observed in T1DM patients; yet, the mechanisms underlying β-cell damage are different from the human disease. Thus, while the construct validity of these models may be limited, their predictive validity is highly relevant.

Environmental induction of hyperglycaemia

To mimic the nutritional determinants of T2DM, several authors adopted a differential strategy entailing the administration of HFD. In these instances, experimental subjects are exposed to an HFD nutritional regime to induce insulin resistance. This regime has been sometimes associated with the concurrent administration of a moderate dose of STZ to reduce β-cell capacity (Reed et al., 2000), a combination resulting in hyperglycaemia, hyperinsulinemia and insulin resistance (Yorek, 2016). The use of HFD to induce insulin resistance and to produce mild or moderate insulin deficiency may represent a valid experimental model of T2DM, whereby it can provide relevant information regarding many of the complications associated with human diabetes. HFD has also been utilized to model chronic inflammation, which is an important pathogenic mechanism of T2DM. Chronic overfeeding triggers inflammation, which leads to alterations in peripheral insulin receptor-associated signalling and thus reduces the sensitivity to insulin-mediated glucose clearance. These events ultimately result in elevated fasting glucose and insulin concentrations as well as in a reduction in glucose tolerance, all of which constitute relevant indicators of insulin resistance (Heydemann, 2016; Nagy and Einwallner, 2018). Moreover, a long-term HFD induces metabolic disorders, oxidation, inflammation, changes in islet size, and irregular secretory functions in the pancreas (Wu et al., 2022; Zhao et al., 2022). A strong evidence supporting HFD as a valid methodology to reproduce the complications associated with T2DM is the fact that it induces telencephalic insulin resistance associated with systemic hyperglycaemia (Cefalu, 2006). This is often associated with a chronic hyperactivation of cortical and hippocampal neurons which may ultimately predispose toward cognitive impairments.

An alternative manipulation adopted to modulate metabolic functions capitalised upon psychosocial stress as a strategy to induce hyperglycaemia. Specifically, chronic psychosocial stress in mice has been reported to increase peripheral and central glucose concentrations and, subsequently, to relate to the emergence of stress-induced cognitive impairments (Van Der Kooij et al., 2018b). It has been suggested that psychosocial and metabolic stress share common underlying mechanisms with glucose dysregulation having a central role. These findings have been associated with the metabolic consequences of environmental stressors in our species, wherein chronic stressors, low socioeconomic status, severe mental health problems, or aggressive behaviour have been shown to increase the risk of T2DM (Hackett and Steptoe, 2016; Winchester et al., 2016). Accordingly, animal models have shown that exposure to experimental stressors may anticipate the onset of chronic subclinical inflammation (Black, 2003). Additionally, animal models that mimic T2DM and T2DM-related metabolic conditions suggest that insulin resistance may lead to chronic inflammation, which may in turn induce cognitive decline (Kelly and Ismail, 2015). It remains to be determined, however, whether central glucose dysregulation is linked to stress-induced cognitive impairments or whether abnormal glucose metabolism may contribute to individual susceptibility to the adverse consequences of chronic stress.

Genetic determinants of hyperglycaemia

Animal models with naturally occurring mutations have traditionally constituted a unique resource potentially mimicking the construct validity of the disease whereby: (i) they become spontaneously diabetic; and (ii) the course of the disease is markedly influenced by genetic background. The Lepr $^{db/db}$ mice represent one of the most widely studied genetically-induced experimental models of diabetes. They are homozygous for the spontaneous mutation of the leptin receptor (Lepr) that is involved in food intake, energy expenditure, and body weight (Berger et al., 2021). Mice carrying this spontaneous mutation exhibit obesity, chronic hyperglycaemia, pancreatic β-cell atrophy, and hypoinsulinemia. Mutations in leptin receptor have been shown to cause early-onset severe obesity and insulin resistance in mice and humans (Wang et al., 2014). While, in humans, it is difficult to disentangle whether insulin resistance precedes or is secondary to the development of obesity, in mice, the temporal association of these symptoms can be prospectively investigated. Studies conducted in Lepr $^{db/db}$ mice seem to suggest that the onset of insulin resistance anticipates the onset of obesity. Thus, Lepr $^{db/db}$ mice have a natural history of the disease similar to that observed in humans whereby they become hyperinsulinemic early in life (within 2 weeks of age) and develop obesity by 3 to 4 weeks. Hyperglycaemia, associated with a β-cell failure, becomes manifest at age 4 to 8 weeks (Bates et al., 2005); this is followed by a compensatory hyperplasia of the islet of Langerhans which keeps being associated with hyperinsulinemia until the latest stages of life (18 to 20 months)("000697 - B6 db Strain Details"). Another genetically engineered diabetic and obese model is the KKay mouse. This mouse was generated by transferring the Ay gene (conferring these mice an unusual yellow coat colour) onto a glucose-intolerant mouse strain (KK). While mice of the KK strain develop diabetes of polygenic origin, the Ay mutation leads to obesity as a function of the *agouti* protein being expressed in incorrect locations ("002468 - Strain Details"). By approximately two months of age, due to insulin resistance, KKay mice develop the following diabetes-like abnormalities: hyperglycaemia, hyperinsulinemia, glucose intolerance, and obesity. The KKAy mice have excessively large pancreatic islets and degranulated pancreatic β-cells. Their obesity is partially due to fat cell hypertrophy, caused by a drop in dopamine and noradrenaline in the hypothalamus ("The Characteristics of KKAy Mice - Maze Engineers"). Due to these features, these mice resemble both the early (β-cells impairment in the pancreas and hyperglycaemia)

and the late stages of diabetes (β-cells can no longer release insulin and insulin replacement therapy is required).

The Akita strain ("003548 - Akita Strain Details") constitutes an additional experimental model of spontaneous diabetes. These mice are characterised by a mutation at the level of the Ins2 gene, one of the two genes encoding for insulin in mice (the second one being Ins1). A mutation in the Ins2 gene leads to incorrect folding of the insulin protein, which in turn results in toxicity in pancreatic β-cells, reducing β-cell mass and insulin secretion. Heterozygous Ins2Akita mice develop insulin dependent diabetes, including hyperglycaemia, hypoinsulinemia, polydipsia, and polyuria within four weeks of age, thus representing a valid experimental model of T1DM. Just as in humans men are more often affected than women, so also in rodents diabetes-like abnormalities appear more frequent in males than in females (Tramunt et al., 2020). Accordingly, in Akita mice, the phenotype is more severe in males than females ("003548 - Akita Strain Details").

Other authors engineered mouse models based on different components of the insulin signalling pathways. Specifically, Tanokashira and collaborators (Tanokashira et al., 2021) capitalised upon the role of the insulin receptor substrate-2 (Irs-2), which plays a fundamental role in metabolism and growth of every tissue. Irs2 knockout mice exhibit a progressive development of a T2DM-like phenotype: while they show hyperglycaemia as early as three days of age, they become diabetic by 10 weeks when they exhibit reduced β-cell mass and insulin resistance in skeletal muscle and liver ("004421 - IRS-2 KO Strain Details").

Diabetic rats also represent an important research tool. For example, the Zucker diabetic fatty rat (ZDF) is usually used as a model for the study of T2DM associated with obesity ("Zucker Rat | Charles River"). Like db/db mice, ZDF rats present a mutation on the leptin receptor, which induces obesity and hyperglycaemia within the first few months of age. The diabetic like features exhibited by ZDF rats appear to be associated with an inability to increase β-cell mass; this results in an insufficient insulin secretion, which ultimately fails to compensate for the obesity-dependent insulin resistance (Clark et al., 1983). Thus, although there are similarities between the human condition and the abnormalities exhibited by ZDF rats, the latter may be characterised by limited degree of construct validity whereby humans with T2DM do not have inadequate β-cell proliferation in early life (Garnett et al., 2005). The Goto-Katazaki (GK) (Guest, 2019) rat is another model used for the study of diabetes. The GK rat is non-obese, has a decreased β-cell mass, and is characterised by liver and skeletal muscle-insulin resistance. Due to impaired insulin secretion, fasting blood glucose concentrations are also slightly increased. Disease progression of this rat has been associated with chronic inflammation and hence utilized in the study of pathophysiology and therapeutic studies of diabetes (Xue et al., 2011). An excess adipose tissue is linked to chronic inflammation as a consequence of the attraction of macrophages in the adipose tissue. It is this increased macrophage infiltration that in part suggests a link between obesity, inflammation and the development of diabetes (Surmi and Hasty, 2008).

Hyperglycaemia as a comorbidity in experimental models of cognitive disturbances

While most of the reviewed studies exploited experimental models of metabolic disturbances induced via alterations at some level of the insulin signalling pathways, some others capitalised upon the known association between T2DM and cognitive impairments and late-onset AD (Hamzé et al., 2022; Kandimalla et al., 2017; Pardeshi et al., 2017; Watanabe et al., 2015). These pathologies have been reported to share several pathophysiological features and common risk factors (Devi et al., 2012; Niedowicz et al., 2014). In mice without pre-existing AD-like symptoms, the induction of diabetes (genetically, pharmacologically or by diet) is associated with an hyperphosphorylation of tau protein (Li et al., 2007; Son et al., 2012). Complementarily, some of the articles reviewed herein used transgenic mice with mutations linked to Alzheimer's disease such as APP/PS1 (Yeh et al., 2015), 3×Tg-AD (Huang et al., 2019) and PS19 (Nakaoku et al., 2019) and observed that they became hyperglycaemic.

Ultimately, although there is not a single animal model recapitulating all the causative factors and associated phenotypic abnormalities observed in our species, the comprehensive consideration of available literature strongly supports the notion that preclinical studies may beget relevant information in the understanding and therapy of insulin-related somatic and mental abnormalities.

Test paradigms to confirm the presence of hyperglycaemia in experimental models

In all of the screened studies, the presence of hyperglycaemia has been revealed via multiple methodologies that differed in terms of reference values, control groups, timing of evaluation, sample collection, and presence of fasting. Therefore, while these differential approaches captured a welcome heterogeneity in the study of complex phenomena, they nonetheless reduced the capability to cross compare different studies. Within this realm, some considerations may be sensible: for example, the use of the same approach to test blood glucose concentrations for both control and treated groups (e.g., either a non-fasting state or a fasting state) shall benefit this field of investigation; likewise, a clear definition of hyperglycaemia shall be useful. Indeed, while the threshold of hyperglycaemia in our review has not always been constant and unique, the spectrum of thresholds used is close to what happens in our species. Generally, the blood glucose concentration threshold was >250 mg/dL in non-fasting conditions and >150 mg/dL in fasting conditions. One important benefit of a standardized reference value is the fact that it may align and adjust the experimental techniques across laboratories thus favouring the reproducibility and external validity of these studies.

Test paradigms to confirm the presence of cognitive impairments in experimental models

A different consideration pertains to the test paradigms required to assess individual cognitive capabilities, for which different methodologies exist and that for which a univocal standard is neither feasible nor necessarily advisable. As mentioned above, the main aim of this review was to assess whether

hyperglycaemic rodents exhibited alterations in working and spatial memory, and attention. Of these parameters, spatial memory has received the highest level of interest by this scientific field. This is substantiated by the fact that of the 91 studies, nearly all investigated this phenotype and all of them reported consistent impairments. It is important to emphasize that spatial memory has been addressed via different experimental paradigms (i.e. Morris water maze, Barnes maze, Novel object recognition, T- Y- and Radial-arm-maze). The convergence of results adopting different methodologies, both in terms of inducing hyperglycaemia and the behavioural readout employed, strengthens the association between hyperglycaemia and spatial memory, whereby it has been observed under heterogeneous conditions (Richter et al., 2010). As discussed elsewhere, heterogenized experiments have been reported to yield more stable results and to be characterized by a reduced number of false positives compared to homogenized experiments (Macrì and Richter, 2015). These findings support the view that alternative experimental strategies may indeed enhance the reproducibility and translational value of preclinical animal research.

Insulin signalling and cognition: candidate biological determinants

As we reviewed, hyperglycaemia – as a proxy of insulin-related metabolic dysfunctions – led to consistent impairments in the cognitive domains of interest. There are strong preclinical, epidemiological and clinical evidence (Biessels et al., 2008; Rom et al., 2019; Van Den Berg et al., 2010; Zhang et al., 2021) in support of the association between diabetes and cognitive dysfunctions, which may concern one or different domains, including processing speed, executive function, learning and memory (Arnoriaga-Rodríguez et al., 2020; Backeström et al., 2021; Omladič et al., 2020; Sadanand et al., 2016; Sattar et al., 2017). Diabetes can be viewed as a metabolic disorder resulting in accelerated cognitive ageing in terms of dementia and cognitive decline. Ultimately, diabetes-related cognitive impairments may be viewed as another long-term complication of diabetes. There is also evidence suggesting that hyperglycaemia *per se* has detrimental effects on cognitive function, whereby acute hyperglycaemia has been associated with poor cognitive outcomes, potentially as a function of accumulation of reactive oxygen species in the brain.

People with long-standing diabetes and no other diabetes-related complications have poorer working memory (Awad et al., 2017; Gallardo-Moreno et al., 2022). This finding has been related to the fact that hyperglycaemia *per se* may induce structural abnormalities in the prefrontal cortex (Lyoo et al., 2013), a brain region involved in working memory. Additionally, hyperglycaemia has been associated with changes in different regions of the brain, including the hippocampus (Nevo-Shenker and Shalitin, 2021). Accordingly, hyperglycaemia, in young patients with T1DM, has been shown to influence long-delay spatial memory months after that first diagnosis (Semenkovich et al., 2016). Likewise, changes in hippocampal synaptic plasticity and subsequent impairments in spatial memory, are well documented in animal models of T1DM and T2DM (Soares et al., 2013). Whereas numerous clinical studies (Broadley et al., 2017; Pappas et al., 2019; Redondo et al., 2016; Sattar et al., 2017; Zhao et al., 2020) have demonstrated a correlation between executive functions and diabetes, only in two articles of the present review has this domain been investigated. Thus, further preclinical studies are needed to elucidate the mechanism underlying the association between diabetes and executive function.

A number of possible mechanisms to explain the association between dysglycaemia, hyperglycaemia and cognitive dysfunction have been suggested. As reported above, one hypothesis is that chronic exposure to elevated glucose concentrations may accelerate cognitive decline (Awad et al., 2004; Messier et al., 2011). Moreover, impaired insulin signalling in the brain may represent a highly promising research avenue. Specifically, while having an important function in glucose transport, the highly abundant insulin receptors in the brain have been implicated in cognitive processes. Several observations suggest that cognitive decline is a consequence of insufficient insulin action in the brain, either due to insulin resistance, insulin deficiency or both. Insulin signalling in the brain has important roles in brain physiology and cognition (Biessels and Reagan, 2015).

Caveats associated with risk of bias

In order to evaluate the degree of confidence with which the conclusions of our work can be extrapolated on a large scale, we carefully assessed the RoB associated with several methodological aspects. Had we identified a widespread elevated RoB, our considerations would be substantially devalued. Yet, the elevated RoB was observed only in a fraction of the domains relevant to the scopes of our review (detailed in the following lines). Specifically, according to SYRCLE's protocol, the overall high RoB identified in the studies considered in the present review was 5.27%. This value was computed as the average of the instances (percent) in which we identified an elevated RoB for a given category. In particular, we observed instances of elevated RoB in the following items: (1) "Baseline characteristics": while we considered hyperglycaemia as a baseline value of interest, two articles of the review did not explicitly report whether this prerequisite had been met but rather referred to the already available evidence that the animal model of interest was characterised by hyperglycaemia; (2) "Performance bias: blinding" and "Detection bias: blinding": in few instances we observed that blinding could not be guaranteed whereby the individual who planned the study also performed the experiments and analysed the data; (3) "Attrition bias: incomplete outcome data": with respect to this item, we observed that few studies failed to report in the analysis all the experimental subjects used in the experiment without providing an explicit explanation for these attrition rates; (4) "Other source of bias": we took into account studies in which the solution administered to the control group (vehicle) was not specified; (5) "Reporting bias": with respect to this parameter, we attempted to identify whether the experiments reported in the published experiments matched those that were officially planned/registered. In this specific case, the analysis was limited to the articles published after 2021, when the Animal Study Registry (Olevska et al., 2021) a platform wherein animal studies can be registered) has been launched. We note, however, that this

parameter is difficult to assess whereby protocols for animal studies are not yet mandatorily registered in central, publicly accessible database (Olevska et al., 2021). As a consequence, be it due to the absence of enforcement and/or limited availability of repositories, none of the studies considered in the present review had been previously registered. We believe that this aspect warrants particular consideration by the preclinical scientific community. As a matter of fact, while on the one hand we deem this aspect sufficiently relevant to be included in the SYRCLE's protocol, on the other hand, we apparently have limited interest and tools to implement it on a large scale.

An additional warning to our community can also be derived from the unclear overall risk of bias, which in the present review attained a value of 52.09%. While an unclear risk of bias does not directly denote inappropriate study planning/execution/reporting, it nonetheless hinders the possibility to thoroughly grasp its fundamental details. We posit that the reporting of methodological details in animal studies shall considerably improve, and that the promotion of high-quality standards for registration and reporting of animal studies shall represent a desired goal in preclinical research.

Conclusions

Our systematic review strongly supports the view that hyperglycaemia in experimental models of metabolic dysfunctions is associated with cognitive impairments. It is thus plausible that hyperglycaemia, as a core feature of diabetes, disturbing insulin signalling and favouring insulin resistance, not only affects systemic metabolism, but also directly impacts the brain, by disturbing cerebral insulin pathways and the associated cognitive functions. Complementarily, although this was not the core aim of our study, hyperglycaemia has been observed in experimental subjects that were originally selected based on their known cognitive impairments and not on their metabolism. Therefore, just as hyperglycaemia may constitute a risk factor for cognitive impairments, so also the latter can influence the former, ultimately binding diabetes and cognition in a recurrent

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N	Ref.	Type of experimental manipulation adopted to induce hyperglycaemia		Details regarding the experimental manipulation	Details on the assessment of hyperglycaemia	Variation of glucose metabolism-/insulin signalling related parameters	Species	Strain	Sex [f; m]	Age and/or weight at the beginning of the study	N of subjects per group {n for behavioural parameters; n for glucose metabolism/ insulin parameters}
1	(Georgy et al., 2013)	pharmacological modulation	STZ	single i.p. injection of 40 mg/kg streptozotocin; 4 weeks of diabetes induction by STZ before testing	higher glucose concentrations compared to predefined threshold (> 210 mg/dL); with fasting	increase in serum glucose (315.6 vs 104.1 mg/dL) in STZ compared to CTRL at the end of the experiment (i.e., 8 weeks after the initial STZ administration)	rat	Sprague Dawley	m	200-240 g	20-22; n/a
2	(Arnold et al., 2014)	environmental manipulation	HFD	extreme high fat diet (60% kcal from fat) for 17 days and moderate HFD 45% kcal fat diet for 8 weeks	higher morning glucose concentrations both in extreme HFD at 17 days (HFD 210.4 mg/dL, CTRL 167.2) and moderate HFD (biweekly between 08:00 and 10:00) compared to control	glucose tolerance tests (performed at 8 weeks and 4 days) were markedly abnormal indicating diabetes in HFD, compared to CTRL	mouse	C57BL/6J	m	8 weeks old	5, 5, 10, 10; 5, 5, 10, 10
3	(Joshi et al., 2021)	pharmacological modulation	STZ	single intra-femoral injection of 50 mg/kg of streptozotocin	n/a	n/a	rat	Wistar	m	8–10 weeks old, 250–300 g	12; n/a
4	(Remor et al., 2019)	pharmacological modulation	STZ	single i.p. injection of 55 mg/kg of streptozotocin after a fasting period of 14 h; 10 and/or 60 days of diabetes induction before testing	higher glucose concentrations compared to predefined threshold (> 200 mg/dL) assessed 4 days after STZ injection	increase in serum glucose in STZ compared to CTRL at 15, 45 and 60 days after STZ injection: nondetectable plasma insulin levels in fasted STZ	rat	Wistar	m	60 days of life, 250–300 g	10; 7-9
5	(Wu et al., 2014)	pharmacological modulation	IS-SS	1.7% isoflurane (IS) or 2.4% sevoflurane (SS) for 4 hours; 2 weeks of hyperglycaemia induction by anaesthesia before testing	higher glucose concentrations compared to controls (109.9 mg/dL) in IS (189.2 mg/dL) and in SS (174.1 mg/dL) respectively after 2 hours and 1 hour of anaesthesia	higher glucose concentrations compared to controls (109.9 mg/dL) in IS (169.9 mg/dL) after 4 hours of anaesthesia and in SS (172.9 mg/dL) after 3 hours and (185.0 mg/dL) after 4 hours of anaesthesia	rat	Sprague Dawley	m	PND 14	17, 35, 37; n/a
6	(Moreira et al., 2007)	strain difference	Goto-Kakizaki	no manipulation, spontaneously diabetic	n/a	n/a	rat	Wistar, Goto-Kakizaki	m	16 weeks old, 280–350 g	8,6; n/a

7	(Rodríguez et al., 2016)	environmental manipulation	altered drinking water with arsenic	50 mg of inorganic arsenic (iAs)/L of drinking water for three months	higher glucose concentrations compared to controls; with fasting (12 hours), assessed at second month of iAs treatment	increased in blood glucose levels at 30 min after an i.p. injection of 2 grams of glucose/kg, in the intraperitoneal glucose tolerance test	mouse	C57BL/6	m	35 g	11; 12
8	(Biessels et al., 1996)	pharmacological modulation	STZ	experiment 1: single intravenous injection of 40 mg/kg of streptozotocin; experiment 2: single intravenous injection of 40 mg/kg of streptozotocin + subcutaneous release insulin implants at a dose of 1 IU per day	blood glucose levels >15.0 mmol/1 in all STZ-injected animals, 4 days after the injection	experiment 1: increase in blood glucose levels (25.6 vs 5.5 mmol/1) in STZ compared to CTRL at the end of the experiment; experiment 2: increase in blood glucose levels (18.9 vs 5.6 mmol/1) in STZ compared to CTRL at the end of the experiment	rat	Wistar	m	300 g	Experiment 1: 10; 10 Experiment 2: 10; 10
9	(Madhavadas et al., 2016)	pharmacological modulation	MSG	neonatal rat pups were injected with MSG (4 mg/g body weight) once daily for 14 consecutive days after birth then tested at 17 months of age	higher blood glucose concentrations compared to controls	after three months of neonatal MSG administration, it was an increase in glucose levels compared to CTRL	rat	Sprague-Dawley	m	neonatal	8; 8
10	(Madhavadas and Subramanian, 2015)	pharmacological modulation	MSG	neonatal rat pups were injected with MSG (4 mg/g body weight) once daily for 14 consecutive days after birth then tested at 18 months of age	higher blood glucose concentrations compared to controls; fasting not specified; at 18 months of age	at 18 months old serum glucose levels of MSG mice were higher than CTRL (313 vs 122.5 mg/dL)	rat	Sprague-Dawley	m	neonatal	6; 6
11	(Tanokashira et al., 2018)	environmental manipulation	HFD	HFD (60% kcal from fat) from 4 to 29 weeks of age	higher blood glucose concentrations compared to controls; with fasting (6 hrs)	elevated levels of fasting glucose and plasma insulin compared to CTRL at 25 weeks of age	mouse	C57BL/6J	m	4 weeks old	8; 12
12	(W. Wang et al., 2019)	pharmacological modulation, environmental manipulation	STZ, HFD	DIO mice: HFD (60% kcal from fat) for 32 weeks. C57BL/6J mice: single i.p. injection of 150 mg/kg of streptozotocin, after an overnight fasting. Test performed at 34-36 week (HFD) and 10 weeks (STZ)	HFD mice: higher blood glucose concentrations compared to controls, at 35 weeks of age; STZ mice: higher blood glucose concentrations compared to controls, after 2 weeks of STZ injection	at 35 weeks of age blood glucose concentration of HFD mice were higher compared to CTRL; after 2 weeks of STZ injection, STZ mice exhibited elevated blood glucose levels (>400 mg/dL).	mouse	C57BL/6J	m	HFD: 4 weeks old; STZ: 10 weeks old	HFD: 14; 5 STZ: 18-19; 5
13	(Tanokashira et al., 2021)	transgenic approach	Irs2-deficient mice	generation and routine genotyping of the Irs2-deficient mice maintained on a C57BL/6J genetic background after more than six backcrosses	higher blood glucose concentrations compared to controls	$Irs2^{-/-}$/6J males developed hyperglycaemia after 9 weeks, the $Irs2^{-/-}$/6J males displayed higher fasting insulin levels and insulin resistance during the insulin tolerance test compared to controls	mouse	$Irs2^{-/-}$/6J, C57BL/6J	m	4 weeks old	10-15; 8-11

14	(Du et al., 2014)	pharmacological modulation	STZ	single i.v. injection of 150 mg/kg of streptozotocin	higher blood glucose concentrations compared to controls	increase in blood glucose levels (18.21 vs 6.71 mM) and decreased serum insulin levels (5.36 vs 14.21 mIU/L) in STZ compared to CTRL	mouse	ICR	m	8-10 weeks old; 20-25 g	7-8; 8-10
15	(Fang et al., 2017)	pharmacological modulation	STZ	i.v. injection of 150 mg/kg of streptozotocin	higher blood glucose concentrations compared to predefined threshold (>11.0 mmol/L)	decrease in serum insulin in STZ mice compared to CTRL (17.41 vs 36.20 mIU/L)	mouse	ICR	m	22-25 g	8; 7
16	(Tabatabaei et al., 2016)	pharmacological modulation	STZ	i.p. injection of 60 mg/kg of streptozotocin. All females were ovariectomized	higher blood sugar concentrations compared to predefined threshold (>300 mg/dL)	significant increase in Fasting blood sugar in STZ mice compared to CTRL	rat	Wistar	f	190-200 g	10; 10
17	(Rababa'h et al., 2019)	pharmacological modulation	STZ	single i.p injection of 50 mg/kg of streptozotocin	higher blood glucose concentrations compared to predefined threshold (≥ 300 mg/dL); with fasting	increase in serum glucose level in STZ mice compared to their corresponding control	rat	Wistar	m	200-300 g	9-10; 9-10
18	(Babri et al., 2013)	pharmacological modulation	STZ	single i.p. injection of 60 mg/kg of streptozotocin	higher blood glucose concentrations compared to predefined threshold (> 300 mg/dL); with fasting	n/a	rat	Wistar	m	250-300 g	7; n/a
19	(Mirshekar et al., 2011)	pharmacological modulation	STZ	single i.p. injection of 60 mg kg of streptozotocin	higher serum glucose concentrations compared to predefined threshold (> 250 mg/dL)	diabetic rats had an elevated serum glucose level compared to control rats after 4 and 8 weeks of treatment	rat	Wistar	m	10-12 weeks old; 215-285 g	8; 8
20	(Baranowska et al., 2020)	pharmacological modulation	STZ	single i.p. injection of 65 mg/kg of streptozotocin	higher urine glucose concentrations compared to controls	increase in plasma glucose concentration in STZ rats compared to CTRL (440±65 vs. 132±16 mg/dL)	rat	Wistar	m	200 g	10, 11; 10, 11
21	(Utkan et al., 2015)	pharmacological modulation	STZ	single i.p. injection of 50 mg/kg of streptozotocin, STZ-treated rats received 5% glucose solution instead of water for the next 24 h to reduce the death risk due to hypoglycaemic shock	higher blood glucose concentrations compared to predefined threshold (> 200 mg/dL); with fasting	plasma levels of glucose in diabetic groups were significantly higher than those in control (424.4±15.6 vs 115.7±6.1 mg/dL)	rat	Wistar	m	250- 300 g	7; 7

22	(Taylor et al., 2015)	pharmacological modulation	STZ	single i.p. injection of 100 mg/kg, of streptozotocin, on 2 consecutive days, in food deprived mice	higher blood glucose concentrations compared to predefined threshold (>15 mM/L)	at the end of the 8-week experiment, diabetic mice had significantly elevated fasting blood glucose levels relative to vehicle treated non-diabetic mice	mouse	C57BL/6J wild-type or YFP-H line mice	m	8 weeks old	n/a
23	(Yang et al., 2014)	pharmacological modulation	STZ	single i.p. injection 65 mg/kg of streptozotocin	higher blood glucose concentrations compared to predefined threshold (>16.7 mmol/L); no details on fasting	n/a	rat	Sprague-Dawley	m	220-250 g	10; 10
24	(Momeni et al., 2021)	pharmacological modulation	STZ	i.p. injections of 50 mg/kg of streptozotocin, for three days	higher blood glucose concentrations compared to predefined threshold (>15 mM); no details on fasting	n/a	mouse	C57BL/6J	m	4-6 weeks old; 20-22 g	13-14; n/a
25	(de Cossío et al., 2017)	transgenic approach	C57BLKS/J - $lepr^{db}/lepr^{db}$	db/db mice are deficient for functional leptin receptor and consequently show severe obesity associated with hyperphagia, altered lipid/carbohydrate metabolism, and several indicators of T2D	impaired glucose tolerance (13 h fasted) and insulin sensitivity (non-fasted) in db/db mice after 9 weeks of treatment	n/a	mouse	C57BLKS/J -$lepr^{db/+}$, C57BLKS/J - $lepr^{db}/lepr^{db}$	m	5 weeks old	13-14; 13-14
26	(Dharavath et al., 2019b)	environmental manipulation	HFD + fructose drink solution	high fat-low protein diet (HFLPD) and 15% oral fructose solution via drinking water for 24 weeks	higher blood glucose concentrations compared to controls; with fasting (8 hours)	fasting serum glucose levels and the percent glycosylated Hb were found to be significantly elevated after feeding the animals with HFLPD for 4 weeks, and a similar trend observed till the 24th week of the study	rat	Wistar	f	8–10 weeks old	6; 6
27	(Skapare et al., 2012)	strain difference	Goto-Kakizaki (diabetes), Zucker fa/fa (obesity)	Goto-Kakizaki (diabetes), Zucker fa/fa (obesity)	at 24 weeks, plasma glucose levels were 1.5 x higher than respective controls in the Goto-Kakizaki rats, impaired glucose tolerance in Goto-Kakizaki and Zucker fa/fa rats vs respective controls	n/a	rat	Goto-Kakizaki, Wistar Kyoto (diabetes), $Zucker^{fa/fa}$, Zucker lean (obesity)	m	8 weeks old	12; 12
28	(Malone et al., 2008)	pharmacological modulation	STZ	single i.p. injection of 50 mg/kg of streptozotocin	higher blood glucose concentration compared to predefined threshold (blood glucose levels >200 mg/dL 3 d after STZ administration); no details on fasting	n/a	rat	Wistar	m	4 weeks old; 100 g	20; n/a

29	(Treviño et al., 2015)	environmental manipulation	HFD	the hypercaloric diet (71.4% carbohydrates, 5.8% fat, 7.3% protein) for 90 days.	higher blood glucose concentration compared to controls; with fasting (5 h)	impaired oral glucose tolerance test with increased levels of 73.4% (30 min), 50.9% (60 min), and 58.9% (90 min) as compared to the controls, with a significant increase in the area under the curve of 60.4%	rat	Wistar	m	70-100 g	14; 14
30	(Nurdiana et al., 2017)	pharmacological modulation	STZ	injection of 60 mg/kg of streptozotocin	higher blood glucose concentration compared to predefined threshold (blood glucose levels >11 mmol/L a week after STZ administration); with fasting	n/a	rat	Sprague-Dawley	m	4 weeks old; 80 g	6; 6
31	(Hardigan et al., 2017)	pharmacological modulation	STZ	i.p. injection of 50 mg/kg of streptozotocin, for 5 days consecutively	higher blood glucose concentration compared to predefined threshold (HbA1c% >8.0%); with overnight fasting	n/a	mouse	C57BL/6J	m	10 weeks old	12-13; 7-17
32	(de Senna et al., 2017)	pharmacological modulation	STZ	single i.v. injection into the tail vein of 50 mg/kg of body weight of streptozotocin, after a 6h fasting period. 20 days of diabetes induction by STZ before testing	higher blood glucose concentration compared to predefined threshold (> 300 mg/dL), 48 h post injection; with fasting 5 h	blood glucose concentrations were significantly higher in STZ groups compared to CTRL groups, 48 h after diabetes induction (96 vs 375 mg/dL) and after 9 weeks of STZ induction (106 mg/dL vs 534 mg/dL)	rat	Wistar	m	3 months old; 270–400 g	15, 13; 15, 13
33	(Wu et al., 2012)	pharmacological modulation	STZ	single i.p. injection of 150 mg/kg of streptozotocin	higher blood glucose concentration compared to predefined threshold (> 300 mg/dL), 3 days after injection of STZ; with fasting (an overnight)	plasma glucose levels were significantly elevated in STZ-treated animals after day 21 days of STZ injection (120.75 vs 502.38 mg/dL)	mouse	ICR	m	20-22 g	8; 8
34	(Lin et al., 2018)	pharmacological modulation	STZ	single i.v. injection of 65 mg/kg body weight of streptozotocin, after overnight fasting, three weeks of diabetes induction by STZ before testing	higher blood glucose concentration compared to predefined threshold (>200 mg/dL), one week after injection of STZ	n/a	rat	Sprague-Dawley	m	180-230 g	6; 6
35	(Collison et al., 2012)	environmental manipulation	altered drinking water with aspartame	ad libitum drinking water containing 0.25 g/L aspartame	higher blood glucose concentration compared to controls	n/a	mouse	C57BL/6J	m, f	6 weeks old	12; 12
36	(Zhou et al., 2015a)	pharmacological modulation	STZ	single i.p. injection of 150 mg/kg of streptozotocin, after 12 h fasting	higher blood glucose concentration compared to predefined threshold (≥16.7 mmol/L); fasting not specified, assessed 72 h after STZ injection	at the end of the study blood glucose levels were higher in the STZ group than in the CTRL group	mouse	ICR	m	18-20 g	12; 12

37	(Zhou et al., 2017)	pharmacological modulation	STZ	single i.p. injection of 150 mg/kg of streptozotocin, after 12 h fasting	higher blood glucose concentration compared to predefined threshold (≥16.7 mmol/L); fasting not specified, assessed 3 days after STZ injection	the blood glucose levels in the STZ group were higher than those in the CTRL group	mouse	ICR	m	18-20 g	18; 12
38	(Huang et al., 2012)	pharmacological modulation	STZ	single i.p. injection of 200 mg/kg of streptozotocin (STZ), non-fasting	higher blood glucose concentration compared to predefined threshold (>200 mg/dL); fasting not specified, assessed 10 days after STZ injection	blood glucose were measured on days 1, 10, 32 and 39, the results show that an acute high dose of STZ induced a chronic hyperglycaemic condition	mouse	C57BL/6J	m	6-8 weeks old	15–20; 15-20
39	(Huang et al., 2007)	pharmacological modulation	STZ	single i.p. injection of 200 mg/kg of streptozotocin, non-fasting. 16-22 days of diabetes induction by STZ before testing	higher blood glucose concentration compared to predefined threshold (>200 mg/dL); fasting not specified, assessed 10 days after STZ injection	blood glucose concentrations were measured on days 1, 10, 15, and 23, blood glucose levels were significantly increased at day 10, 15, and 23 compared to the level of day 1, in STZ mice	mouse	C57BL/6J	m	6-8 weeks old	9-12; 9-12
40	(Huang et al., 2019)	pharmacological modulation	STZ	i.p injection of 100 mg/kg of streptozotocin on days 1, 2, 8, and 9, after 6 h fasting. 34-42 days of diabetes induction by STZ before testing	higher blood glucose concentration compared to predefined threshold (≥200 mg/dL); fasting not specified, assessed on day 14	STZ increased the blood glucose levels 14 days after the STZ injection	mouse	3×Tg-AD	m	6 months old	15, 8; 15, 8
41	(Lin et al., 2017)	environmental manipulation	HFD	high-fructose-high-coconut oil diet for 20 weeks. 20 weeks of diets before testing	at week 20 the rats fed the HFD had significantly higher glucose and insulin compared to the control group ($p < 0.05$)	fasting blood glucose levels of the HFD group was generally higher and significantly increased starting at week 16 compared to animals on the control diets	rat	Wistar	m	6 weeks old; 200 g	8, 12; 8, 12
42	(He et al., 2020)	pharmacological modulation, environmental manipulation	STZ+HFD	i.p injection of 85 mg/kg of streptozotocin twice within 72 h, after a 3-week high-fat diet feeding	higher blood glucose concentration compared to predefined threshold (>11.6 mmol/L); fasting not specified, assessed 6 hours after the STZ injection	a marked increase in fasting blood glucose level was observed in HFD/STZ mice, oral glucose tolerance test: a significant increase in glucose concentration in HFD/STZ mice at all time points	mouse	C57BL/6J	m	4 weeks old	10, 10; 7, 7
43	(Zhou et al., 2018)	pharmacological modulation	STZ	single i.p. injection of 150 mg/kg of streptozotocin; long-acting insulin glargine was administered subcutaneously to mice from the seventh day of STZ injection, until the end of experiment (starting dose of 2 IU/kg and then the dose was adjusted according to the glucose levels)	higher blood glucose levels compared to predefined threshold (>16.7 mmol/L), assessed after 3 and 7 days of STZ injection	after STZ injection, STZ mice presented significantly higher values of glycemia compared to CTRL	mouse	C57BL/6J	m	20-25 g	12; 12

44	(Sibiya and Mabandla, 2017)	pharmacological modulation	STZ	single i.p. injection of 60 mg/kg of streptozotocin	higher blood glucose concentration compared to controls; without fasting	blood glucose concentrations were significantly higher in STZ groups compared to control groups at week 5	rat	Sprague-Dawley	m	250-300 g	6; 6
45	(Kumar and Maqbool, 2020)	pharmacological modulation	STZ + nicotinamide	injection of 65 mg/kg of streptozotocin after nicotinamide (110 mg/kg in normal saline, i.p.) injection (dose volumes 3 mL/kg). Glucose solution (10 %) was provided for the next 24 h to avoid acute hypoglycaemia in rats	higher blood glucose concentration compared to a predefined threshold (200 mg/dL); with fasting (overnight), assessed on day 5	blood glucose concentrations were significantly higher in STZ groups compared to control groups, at day 5 and day 26	rat	Wistar	m	180-200 g	6; 6
46	(Choeiri et al., 2005)	transgenic approach	Ins2C96Y Akita mice	no manipulation, spontaneously diabetic	higher blood glucose concentration compared to controls; with fasting (18 h)	already at age of 7 weeks, Akita mice had higher fasting blood glucose levels than their corresponding CTRL mice	mouse	C57BL/6 wild type (CTRL) and Ins2C96Y Akita	m	6–7 weeks old	6; 6
47	(Marissal-Arvy et al., 2018)	pharmacological modulation	STZ	i.p. injection of 65 mg/kg of streptozotocin/day for 2 days. 3 weeks of diabetes induction before testing	higher blood glucose concentration compared to a predefined threshold (>200 mg/dL); fasting not specified, assessed 3 days after STZ injection	n/a	rat	Sprague-Dawley	m	3 weeks old; 50–55 g	8; 8
48	(Braga et al., 2021)	environmental manipulation	HFD	the animals from the HFD group received, for twelve weeks, ad libitum, a high-fat diet (12 kJ% protein, 27 kJ% carbohydrates and 61 kJ% lipids)	higher blood glucose concentration compared to controls, with fasting (6 h)	after 4 weeks: the HFD not altered basal glucose levels, significant increase in glucose levels of HFD group a T60. After 8 weeks: the HFD group displayed a significant increase in the basal glucose levels; significant increase in glucose levels of HFD group a T60, significant increase in the AUC of HFD compared to CTRL. After 12 weeks: significant increase in the basal glucose levels in mice from HFD group; significant increase in glucose levels of HFD a T30 and T60; significant increase in the AUC of HFD compared to CTRL.	mouse	C57BL/6	f	12 months old; 22-27 g	10, 11; 10, 11

49	(Van Der Kooij et al., 2018a)	environmental manipulation	chronic social defeat stress	social defeat in the home cage of the aggressor mouse lasting 10 s of aggressive encounter; these episodes were repeated three times with different. Following the triple social defeat, C57 mice were housed overnight with their opponent separated by the metal grid. The social defeat was repeated for 10 consecutive days	higher blood glucose concentration compared to controls, with fasting (1 h)	glucose levels were significantly increased on the morning, 2 days post-CSD in comparison with controls, in a glucose tolerance test performed 9 days post-CSD in fasted animals, glucose levels were increased at T30 compared with control mice	mouse	C57BL/6	m	8 weeks old	10, 23; 10, 24
50	(Bhutada et al., 2010)	pharmacological modulation	STZ	i.p. injection of 60 mg/kg of streptozotocin; streptozotocin-treated rats received 5% of glucose solution instead of water for 24 h after injection of streptozotocin to reduce death due to hypoglycaemic shock	higher blood glucose concentration compared to predefined threshold (250 mg/dL); with fasting (3 h), assessed 48 hours after STZ injection	treatment 1: 38 days after STZ injection, plasma glucose levels were highly elevated in STZ rats as compared to CTRL rats (111.6 vs 401 mg/dL); treatment 2: at the end of the experiment, plasma glucose levels were highly elevated in STZ rats as compared to CTRL rats (101.8 vs 400.33 mg/dL)	rat	Wistar	m	200–225 g	Treatment 1: 12; 12 Treatment 2: 6; 6
51	(Pathan et al., 2008)	environmental manipulation	HFD	the high fat diet (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) was administered ad libitum, for a period of 5 weeks. Each rat in high fat diet group consumed 105 kcal/day	after 4 weeks of experimental diet feeding, an increase in plasma glucose was observed in high fat diet fed rats (glucose level mg/dL 128.47 ± 2.45) compared to control (glucose level mg/dL 99.86 ± 2.76)	n/a	rat	Sprague-Dawley	m	150–190 g	8; 6
52	(Esmaeili et al., 2017)	pharmacological modulation	STZ	single i.p. injection of 65 mg/kg of streptozotocin, for three successive days	higher blood glucose concentration compared to predefined threshold (blood glucose levels exceeded 7.8 mmol/L), seven days after STZ injection; with fasting	n/a	rat	Wistar	m	200–300 g	8; 10
53	(Ren et al., 2013)	pharmacological modulation	STZ	single i.p. injection of 55 mg/kg of streptozotocin, for 15 days, after 12 h fasting	higher blood glucose concentration compared to predefined threshold (blood glucose levels of 16.7 mM) at 72 hours after streptozotocin injection; with fasting	fasting blood glucose levels were significantly decreased in control group compared with the diabetes mellitus group, at 4, 9 weeks and 80 days after streptozotocin injection	rat	Sprague-Dawley	m	10 weeks old	15; 15

54	(Yeh et al., 2015)	pharmacological modulation, environmental manipulation	STZ+HFD	HFD (60% energy from fat) for 2 weeks + i.p. injections of 50 mg/kg of streptozotocin. The HFD continued until mice were killed after 11 or 22 weeks of dietary	increased in fasting blood glucose level in HFD group vs CTRL at 4, 11 and 22 weeks	increased in fasting blood glucose level in HFD group vs CTRL at 4, 11 and 22 weeks	mouse	APP/PS1 transgenic mice and their WT littermates	m, f	10 weeks old	13, 4, 7, 9 for NCD WT, NCD AD, HFSTZ WT, and HFSTZ AD respectively; 21, 15, 25, and 18 for NCD WT, NCD AD, HFSTZ WT
55	(Pei and Sun, 2018)	pharmacological modulation	STZ	single i.p. injection of 50 mg/kg of streptozotocin, after overnight fasting	higher blood glucose concentration compared to predefined threshold: blood glucose levels more than or equal to 16.7 mmol/L, 72 h after STZ injection; fasting not specified	n/a	mouse	ICR	m	Adult, 18–20 g	15; 15
56	(Ye et al., 2018)	transgenic approach	C57BLKS/J - $lepr^{db}/lepr^{db}$	diabetic mice	higher blood glucose concentrations compared to controls during 12 weeks of treatment; with fasting (hours not specified)	oral glucose tolerance test blood glucose was conducted after an overnight fasting. mice were orally gavage with glucose solution (1 g/Kg), followed by blood glucose measurement at 0, 30-, 60-, 90-, and 120-min. Glucose total area under the curve in db/db mice was significantly higher than db/m mice	mouse	C57BLKS/J - $lepr^{db}/lepr^{db}$, non-diabetic mice (db/m)	m	7 weeks old	10; 10
57	(Mao et al., 2008)	pharmacological modulation	STZ	single i.v. tail vein injection of 45 mg/kg of streptozotocin	higher blood glucose concentration compared to predefined threshold: plasma glucose level of 15 mmol/L or higher on day 7 following STZ administration, blood glucose was monitored daily and the STZ rats showed initial high blood glucose levels (> 22 mM) that continually increased during the 8-week period; fasting not specified	in the Oral Glucose Tolerance Test, during the 2 h following glucose ingestion, the CTRL rats exhibited smaller area under curve than STZ rats, the diabetic rats exhibited much lower insulin levels (16.7 ± 1.0 lIU/mL) compared to the control rats (39.5 ± 19.3 lIU/mL)	rat	Wistar	m	Adult (155–190 g)	7; 7
58	(T. H. Liu et al., 2020)	pharmacological modulation, environmental manipulation	STZ+HFD	HFD for 3 weeks followed by injection of STZ. The animals were kept on the HFD for the rest of the experimental period. Diabetes was induced through a single i.p. injection of 35 mg/kg streptozotocin, for 7 weeks.	higher blood glucose concentration compared to predefined threshold: serum glucose level was above 200 mg dL^{-1} at week 11; with fasting (12 hours).	glucose levels were assessed for 3 weeks after STZ injection to ensure that diabetes was not reversed. Fasting blood glucose increased to more than 200 mg dL^{-1} over the weeks; in the HFD/STZ groups it was significantly higher than control	rat	Wistar	m	6 weeks old (200–210 g)	6; 6

59	(Patel and Udayabanu, 2014)	pharmacological modulation	Dexamethasone	Dexamethasone (1 mg/kg/day, i.m.) for 12 weeks	higher blood glucose concentrations compared to controls (116.75 %); fasting not specified	n/a	mouse	Swiss albino	m, f	Adult, 24–30 g	8-10; 8-10
60	(J. Liu et al., 2020)	pharmacological modulation, environmental manipulation	STZ+HFD	HFD diet for 8 weeks + i.p. injection of 30 mg/kg of streptozotocin, under fasting conditions	higher blood glucose concentration compared to predefined threshold: blood glucose levels >11.1 mmol/L on the fifth day after STZ injections; with fasting (hours not specified)	fasting blood glucose and insulin were measured at the end of all experiments and showed that the HFD and the STZ injection induced hyperglycaemia and insulin resistance	rat	Sprague-Dawley	m	120-150 g; 5 weeks old	8; 6
61	(Jin et al., 2018)	pharmacological modulation	MSG	monosodium glutamate treated rodents are used as an animal model of T2DM, experimental pups were administered 50% water-soluble MSG by subcutaneous injection at a dosage of 4 mg/g body weight at postnatal days 1, 3, 5, 7, and 9	higher blood glucose concentrations compared to controls at 3 months old; with fasting (12 hours)	MSG exposure during the neonatal period significantly increased levels of fasting blood glucose and fasting insulin, in 3-month-old rats compared with age-matched rats from the control group, the insulin sensitivity index was also reduced in 3-month-old MSG-treated rats compared with age-matched rats from the control group	rat	Sprague-Dawley	m	neonatal	10; 10
62	(H. Wang et al., 2019)	pharmacological modulation	injection of glucose	glucose fluctuation group: i.p. injection of 0.375 mL/kg of glucose (250 g/L) following with an injection of 1U of insulin to make a fluctuating blood glucose level. Chronic HYP group: i.p. injection of 0.375 mL/kg of glucose (250 g/L) to make a continuously high blood glucose level	blood glucose was determined at 8:00, 8:30, 10:00, 11:00, 14:00, 14:30, 16:00, 17:00, and 20:00 to monitor the fluctuation of blood glucose in all groups, blood glucose levels at each time points demonstrated the model was built successfully	n/a	rat	Goto-Kakizaki and Winstar control	f	adult (180–200 g)	10; 10
63	(Babic et al., 2018)	environmental manipulation	cookie pellet with clozapine	12 mg/kg of clozapine for 6 weeks, three times daily at 8-hourly intervals	higher blood glucose concentrations compared to controls at baseline; with fasting (overnight)	blood glucose levels in the clozapine group did not return to a homeostatic level by the end of the Oral Glucose Tolerance Test, indicative of poor insulin response to rising blood glucose levels and suggests the presence of a diabetic phenotype in these rats	rat	Sprague–Dawley	f	200–220 g	11-12; 10-12
64	(Lee et al., 2014)	pharmacological modulation	STZ	i.p. injection of 65 mg kg^{-1} of streptozotocin, with 12 h of fasting at week 1	higher blood glucose concentrations compared to controls at 12 weeks in the Glucose tolerance test; with fasting (overnight)	n/a	rat	Wistar	m	6 weeks old	6; 6

65	(Wu et al., 2020)	transgenic approach	C57BLKS/J - lepr^{db}/lepr^{db}	db/db mouse models of Type 2 diabetes	higher blood glucose concentrations compared to controls at 16 weeks; with fasting (overnight)	for Glucose Tolerance Test, the mice were fasted overnight, and then intraperitoneally injected with glucose at a dose of 2 g/kg body weight, for Insulin Tolerance Test, the mice were fasted for 5 h, and then intraperitoneally injected with insulin at a dose of 0.75 U/kg body weight	mouse	C57BLKS/J - lepr^{db}/lepr^{db}, and non-diabetic db/m	m	12 week old	10; 10
66	(Wang et al., 2015)	pharmacological modulation	STZ	i.p. injection of 60 mg/kg of streptozotocin, on 5 consecutive days in 3-month-old mice, after 5h fasting	higher blood glucose concentration compared to predefined threshold: blood glucose level >250 mg/dL, 2 weeks after initiation of STZ injection (339.07 ± 50.9 mg/dL in non Tg/STZ mice and 342.88 ± 36.2 mg/dL in Tg/STZ mice); fasting not specified	two months later, blood glucose levels for diabetic mice remained significantly high in both Tg (498.09 ± 33.5 mg/dl) and non Tg (517.09 ± 27.6 mg/dl) mice	mouse	C57BL/6J	m	3 month old	6-8; 5-10
67	(Lee and Yang, 2019)	pharmacological modulation, environmental manipulation	STZ+HFD	high-fat chow diet (HFD; 60% kcal fat) + STZ (100 mg/kg)	higher blood glucose concentrations compared to control: 102 ± 11 mg/dL for control group and 425 ± 29 mg/dL for all HFD groups at week 8; with fasting (3 hours)	after an overnight fasting for 16, the animals were fed with glucose solution (2 g/kg body weight) via stomach gavage, at 15, 30, 60, 90, and 120 min after the fasting blood glucose concentrations were significantly increased by HFD diet and STZ injection	mouse	ICR	m	6 weeks old	8; 8
68	(Ren et al., 2019)	pharmacological modulation, environmental manipulation	STZ+HFD	HFD (30% fat, 20% sugar, 15% protein, 2.5% cholesterol, 1% sodium cholic acid and 31.5% custom carbohydrate) for 6 weeks + i.p. injection of 40 mg/kg of streptozotocin	higher glucose concentration compared to control at all weeks; with fasting (8 hours)	oral glucose tolerance test was performed on C57/BL6 mice following overnight-fasting period at the 6th week post-treatment, HFD-STZ group had higher glucose concentration compared to control	mouse	C57BL/6J	m	6-8 weeks old, weighing 20-22 g	6; 6
69	(Noor and Zahid, 2017)	pharmacological modulation, environmental manipulation	STZ+HFD	diabetes was induced by switching the mice to high-fat diet (HFD) and two i.p. injection of 100 mg/kg of streptozotocin, at 6 and 9 weeks of age, after overnight fasting	higher blood glucose concentration compared to predefined threshold: blood glucose level >12 mmol/L, assessed after eight days of STZ injection; with fasting (hours not specified)	n/a	mouse	BALB/c	m	n/a	10; 10
70	(Zhang et al., 2016)	pharmacological modulation	STZ + nicotinamide	i.p. injection of 210 mg/kg of nicotinamide (NTM) and of 60 mg/kg of streptozotocin	higher blood glucose concentration compared to predefined threshold (>250 mg/dL) assessed 2 days after NTM-STZ injection: without fasting	rats confirmed hyperglycaemia status 30 days after NTM-STZ injection	rat	Wistar	m	13 months old	8; 20

71	(Li et al., 2012)	transgenic approach	C57BLKS/J - $lepr^{db}/lepr^{db}$	db/db mice had more tau phosphorylated at S396 and total tau in their hippocampi than their non-diabetic control db+ mice	higher blood glucose concentrations compared to controls at 6 weeks old; with fasting (hours not specified)	the db/db mice had a higher fasting blood glucose level than the control mice when they were 6 weeks old, and that difference continued when the mice were 24 weeks old	mouse	C57BLKS/J - $lepr^{db}/lepr^{db}$, and non-diabetic db/m	m	6 week old	11; 11
72	(Gu et al., 2017)	environmental manipulation	psychological-stress model (PSD) in Zucker diabetic fatty rat	the PSD group was subjected to three stress stimulations: restriction, rotation, and congest	higher blood glucose concentrations compared to controls; with fasting (14 hours)	n/a	rat	Zuker diabetes fatty (ZDF)	m	5 weeks old	3; 3
73	(Ahmed et al., 2020)	pharmacological manipulation	STZ	single i.p. injection of 45 mg/ml of streptozotocin, after 16 hr fasting	higher blood glucose concentrations compared to predefined threshold (10 mM), with fasting (16 hours)	blood glucose in STZ-administered rats ranged from ~15-20 mM, vs ~4-7 mM in vehicle-administered controls	rat	Sprague–Dawley	m	200–220 g	10; 10
74	(Ahmed et al., 2019)	pharmacological manipulation	STZ	single i.p. injection of 45 mg/kg of streptozotocin, following 16 hr fasting	higher blood glucose concentrations compared to predefined threshold (7.0 mM); with fasting (hours not specified)	in STZ-treated hyperglycaemic group there was a gradual increase in fasting blood glucose (>7 mM after 1–3 weeks of STZ induction which drastically elevated to 11.51 and 13.69 mM after 6 and 9 weeks respectively)	rat	Sprague–Dawley	m	180-220 g	10; 10
75	(Liu et al., 2022)	transgenic approach	C57BLKS/J - $lepr^{db}/lepr^{db}$	db/db mouse models of Type 2 diabetes	hyperglycaemia not measured and was instead assumed based on the db/db model	n/a	mouse	db/db	m	12 weeks old, 25-49 g	9; 9
76	(Diegues et al., 2014)	pharmacological modulation	Alloxan	single i.v. injection of 32 mg/kg of ALX	higher blood glucose compared to threshold (14-35 mmol/L)	glucose levels in control groups ranged between 7.4-7.7 mmol/L, vs glucose levels in diabetic groups which ranged from 21-25 mmol/L after 6 weeks	rat	Wistar	m	38 days old, 175-200 g	10; 10
77	(Kamsrijai et al., 2020)	pharmacological manipulation	STZ	single i.p. injection of 60 mg/kg of streptozotocin, on the 15th day of the experiment	higher glucose concentration compared to threshold (>300 mg/dL); with fasting (12 hours)	STZ-administered groups had higher fasting blood glucose (~430 mg/L) compared to control groups (180-190 mg/L)	rat	Wistar	m	6 weeks old, 160-180 g	9; 9
78	(Rejdak et al., 2001)	environmental manipulation	oral administration of glucose	oral administration of 40% glucose solution (4 g/kg), 30 min before the experiment	higher blood glucose concentrations compared to controls (272.3 ± 46.1 mg/dL); without fasting	hyperglycaemic group had higher blood glucose (272.3 ± 46.1 mg/dL) compared to controls	mouse	Swiss	m	20–30 g	20-35; 20-35

79	(Lazcano et al., 2014)	pharmacological manipulation	STZ	single i.p. injection of 80 mg/kg on the 15th day of the experiment	higher blood glucose concentrations compared to predefined threshold (200 mg/dL); without fasting	control group blood glucose levels were 86.01 ± 1.3, 82.81 ± 1.6, 77.81 ± 2.5, 81.9 ± 1.43, and 77.81 ± 2.5 mg/dL, whereas diabetic group were 82.25 ± 2.4, 264.10 ± 7.11, 299.80 ± 7.19, 348.8± 12.1, and 363.2± 11.4 mg/dL	rat	Wistar	m	180-220 g	7; 7
80	(Cai et al., 2020)	pharmacological modulation, environmental manipulation	STZ+HFD	single i.p. injection of 35 mg/kg of streptozotocin, followed by administration of high fat diet for 8 weeks	higher blood glucose concentrations compared to controls (~300 mg/dL vs ~120 mg/dl); with fasting (6 hours)	animals in experimental group exhibited an increasing fasting blood glucose level over 8 weeks ranging from ~150 mg/dL in week 1 to ~300 mg/dL in week 8, animals in the control group showed fasting blood glucose ranging from ~120 mg/dL in week 1 to 140 mg/dL in week 8	rat	Sprague–Dawley	m	18 weeks old, 250 g	10; 10
81	(Wen et al., 2020)	transgenic approach	C57BLKS/J - $lepr^{db}/lepr^{db}$	db/db mice utilised as a model of Type 2 diabetes and obesity	higher blood glucose concentrations compared to controls, with fasting (4 hours)	n/a	mouse	C57BLKS/J - $lepr^{db}/lepr^{db}$	m	8 months old	10; 10
82	(Li et al., 2018)	strain difference	KKAy mice	KKAy mice, a genetic model of type 2 diabetes with obesity and insulin resistant hyperglycaemia	higher blood glucose concentrations compared to predefined threshold (random blood glucose ≥ 11.1 mmol/L or fasting blood glucose ≥ 7.0 mmol/L), with fasting	KKAy group had higher random blood glucose levels in week 4, 8 and 12 of the experiment of ~30 mmol/L, 25 mmol/L and 25 mmol/L respectively vs ~8 mmol/L in controls at all timepoints, the KKAy group also had higher fasting blood glucose in week 4, 8 and 12 of ~22 mmol/L, 20 mmol/L and 22 mol/L respectively vs ~8 mmol/L in controls at all timepoints	mouse	KKAY	m	30 ± 5 g	6; 6
83	(Ahmadi et al., 2017)	pharmacological modulation	STZ	single i.p. injection of 55 mg/kg of streptozotocin	higher blood glucose concentrations compared to predefined threshold (250 mg/dL), with fasting (overnight)	serum glucose levels in control non-diabetic mice were approximately ~120 mg/dL vs ~450 mg/dL in diabetic mice (exceeding the 250 mg/dL threshold set based on clinical criteria for chronic hyperglycaemia)	Rat	Wistar	m	270–300 g	7; 7
84	(Wirt et al., 2021)	pharmacological manipulation	STZ	i.p. injection of staggered and low doses of streptozotocin at 20 mg/kg/ml following 6 hours fasting on day 1, 2, 3, 14, 15, 35 and 36	higher blood glucose concentrations compared to predefined threshold (≥250 mg/dL); with fasting	animals in the experimental group exhibited a sustained fasting blood glucose reading >250 mg/dl compared to vehicle-administered controls which did not reach this threshold	rat	Long-Evans	m	8-12 months old, 400-550 g	3, 5; 3, 5
85	(Nakaoku et al., 2019)	environmental manipulation	HFD	mice fed high-fat diet for 5.5 months (60% fat, 20% carbohydrate, and 20% protein)	higher blood glucose concentrations compared to controls; with fasting (6 hours)	n/a	Mice	PS19	m	6 weeks old	15-17; 15-17

86	(Delkhosh-Kasmaie et al., 2018)	pharmacological manipulation	STZ	single i.p. injection of 55 mg/kg of streptozotocin, after 12 hr fasting	higher blood glucose concentrations compared to predefined threshold (250 mg/dL); with fasting (12 hours)	blood glucose levels in control group were 79.2 ± 3.61, 76.8 ± 4.03 and 86 ± 3.34 mg/dL on days 15, 25 and 35 of the experiment, respectively, blood glucose concentrations reached to 394.5 ± 22.32 and 406.8 ± 19.38 and 420 ± 21.71 mg/dL on days 15, 25 and 35 after induction of diabetes, respectively	rat	Wistar	m	180-210 g	6; 6
87	(Lupien et al., 2003)	pharmacological modulation	STZ	anesthetized under 5% isoflurane, 95% oxygen for 1 min, and injected s.c. with 50 mg/kg of streptozotocin, overnight fasting	higher blood glucose concentrations compared to predefined threshold (360 mg/dL), without fasting	blood glucose levels were significantly higher in diabetic mice with vehicle and IGF-1 (515 ± 73, 495 ± 99 mg/dL) vs non-diabetic control (125.0 ± 11 mg/dL)	rat	Wistar	m	10 weeks old	7-12; 7-12
88	(Heng et al., 2011)	pharmacological modulation	STZ	single i.p. injection of 60 mg/kg of streptozotocin	higher blood glucose concentrations compared to predefined threshold (16.7 mmol/L); without fasting	blood glucose level in the diabetic group was significantly higher than the control group (~20 mmol/L vs ~5 mmol/L), by the end of week 5, blood glucose levels in the diabetic group remained significantly elevated	rat	Sprague–Dawley	m	250-280 g	20; 20
89	(Jash et al., 2020)	pharmacological modulation	STZ	i.p. injection of 120 mg/kg of streptozotocin	higher blood glucose concentrations compared to predefined threshold (300 mg/dL); with fasting (4 hours)	glucose levels persistently higher and above threshold in diabetic group	mouse	C57BL/6J	m	4 weeks old	6; 6
90	(Rajab et al., 2017)	pharmacological modulation	STZ	i.p. injection of 55 mg/kg of streptozotocin, daily for five days	higher blood glucose concentrations compared to predefined threshold (280 mg/dL); without fasting	non-fasting blood glucose measurements (22.1 mM) which were significantly higher than the control mice (8.1 mM)	mouse	BALB/C	m	20-25 g	12-14; 12-14
91	(Sharifzadeh et al., 2017)	pharmacological modulation	STZ	single i.p. injection of 60 mg/kg streptozotocin	higher blood glucose concentrations compared to predefined threshold (250 mg/dL); with fasting	streptozotocin-induced diabetic rats showed consistent fasting hyperglycaemia throughout the study	rat	Wistar	m	250-300 g	8-10; 8-10

Table 1. Details regarding the experimental manipulation to induce hyperglycaemia and the experimental subjects (f: female; m: male).

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CHAPTER II

Brain specific kcnq1 knockout impairs cognitive capabilities in mice

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Abstract

The *KCNQ1* gene has been identified as a susceptibility gene for metabolic disorders like type 2 diabetes, obesity, and metabolic syndrome, as well as cognitive dysfunctions such as Alzheimer's, Parkinson's disease, and obsessive-compulsive disorder. KCNQ1 is a voltage gate potassium channel, which is expressed everywhere in the body including the pancreas and the brain, and has a modulatory role in both peripheral and central insulin signalling. Besides influencing peripheral energy metabolism, insulin signalling has been reported to modulate brain function and to have a role in the onset of numerous neurological and psychiatric disturbances. Therefore, KCNQ1 has been proposed as a potential target underlying the comorbidity between insulin-related mental and somatic disorders. Herein, to investigate whether the effects of kcnq1 on insulin-related comorbidity are primarily localized in the periphery or in the central nervous system, we conducted behavioural and physiological assessments of a conditional knockout mouse model with brain-specific deletion of *kcnq1* during lifespan (adolescence and adulthood). The cognitive domains investigated included attention, working memory, anxiety-like behaviours, and recognition memory, together with insulin sensitivity, glucose tolerance, and general metabolism. Our findings suggest that *kcnq1* deletion in the brain significantly influences cognitive abilities in rodents. Specifically, the absence of the gene leads to a consistent deficit in spontaneous alternation and attentional set-shifting capabilities, indicating impairments in the executive functions. While cognitive impairments were observed in both ages, the metabolic dysfunction was more pronounced in adolescence. Adolescent *kcnq1* knockout mice exhibited distinct lipid metabolic patterns compared to controls. Overall, our results support the idea that KCNQ1 plays an intricate role in the interaction between insulin signalling and neurological conditions, being also influenced by the developmental stage.

Introduction

Dysregulation of insulin signalling extends beyond its traditional association with metabolic disorders such as type 2 diabetes, metabolic syndrome, and obesity. Recent research has identified its involvement in a spectrum of conditions including neurodegenerative disorders like Alzheimer's (AD) and Parkinson's disease [1], as well as compulsivity-linked neurodevelopmental disorders such as obsessive-compulsive disorder (OCD) [2], autism spectrum disorders (ASDs) [3], and attention deficit hyperactivity disorders (ADHD)[4]. As a result, the co- and multimorbidity associated with a dysregulation of insulin signalling cover a large spectrum of pathologies. All of them pose a significant health, societal, and economic burden worldwide. More than 20% of the global population is affected by diseases linked to altered insulin signalling and these diseases are associated with enormous healthcare costs, exceeding $1.2 trillion annually on a global scale [5]. The growing prevalence of insulin-related diseases, particularly type 2 diabetes, has reached epidemic proportions in many countries. Sedentary lifestyles, poor diets, and rising obesity rates contribute to this issue, especially in developed and developing nations [6]. At the same time, more than 55 million people worldwide live with dementia. Alzheimer's disease accounts for 60-70% of all dementia cases with a global economic impact that is estimated to be over $1.3 trillion annually [7].

Both diabetes and dementia are age- and biological sex- related conditions. They generally become increasingly prevalent as individuals grow older, while women with diabetes are at a higher risk of developing Alzheimer's disease than men [7]. Even if insulin signalling has long been associated with glucose metabolism, it also exerts a major role in the central nervous system (CNS), wherein its receptors are highly expressed [8]. Insulin can either derive from the periphery crossing the blood brain barrier [9], or via direct synthesis by neurons [10]. 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 [2,3,8,11]. Synaptic dysfunction caused by altered central insulin signalling (potentially as a consequence of peripheral insulin dysregulation) results in deficits in learning, memory, and executive function [12,13], which may contribute to OCD, ADHD, as well as AD, either directly or by promoting cognitive/behavioural rigidity and impairing the ability to adopt new behavioural strategies [4].

A detailed understanding of the signalling of key molecules implicated may shed light on the intricate interplay between insulin signalling and neural function. In that context, KCNQ1, a voltage-gated potassium channel, is a key candidate potentially linking metabolism and cognition. Expression of *kcnq1* was detected in the hippocampus, the cortex, the dentate gyrus, the hypothalamus, and in the pyramidal cells of the CA1-3 regions [14]. It modulates insulin secretion locally within neurons, exerting paracrine or autocrine effects [15]. It is also involved in the dopaminergic system whereby it modulates neuronal excitability by dopamine release [16]. Simultaneously, several studies indicate that *KCNQ1* is expressed in pancreas revealing a role of that gene in insulin-signalling [17,18]. A deletion in *kcne2* (a KCNQ1 co-assembling protein) in mouse models has been shown to result in altered insulin

secretion [19]. Additionally, the absence of *kcne2* induces extensive changes in the pancreatic transcriptome that are consistent with various aspects of T2DM [19]. Interestingly, *kcnq1*$^{-/-}$ mice exhibit enhanced insulin sensitivity and insulin release, leading to altered plasma insulin concentrations [20].

KCNQ1 has been proposed as a susceptibility gene for type 2 diabetes [21]. Independent clinical studies have shown that single nucleotide polymorphisms within *KCNQ1* are strongly associated with diabetes mellitus across diverse populations, including those from Africa [22], America [23,24], East Asia [25–27], Europe [28–30], and Latin America [28]. *KCNQ1* has also been implicated in Alzheimer's disease [1] and OCD [31]. Moreover, the implication of *KCNQ1* gene in insulin signalling is further supported by Romano Ward Syndrome (RWS) patients, wherein mutations in *KCNQ1* contribute to both cardiac anomalies and insulin resistance [32]. Additional evidence derive from preclinical research; mouse models deficient in *kcnq1* (a model for RWS) exhibit deficits in repetitive behaviour and cognitive rigidity, mirroring clinical observations in human patients [33]. Ultimately, KCNQ1's dual role in metabolic regulation and neuronal excitability may constitute a link in the metabolic-cognitive feedback loop. Just as somatic insulin disorders are associated with cognitive decline (for example, chronic hyperglycaemia can impair brain function, leading to cognitive deficits [34]), so also mutations or dysfunctions in *KCNQ1* can trigger a cascading effect, beginning in the central nervous system and subsequently disrupting both metabolic balance and cognitive performance.

To isolate the potential role of central KCNQ1 on insulin-related multimorbidity, we conducted behavioural, physiological, and metabolic tests in a conditional (brain-specific) *kcnq1* knockout mouse model. In those experimental subjects, *kcnq1* expression is decreased in a time- and tissue-specific manner via Tamoxifen administration. Specifically, we administered Tamoxifen to decrease the expression of *kcnq1* in the CNS during adolescence and adulthood in both sexes. We adopted an ontogenetic perspective in order to study the developmental nature and time course of insulin multimorbidity. The use of the conditional *kcnq1* knockout model provides insights into the importance of central insulin dysregulation as a causal mechanism underlying brain insulinopathies. By elucidating the role of KCNQ1 in insulinopathies, this research aims to address the complex interplay between insulin signalling and neurological conditions.

Materials and Methods

Ethics statement

Experiments were conducted at the Centre for Behavioural Sciences and Mental Health (SCIC) of the National Institute of Health (Rome, Italy) with a protocol approved by the Ministry of Health (license no. 216/2020-PR to SM) in full compliance with Legislative Decree 26/2014 on the protection of animals used for scientific purposes. Every effort was made to minimize the suffering and the number of animals used.

Subjects

Experimental subjects (conditional *kcnq1* knockout mice and relative controls) derived from the interbreeding between ten males and six females Kcnq1$^{flox/flox}$/Nestin-CreERT2$^{+/-}$ mice (C57BL/6 genetic background) acquired from Genoway laboratories (Lyon, FR). Mice derived from an interbreeding of homozygous *kcnq1* conditional KO mice (Kcnq1 flox/flox developed as part of the genOway/JAR/TAC1 project) with double heterozygous *Kcnq1* conditional KO/Nestin-CreERT2 mice (C57BL/6-Tg(Nes-cre/ERT2)KEisc/J; JAX #016261). Mice were housed in same-sex and same-genotype pairs per cage and 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), aside from tests requiring food restriction. Following two weeks of acclimatization, males and females were paired for reproduction. The mating involved pairing two females with a male for five days. After assessed pregnancy, females were separated from the male and single-housed. Pups remained with the mother until weaning, which took place 21 ± 1 days after delivery. Then, pups were marked and housed in same-sex and same-group pairs. Experimental assessments were performed in a dedicated room adjacent to the housing room, minimizing fluctuations in light, temperature, sound, and other environmental factors. All the subsequent pairings were conducted in the same way, considering the genotype of the parents to achieve the appropriate number of subjects for experimental use. Thus, Kcnq1$^{flox/flox}$/Nestin-CreERT2$^{+/+}$ females were mated with Kcnq1$^{flox/flox}$/Nestin-CreERT2$^{+/+}$ and/or Kcnq1$^{flox/flox}$/Nestin-CreERT2$^{+/-}$ males to reach Cre-expressing pups, and Kcnq1$^{flox/flox}$/Nestin-CreERT2$^{-/-}$ females were paired with Kcnq1$^{flox/flox}$/Nestin-CreERT2$^{-/-}$ males to get subjects lacking Cre expression. Homozygous and heterozygous Nestin-CreERT2 subjects were brain-specific inducible Cre-expressing mice in which the gene *kcnq1* was temporally and spatially downregulated through tamoxifen injections. *Nestin-Cre/ERT2* transgenic mice express a CreERT2 fusion protein under the control of the rat *nestin* promoter. Nestin is an intermediate filament protein expressed mainly in radial glia and neural stem cells during development. Cre-ERT2 fusion gene activity is

inducible; observed at high levels following tamoxifen administration. When Nestin-Cre/ERT2 transgenic mice are bred with mice containing *loxP*-flanked sequences, Cre-mediated recombination results in a deletion of the floxed gene (*kcnq1* in our mice). Specifically, the Nestin-Cre/ERT2 transgene directs *Cre* expression in the nestin-expressing cells in the brain, which are generally restricted to the sub ventricular and sub granular zones. The Cre-ERT2 fusion protein consists of a Cre recombinase fused to a mutant form of the human estrogen receptor. It normally does not bind its natural ligand (17β-estradiol) at physiological concentrations but it binds the synthetic estrogen receptor ligands 4-hydroxytamoxifen (OHT or tamoxifen). Restricted to the cytoplasm, Cre-ERT2 can only gain access to the nuclear compartment after exposure to tamoxifen.

Tamoxifen injections

To induce Cre activity, intraperitoneal (i.p.) tamoxifen injections were performed at the 4th week or the 22nd week after birth (for mice to be tested in adolescence or adulthood, respectively), twice a day for five days. Behavioural and metabolic tests were performed at least 10 days after the last injection. The tamoxifen (10 mg/mL) solution was reconstituted in a mix of ethanol and sunflower oil (1:10, v/v) and then incubated at 37 °C for 2 hours and 30 minutes. Aliquots of 5 ml were prepared and stocked at -20°C. A new tube was defrosted and mixed before each injection. A solution of ethanol and sunflower oil (1:10 v/v) was prepared as vehicle for the negative control groups. Mice were injected intraperitoneally twice daily, with a 12 hours' time gap for 5 consecutive days for a total of 10 injections. The volume to be injected was calculated on the body weigh the animals. Two different doses were injected: 1 mg/100 µl/20 g bodyweight (=50 mg/kg) for the first 5 injections and 1.5 mg/150 µl/20 g bodyweight (=75 mg/kg) for the second 5 injections. Mice were then left undisturbed for 10 days to recover and to allow the excision to occur.

Experimental plan

The experimental plan entailed:

- two genotypes (Kcnq1$^{flox/flox}$/Nestin-CreERT2^{+} in which the gene *kcnq1* was floxed and Cre recombinase was expressed and Kcnq1$^{flox/flox}$/Nestin-CreERT2^{-} who did not hold the transgene);
- three groups: knockout (Kcnq1$^{flox/flox}$/Nestin-CreERT2^{+} treated with tamoxifen as the experimental knockout model), ctrl1 (Kcnq1$^{flox/flox}$/Nestin-CreERT2^{+} treated with vehicle to assess the role of tamoxifen), and ctrl2 (Kcnq1$^{flox/flox}$/Nestin-CreERT2^{-} treated with tamoxifen to evaluate any effect of the Cre construct);
- both sexes;
- two developmental stages (adolescents aged 6 to 8 weeks after birth and adults aged 24 weeks after birth);
- four test batteries (A: entailing the attentional set-shifting task; B: entailing the Barnes maze test, the intra-peritoneal glucose tolerance test (IPGTT), and the insulin sensitivity test (IST); C:

entailing the T-maze test, the open-field test, the novel object recognition test, the elevated zero-maze test, and the marble burying test; D: entailing the metabolic cages) (*figure 1*). Each battery lasted three weeks in order to keep testing within the required developmental stage of the animals.

The complexity of the mice's experimental genotype may have required more than two control groups to assess each genomic construct (i.g. the floxed sequences, the *kcnq1* gene in heterozygosis and in homozygosis). However, the selection of the aforementioned control groups allowed valid and clearly interpretable results, also resting upon the 3Rs principle. Specifically, the Ctrl1 group was used to rule out the possibility that the observed effects were due to tamoxifen, while the Ctrl2 group addressed potential effects related to the Cre construct. Under these conditions, the effect of the knockout can only be ascertained if a significant difference is observed between knockout mice and both control groups.

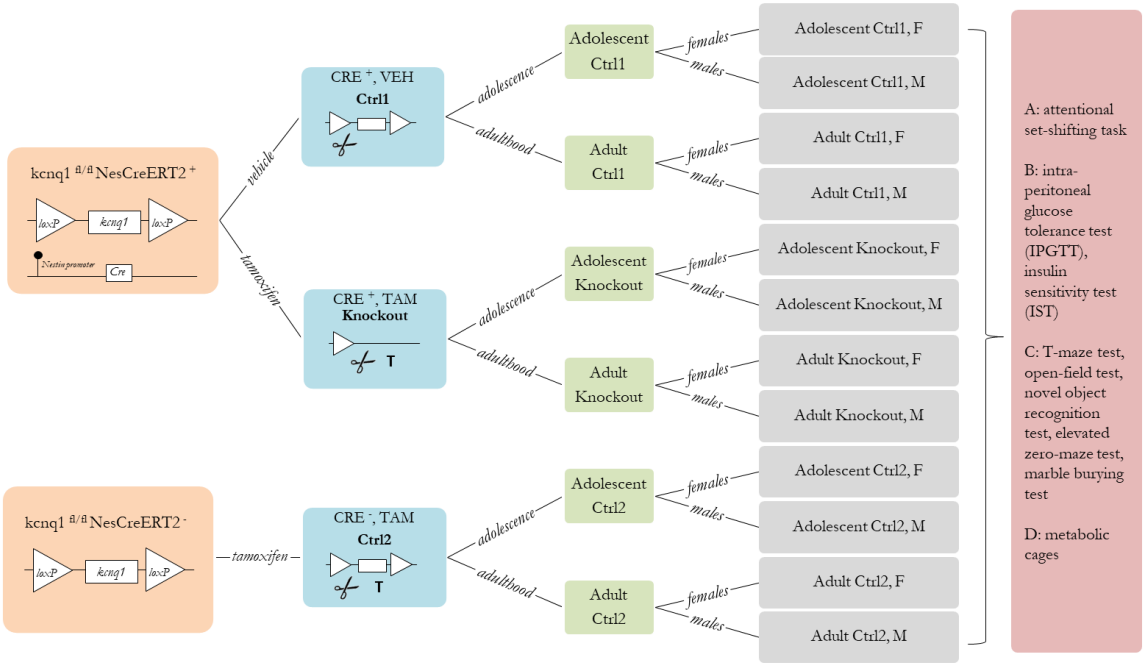

Figure 1. Schematic representation of the experimental plan.

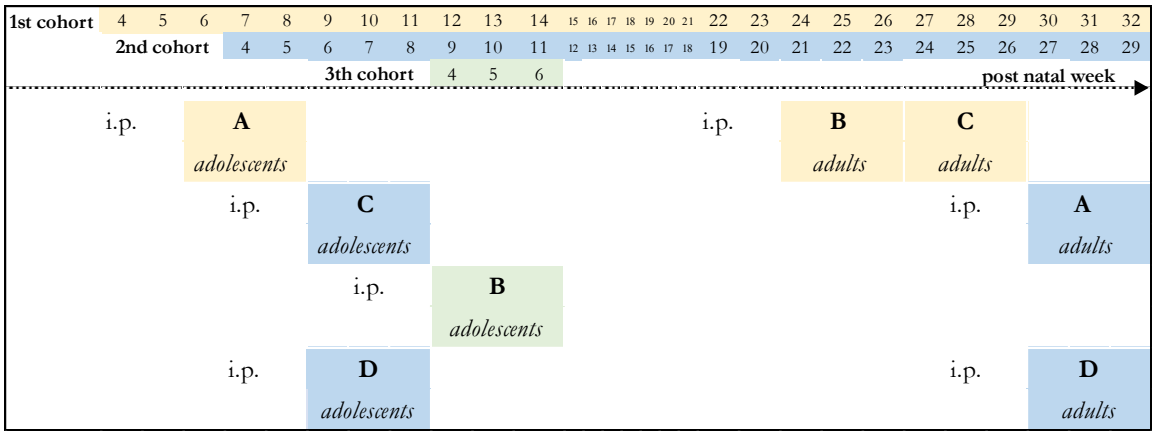

Figure 2. The three experimental cohorts of subjects derived from different interbreeding (indicated by the same colour) to complete the entire experimental plan.

Metabolic tests

Metabolic investigation included intra-peritoneal glucose tolerance test (IPGTT), insulin sensitivity test (IST), and housing in fully automated metabolic cages which provided continuous data on energy expenditure, lipid metabolism, food and water intake, and general locomotion.

Intra-peritoneal glucose tolerance test (IPGTT)

Glycaemia was assessed through blood glucose concentration obtained with "tail-nick" technique, which allows blood collection while respecting animal welfare and minimizing perceived suffering [35]. Blood glucose concentration was determined at time 0 (baseline concentrations before glucose administration), and then 30, 60, 120 and 180 minutes after an intra-peritoneal glucose injection of 2g/kg (D-glucose 10%; Sigma, St. Louis, MO, USA). Mice were tested in the morning (at 9.00 a.m.) after 15-h of food deprivation.

Insulin sensitivity test (IST)

Blood glucose concentration was determined, adopting the "tail-nick" procedure, at time 0 (baseline concentrations before insulin administration), and then 15, 30, 60, 120 and 180 minutes after an intra-peritoneal insulin injection of 0.4 U/kg (Humulin R, Eli-Lilly, 100 U/mL, Indianapolis IN, United States). Mice were tested in the morning (at 11.00 a.m.) after 2-h of food deprivation.

Metabolic cages

Mice were individually tested in metabolic cages (*PhenoMaste*r, TSE System GmbH, Berlin, Germany) which allowed the automatic analyses of baseline metabolism. 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*). Each cage was continuously provided with fresh air from an air pump via the Control Unit. All measurements were taken every 7 minutes (cycle interval) throughout the entire session but the parameter's analyses started after a 24-h of habituation. Values were normalized to body weight. In detail, the following parameters were obtained: (*i*) general locomotion defined as the total number of infrared beam breaks in the X and Y-axes; the software registers the number of beam interruptions caused by the animal's movement, with a grid of 2-dimensional infrared sensors placed around the cage and located every 5 mm; (*ii*) actual speed (cm/s) with reference to the cycle interval; (*iii*) food and water intake through the automatic weight of a food hopper and a water bottle located at the top of the cage; the removal of liquid or food pellets from the dispensers was registered by the scale sensors and the alterations were registered by the software; (*iv*) indirect calorimetry measures oxygen consumption and carbon dioxide production

through an open-circuit system and gas-sensing units to calculate resting energy expenditure (EE) as a measure of heat production and respiratory exchange ratio (RER) defined as the volume of carbon dioxide released over the volume of oxygen absorbed during respiration (vCO_2/vO_2). The RER indicates the predominant substrate being metabolized and it can range from 0.7 (utilization of fats as the primary energy source.) to 1.0 (utilization of carbohydrate as the primary energy source). Mice were placed in metabolic cages for 4 days, single housed, with *ad libitum* water and food, on a 12-hours reversed light-dark cycle (lights on at 19:30).

Behavioural tests

The different cognitive domains were analysed through: the Barnes maze test to assess short and long-term spatial memory, T-maze to evaluate the spontaneous alternation behaviour, open-field to assess spontaneous locomotion and anxiety-like behaviour, novel object recognition to analyse recognition memory, elevated zero-maze to evaluate anxiety-like behaviour, and attentional set-shifting task to assess executive functions like attentional and cognitive flexibility. An automated video recording and video-tracking system (ANY-maze software) collected the behavioural measures obtained in of the open-field test, the novel object recognition test, and the elevated zero-maze. All assessments were conducted under red light.

Barnes maze test

The maze (*Barnes Maze 40193*, Ugo Basile® S.r.l., Gemonio, VA, Italy) consisted of a circular platform (60 cm above the floor, with a diameter of 100 cm) with 20 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 in an aversive environment for rodent (a lightly room), providing a motivation to locate the escape box. The experimental protocol consisted of one day of *habituation phase*, five days of *acquisition phase* and two *probe trials*, conducted respectively 24 hours (day 7) and seven days (day 13) after the last day of the *acquisition phase*. 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. These phases allowed the animal to learn the position of the escape box on the apparatus, in relation to spatial cues surrounding the maze in the experimental room. The escape box contained a handful

of sawdust bedding, sampled from the home cage of the experimental subject to increase its rewarding nature.

The first *probe trial* "probe 24 hours" (day 7), performed 24 hours after 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. The latency to enter in the target area was recorded in both phases. At the end of each trial the platform was cleaned with a 30% ethanol/water solution.

T-maze test

The T-maze test was employed to evaluate the spontaneous alternation behaviour and working memory. The apparatus consisted of a T-shaped Plexiglass maze with three lockable equally sized arms (24 cm L x 10 cm W x 8 cm H). Leaving the starting compartment, the subject was allowed to explore and chose one of the arms with a cut-off of two minutes. At the choice of one of the alternative arms, the data (latency to enter and left *vs* right arm) were collected, the door closed and the animal was gently relocated in the starting compartment to perform a second-choice trial. During the second-choice trial, 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. All subjects performed a total of ten sessions (two sessions of two trials per day, with a 3-hour interval), for five consecutive days. The percentage of spontaneous alternations, expressed as the number of alternated trials on the total sessions x 100, was calculated. Cut-off trials were detracted from the total. When the animal completed the session, the maze was cleaned with a 30% ethanol/water solution.

Open field (OF) test

Open field test was performed to assess general locomotor activity and anxiety-like behaviour as the apparatus (*Open Field 47432*, Ugo Basile S.r.l., Gemonio, Italy) consisted of an exposed central area and a protected peripheral zone surrounded by grey PVC walls (40 cm L x 40 cm W x 30 cm H). Mice were placed at the centre of the arena and were allowed to freely explore it for 10 minutes. The ANY-maze software virtually divided the arena in two equally-sized areas of 800 cm^2 (centre and periphery) and recorded the total distance travelled, the average speed and time spent in the different zones. At the end of the test, the arena was cleaned with a 30% ethanol/water solution.

Novel objects recognition (NOR) test

The novel object recognition test was used to assess short and long-term recognition memory. A mouse was presented with two similar objects during the first session, and then one of the two objects was replaced by a new object during a second session. The amount of time taken to explore the new object provides an index of recognition memory. The test involved a habituation phase, which corresponded to the open-field test, an acquisition phase and two test phases, conducted one hour (short-term recognition memory) and 24 hours after the acquisition (long-term recognition memory). During acquisition, the subject was located at the centre of the arena and was allowed to explore two identical objects placed next to two adjacent corners symmetrically equidistant (10 cm) from the walls of the arena. During the test phases, one of the objects already familiarised with was replaced with another one with the same size but different shape and colour. All the testing phases lasted 10 minutes. Shape and material (glass, plastic or metal) of familiar or novel object, as well as the relative position, were counterbalanced and randomized for each experimental subject. The ANY-maze software virtually divided the arena into two equally-sized quadrants surrounding the familiar and the new object. Time spent to explore the new object and the novel object percent preference [(time spent exploring novel object/time spent exploring both objects) x 100] represented the primary dependent variables. When the animal completed the test, the arena was cleaned with a 30% ethanol/water solution.

Elevated zero-maze test

The elevated zero-maze was used to evaluate anxiety-like behaviour; it is based on the natural tendency of rodents to explore an unknown environment and at the same time the fear of open, unprotected, elevated and therefore potentially risky spaces. The apparatus (*Elevated Zero-Maze 40163*, Ugo Basile S.r.l., Gemonio, Italy) consisted of a circular platform of a diameter of 60 cm, raised 62 cm above the floor. Two opposing sectors (5 cm wide) were protected by 16 cm high walls (closed sectors), and the two remaining sectors were unprotected (open sectors). The subject was placed in one of the two closed sectors, individually, and each session lasted 6 minutes. Percent time spent in the open sectors (as an index of anxiety) was analysed. When the animal completed the test, the maze was cleaned with a 30% ethanol/water solution.

Attentional set-shifting task (ASST)

ASST was used to examine attentional capabilities and cognitive flexibility. Animals are required to learn a rule such as the association of an action (digging in different-scented medium) with a reward (a quarter of Cheerios® cereal) and then, upon the achievement of the learning criterion, they are requested to disregard such 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 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, three days before testing, mice were allocated to a food restriction schedule aimed at maintaining 85-90% of their *ad libitum* bodyweight. Such food restriction schedule was kept until the end of the ASST. The test started with the habituation phase in which mice can freely explore the apparatus with empty bowls for five minutes. Then nine trials followed: the first three with reward at the bottom of both bowls, from trial four to six, the reward was on top of the sawdust-filled bowls, and finally, during the last three trials, the cereal was covered by a 2 cm layer of bedding material. The habituation ended when the mouse ate the reward from both bowls. During the testing phase, which lasted five consecutive days, mice performed five different stages in which they had to discriminate, *via* trials and errors, the right stimulus (odour or medium) associated with the reward. The rewarded stimulus changed during the different stages (*figure 3*). A trial started with the mouse placed in the starting compartment, and the sliding door providing access to the rewarded bowls were closed; then, the operator raised the doors to allow access to the two choice compartments. The same trial ended when the animal dug into a bowl (simple contact or sniffing were not considered as digging) and the correctly or incorrectly choice was recorded. Afterwards, mouse was relocated in the starting area, the door closed, and a new trial performed. During the first four trials (exploratory trials), mice were allowed to dig into both bowls in order to learn the right association. In contrast, on subsequent trials, the door was closed after any correct or incorrect choice. All the stages required eight correct responses out of 10 consecutives trials to be considered completed. The five stages proceeded as follows: during the simple discrimination (SD) only one dimension (olfactory) was introduced, and mice had to discriminate between two odours while the medium was always sawdust; during the compound discrimination (CD) a new medium, as confounding stimulus, was introduced, but the rewarded stimuli remained the same of SD; then, following the reversal learning paradigm, during the successive stage (compound discrimination reversal, CDR) the rewarded stimulus was the opposite to the one rewarded during CD; in the intra-dimensional shift (IDS) two new stimuli and odours were introduces but the rewarded one remained the odour; in the last stage (extra-dimensional shift, EDS) the rewarded stimulus was the medium and thus mice had to shift their attention to a previously unrewarded category. 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	Rewarded dimension	Rewarded stimulus	Discriminants
SD	smell	cinnamon	sawdust with *cinnamon* or sage
CD	smell	cinnamon	sawdust or cotton with *cinnamon* or sage
CDR	smell	sage	sawdust or cotton with cinnamon or *sage*
IDS	smell	ginger	crepe-paper or confetti with *ginger* or coriander
EDS	tact	polystyrene	*polystyrene* or tissue-paper with cloves or rosemary

Figure 3. Table shows the arrangement of the provided discriminants and rewarded-stimuli (in *italics*) during all the stages of the ASST; simple discrimination (SD), compound discrimination (CD), compound discrimination reversal (CDR), intra-dimensional shift (IDS), extra-dimensional shift (CDR).

Marble burying test

This test was applied as a measure of compulsive-like behaviours, it used the propensity of rodents to dig and persistently cover harmless objects as an indicator of obsessive compulsive tendencies. Twelve glass marbles were placed in a Plexiglas cage (38cm Lx 21cm Wx 18 cmH, Tecniplast S.p.A.), on the surface of bedding sawdust, arranged in four rows of three marbles each. Animals were individually placed in the cage, and left free to explore it for 30 minutes. At intervals of 10 minutes, the number of buried marbles was counted. A marble was considered buried when covered almost for 75% with bedding. At the end of the test, the mouse was removed and the cage cleaned with a 30% ethanol/water solution.

Statistical analysis

All statistical analyses were conducted using the software StatView 5.0.1 (SAS Institute Inc., Cary, NC, USA). The experimental design entailed three experimental groups ($Kcnq1^{flox/flox}$/Nestin-$CreERT2^{+}$ treated with the vehicle, $Kcnq1^{flox/flox}$/Nestin-$CreERT2^{+}$ treated with tamoxifen, and $Kcnq1^{flox/flox}$/Nestin-$CreERT2^{-}$ treated with tamoxifen) x two sexes (males vs. females) x two developmental stages (adolescence and adulthood) x *n* repeated measures (variable depending on the specific test) and their interactions. Experimental data have been analysed through repeated analysis of variance (ANOVA) for split-plot designs. Age, group, and sex constituted between subject factors and all the other variables constituted within-subject factors. Post-hoc comparisons were performed using Tukey significant difference test. Significance level was set at $p<0.05$. Five subjects were removed from the analyses of the general locomotor activity due to a problem of the metabolic cages' sensors. The number of experimental groups was established as a power $1-b=0.80$ and type I error probability $a=0.05/3=0.016$ in a Student's t test (for independent samples) at 1 tails, in order to highlight a difference between the three groups equal to 25% of the mean value observed in the

control group, (corresponding to a large effect, Cohen's d=0.9 for behavioural test and Cohen's d=1.1 for metabolic analyses). Based on the power analysis performed, 24 animals per group were required, except for the ASST and metabolic test wherein 16 animals per group were needed.

Results

Metabolic analyses

Body weight

As expected, male subjects were heavier than female mice both in adolescence (sex: $F_{1,74}$=69.12; p<0.0001, fig. **4a**) and adulthood (sex: $F_{1,44}$=101.35; p<0.0001, fig. **4a**). However, along the developmental weeks (adolescence: week*group $F_{4,148}$=1.17; p=0.32; adulthood: week*group $F_{4,88}$=0.87; p=0.48), the knockout did not influence the body weight in both sexes (females, fig. **4b**; males fig. **4c**).

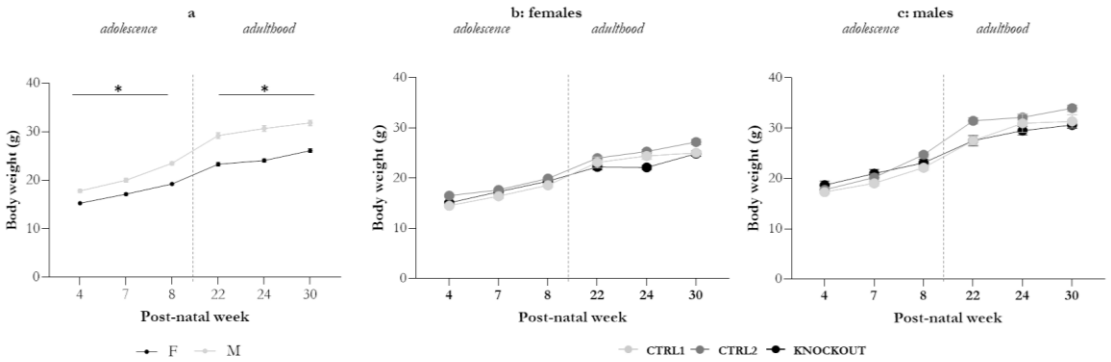

Figure 4. Body weight expressed in grams (mean ± standard error) along the developmental weeks; **a** ***** p<0.05 between adolescent females (n 51) and adolescent males (n 53), ***** p<0.05 between adult females (n 23) and adult males (n 28); **b** females: adolescent ctrl1 (n 18), adolescent ctrl2 (n 14), adolescent knockout (n 19), adult ctrl1 (n 6), adult ctrl2 (n 11), adult knockout (n 6); **c** males: adolescent ctrl1 (n 18), adolescent ctrl2 (n 23), adolescent knockout (n 12), adult ctrl1 (n 7), adult ctrl2 (n 12), adult knockout (n 9).

Food intake

Experimental data show the daily amount of food intake, automatically recorded in metabolic cages. During adulthood (age x group: $F_{2,61}$=5.99; p=0.004; fig. **5**), regardless of sex, knockout mice had a higher daily food intake compared to ctrl2 subjects (p<0.05 in post-hoc test).

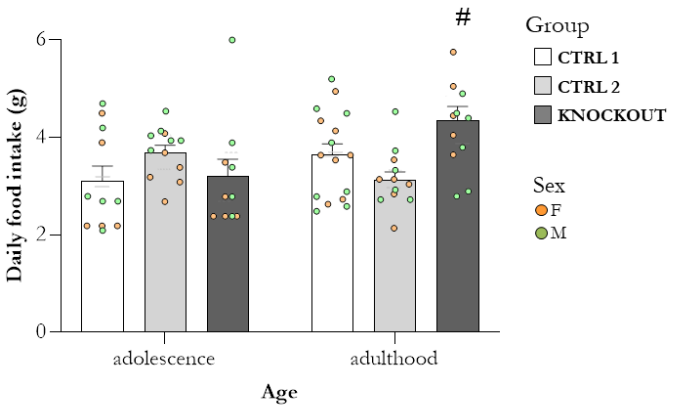

Figure 5. Daily food intake expressed in grams (mean ± standard error) recorded in automatic metabolic cages; **#** p<0.05 in the comparison between knockout (n 12) vs ctrl2 (n 12) in adulthood.

Glucose tolerance test

Glucose administration produced the expected physiological response in all experimental groups whereby blood glucose concentrations where higher than baseline after the injection and gradually decreased back to baseline values three hours later (time points after glucose injection: $F_{4,208}$=44.78; p<0.0001). Moreover, data showed that glucose tolerance was not affected by age ($F_{1,52}$=0.11; p=0.74), and by the absence of the gene both in adolescence ($F_{2,15}$=0.06; p=0.94, fig. **6a**) and adulthood ($F_{2,37}$=0.83; p=0.44 fig. **6b**). Finally, while in adolescence sex had no impact on glucose tolerance ($F_{1,27}$=2.09; p=0.16, fig. **6c**), it affected this parameter in adulthood ($F_{1,37}$=6.63; p=0.01). Specifically, female subjects had a lower blood glucose concentration compared to male mice (time x sex: $F_{8,148}$=2.61, p=0.04; fig. **6d**) 60 and 120 minutes after the injection of glucose (p<0.05 in post-hoc test).

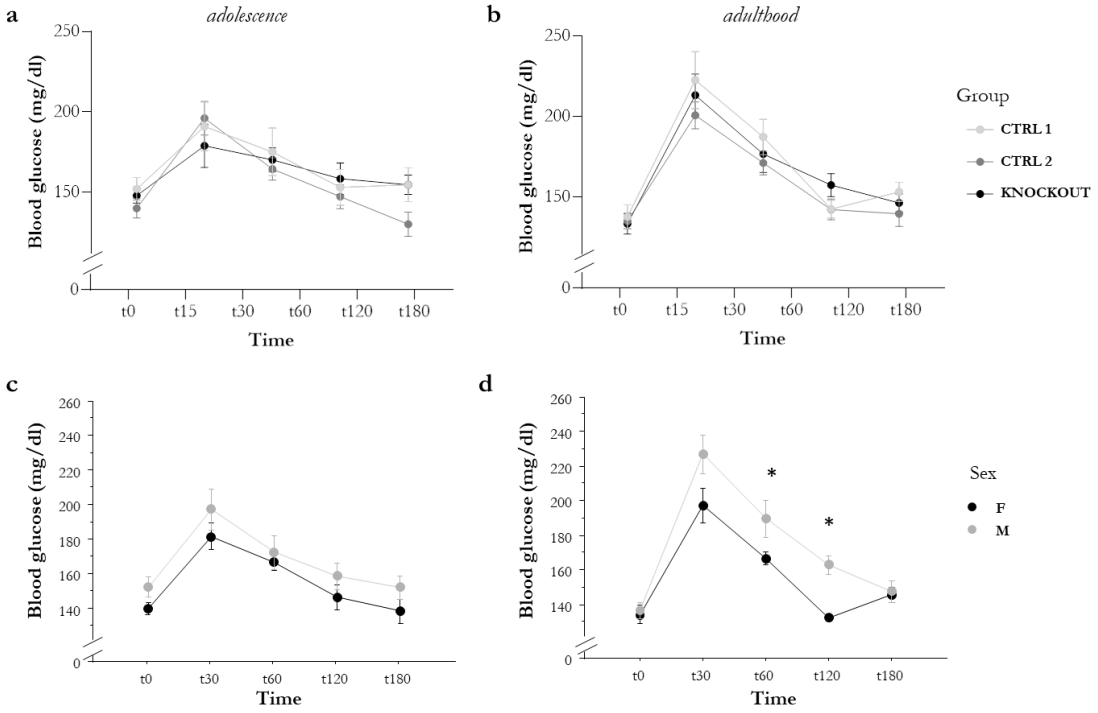

Figure 6. Glycaemia analysed as milligrams of glucose on decilitres of blood (mean ± standard error) at baseline (t0) and 30, 60, 120, 180 minutes after glucose injection, during **a** adolescence and **b** adulthood; blood glucose concentration (average of all time points + standard error) during **c** adolescence and **d** adulthood: ***** $p<0.05$ between adult female mice (n 21) and adult male mice (n 22) (t60, t120).

Insulin sensitivity test

Insulin administration produced the expected physiological response in all experimental groups whereby blood glucose concentrations where lower than baseline after the injection and gradually returned to baseline concentrations three hours later (time points after insulin injection: $F_{5,375}=89,46$; $p<0.0001$). The knockout did not influenced insulin sensitivity in adolescence ($F_{2,33}=0.42$; $p=0.66$, fig. **7a**) and adulthood ($F_{2,42}=0.67$; $p=0.51$, fig. **7b**). However, there was an effect of sex in both developmental stages (fig. **7c-d**). Specifically, female subjects had a lower blood glucose concentration compared to male mice (time x sex: adolescence $F_{5,165}=2.69$; $p=0.02$; adulthood $F_{5,210}=3.32$, $p=0.007$) at the baseline (T0) and 15, 30 minutes after the injection of insulin ($p<0.05$ in the post-hoc test).

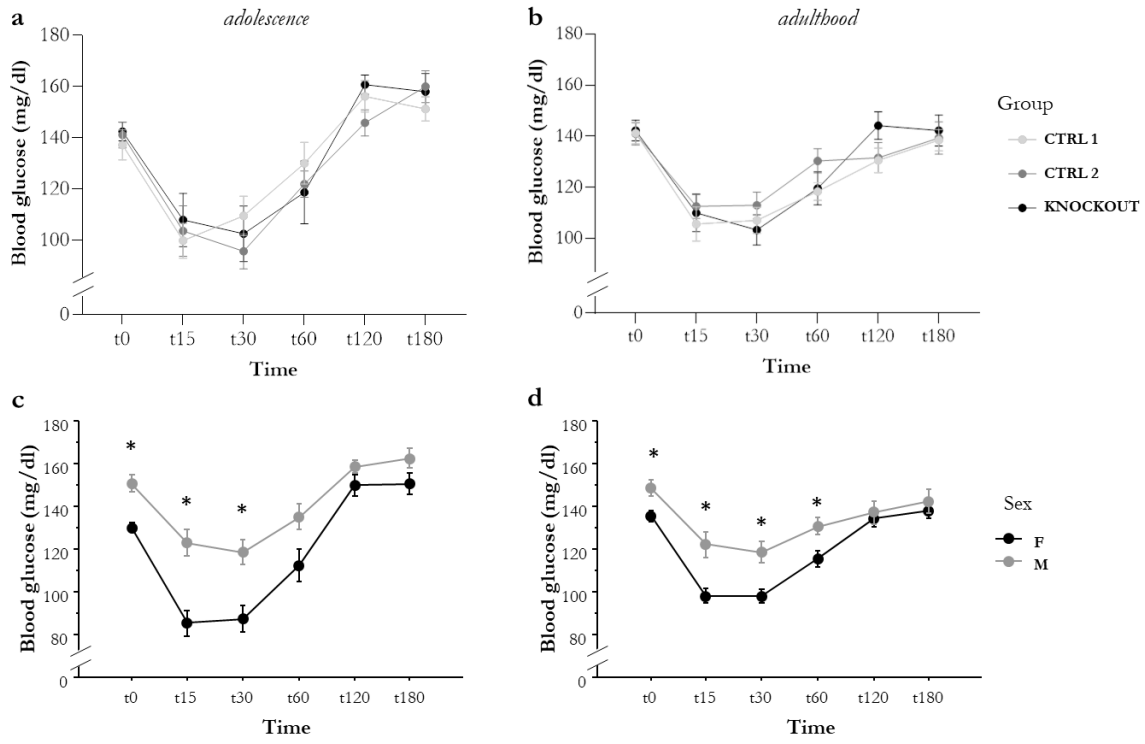

Figure 7. Glycaemia analysed as milligrams of glucose on decilitres of blood (mean ± standard error) at baseline (t0) and 15, 30, 60, 120, 180 minutes after insulin injection, during **a** adolescence and **b** adulthood; blood glucose concentration (average of all time points + standard error) during **c** adolescence: ***** $p<0.05$ in the comparison between female mice (n 20) and male mice (n 19) (t0, t15, t30), and during **d** adulthood: ***** $p<0.05$ between female mice (n 22) and male mice (n 26) (t0, t15, t30, t60).

Metabolic cages

Energy expenditure, general metabolism and general locomotion of mice were automatically assessed in metabolic cages. All these parameters significantly changed during the day, following the natural circadian rhythm of both adolescent and adult mice (time: RER: $F_{48,2976}=55.39$; $p<0.0001$, energy expenditure: $F_{48,2976}=50.07$; $p<0.0001$, locomotor activity: $F_{48,2688}=23.28$; $p<0.0001$, speed: $F_{48,2688}=9.15$; $p<0.0001$).

The value of the respiratory exchange ratio (RER) reflects the energy substrate (carbohydrates vs. fat) used by experimental subjects to produce heat. Adults had a lower RER compared to adolescents (age: $F_{1,62}=20.47$; $p<0{,}0001$, fig. **8**). Moreover, while RER did not differ between groups in adulthood (group: $F_{2,35}=1.48$; $p=0.25$, fig. **9b**), it was lower in knockout mice compared to both control groups in adolescence (group: $F_{2,27}=9.58$; $p=0.0007$; $p<0.05$ in post-hoc tests, fig. **9a**). Specifically, while mice belonging to the two control groups predominantly used carbohydrates (RER ~ 1) as an energy source, knockout subjects also used lipids to produce energy (RER ~ 0.9). Similar to RER, adolescent mice exhibited higher energy expenditure (EE) compared to adult subjects (age: $F_{1,62}=30.43$; $p<0{,}0001$, fig. **10**). However, differently from RER, while in adolescence

any group-dependent difference was found (group: $F_{2,27}$=1.26; p=0.30, fig. **11a**), during adulthood all groups differed from one another (group: $F_{2,35}$=16.67; p<0.0001, fig. **11b**). Specifically, adult knockout mice had higher EE compared to ctrl2 and lower EE compared to ctrl1 (p<0.05 in post-hoc tests). Additionally, ctrl1 mice had higher EE compared to ctrl2 (p<0.05 in post-hoc test). General locomotion decreased throughout development (age: $F_{1,56}$=25.88; p<0.0001, fig. **12**). Specifically, knockout subjects exhibited reduced locomotion compared to both control groups during adolescence ($F_{2,27}$=9.8; p=0.0006, fig. **13a**) but not in adulthood ($F_{2,29}$=0.36; p=0.69, fig. **13b**).

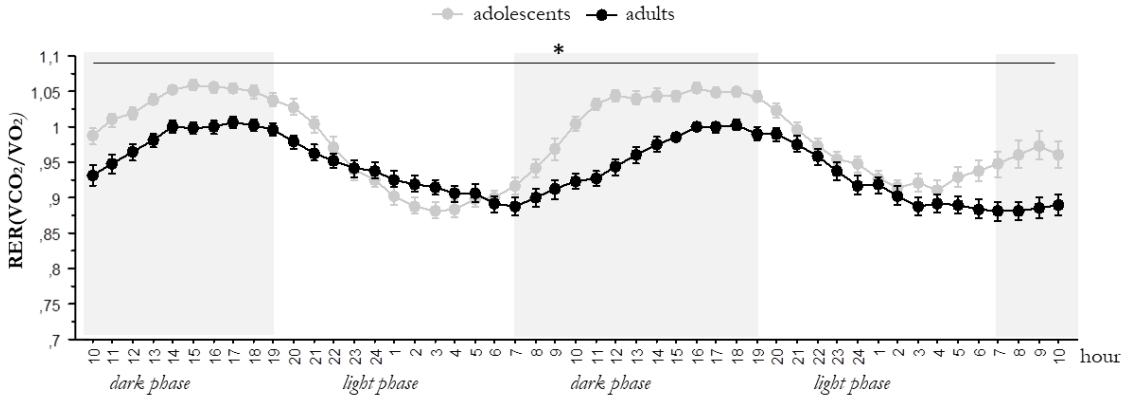

Figure 8. Value of respiratory exchange ratio defined as the volume of carbon dioxide released over the volume of oxygen absorbed during respiration (vCO2/vO2, mean ± standard error); ***** p<0.05 between adolescents (n 33) and adults (n 40).

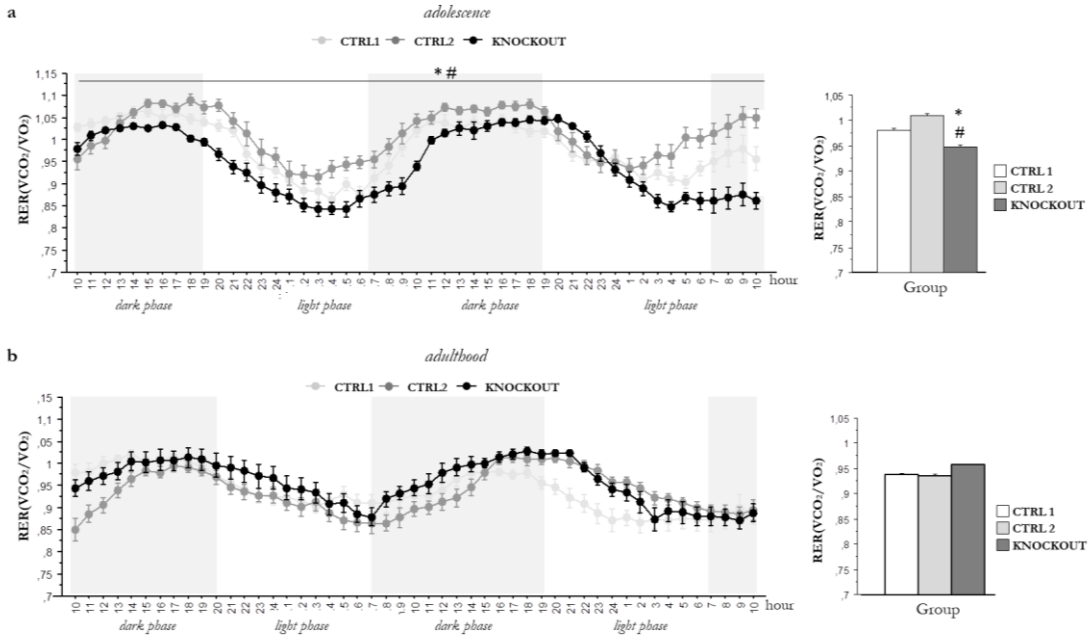

Figure 9. Respiratory exchange ratio expressed as volume of carbon dioxide released over volume of oxygen absorbed during respiration (vCO2/vO2, mean ± standard error); **a** adolescence: * p<0.05 between knockouts (n 10) and ctrl 1 (n 11), **#** p<0.05 between knockouts and ctrl 2 (n 12); p<0.05; **b** adulthood.

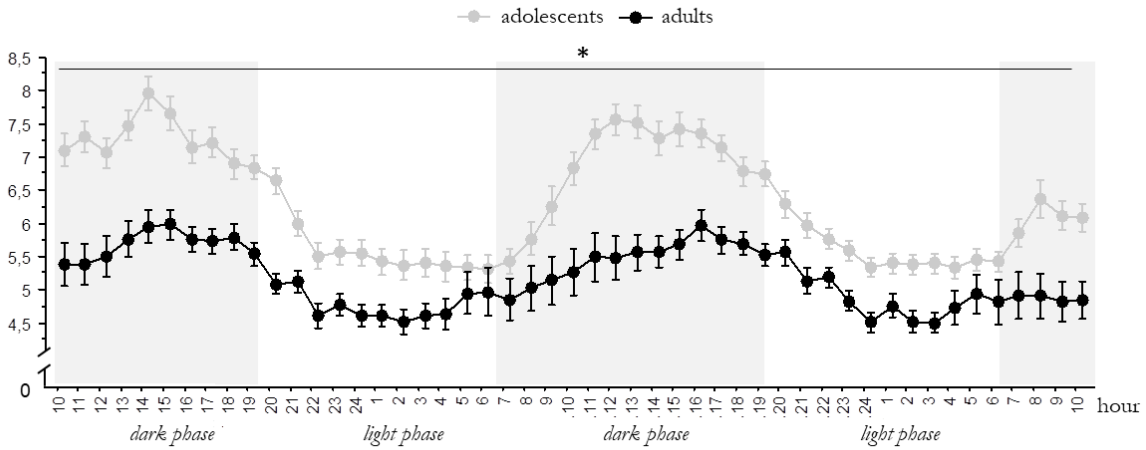

Figure 10. Energy expenditure value (Kcal/h/Kg, mean ± standard error); ***** p<0.05 between adolescents (n 33) and adults (n 40).

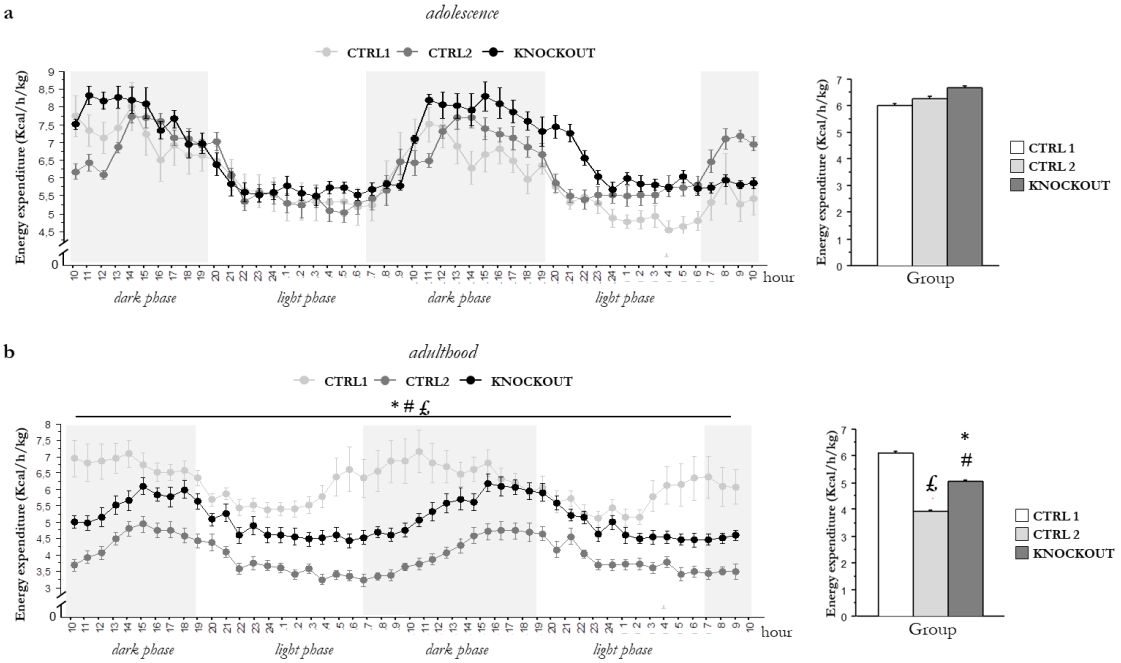

Figure 11. Value of energy expenditure (Kcal/h/Kg, mean + standard error) in **a** adolescence and **b** adulthood; adults: ***** p<0.05 between knockout (n 12) and. ctrl1 (n 17), **#** p<0.05 between knockout and ctrl2 (n 12), **£** p<0.05 between ctrl1 and ctrl 2.

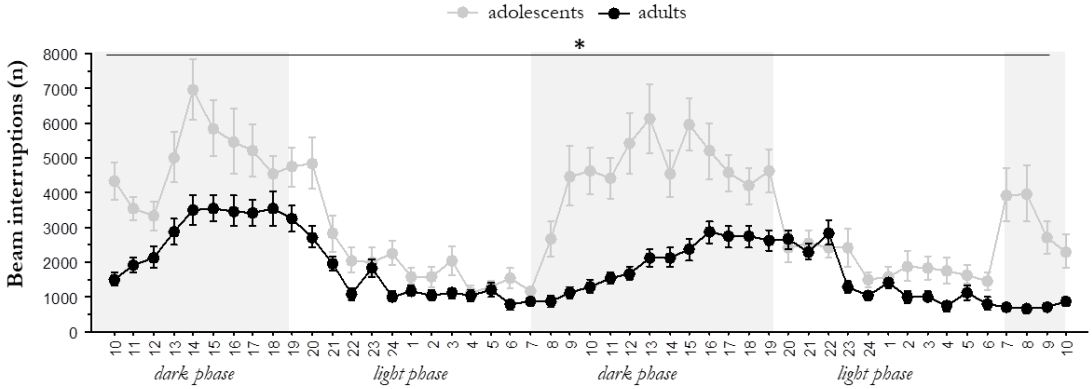

Figure 12. General locomotor activity analysed as number of infrared beam interruptions caused by the animal's movement (mean ± standard error); ***** $p<0.05$ between adolescents (n 33) and adults (n 35).

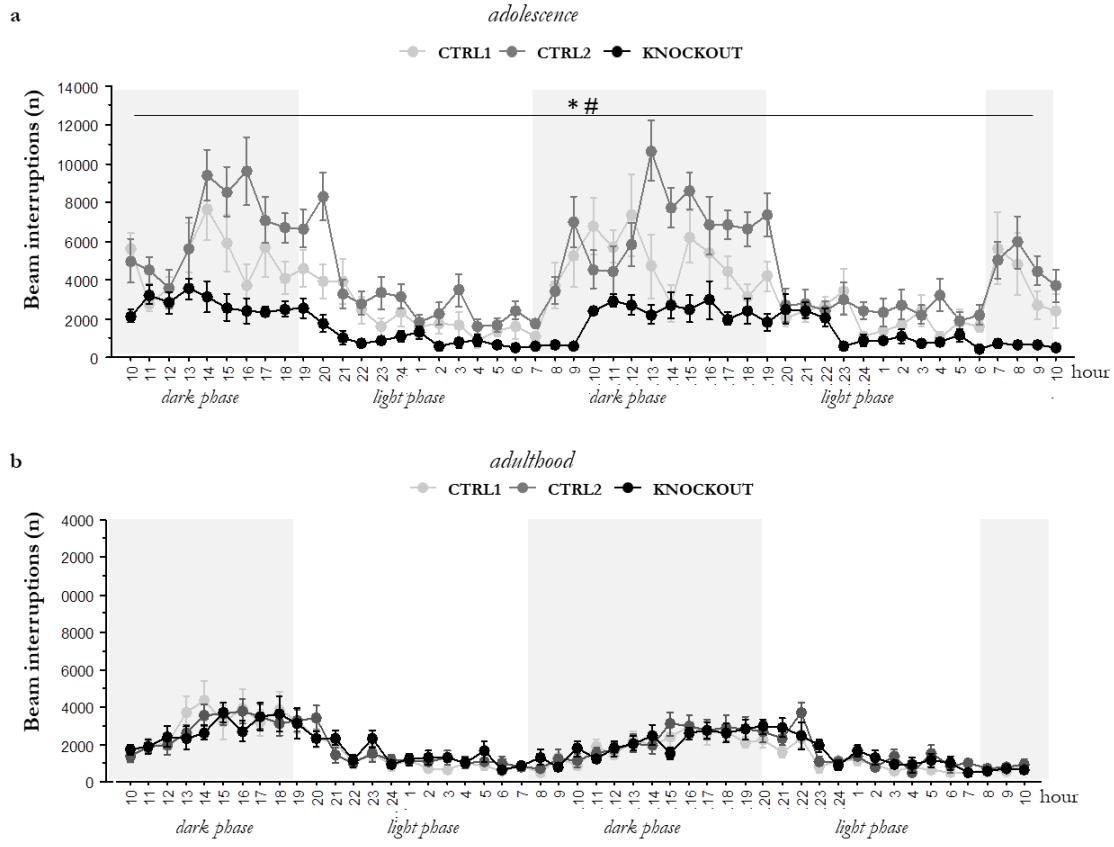

Figure 13. General locomotor activity (mean ± standard error) analysed as number of infrared beam interruptions caused by the animal's movement in automatic metabolic cages during **a** adolescence and **b** adulthood; ***** $p<0.05$ between adolescent knockouts (n 10) and ctrl 1 (n 11), **#** $p<0.05$ between adolescent knockouts and ctrl 2 (n 12).

Behavioural tests

Barnes maze test

The spatial learning domain was influenced by age ($F_{1\text{-}98}$=5.91; p=0.07). Specifically, adolescents required more time than adults to reach the target during the first and the second day of acquisition (p<0.05 in the post-hoc test; fig. **14a**). However, all the animals, regardless of treatment, genotype, or sex, successfully learned the position of the escape box as showed by the latency to reach the target. The parameter significantly decreased over the five days of acquisition ($F_{4,392}$=5.42; p=0.0003; fig. **14b-c**).

The developmental stage also affected the short-term spatial memory (Fig. **15c**). Adolescence mice had a higher latency to arrive at the target area than adult mice (age: $F_{1,98}$=12.46; p=0.0006). Moreover, either during adolescence or adulthood, the knockout and the sex did not influenced the short-term (day 7) (adolescence: group $F_{2,49}$=1.39; p=0.26, sex $F_{1,49}$=0.87; p=0.35, fig. **15a**; adulthood: group $F_{2,49}$=0.91; p=0.41, sex $F_{1,49}$=0.53; p=0.47, fig. **15b**) or the long term memory (day 13) (adolescence: group $F_{2,49}$=1.5; p=0.23; sex $F_{1,49}$=1.47; p=0.23; adulthood: group $F_{2,48}$=0.29; p=0.75; sex $F_{1,48}$=1.12; p=0.29).

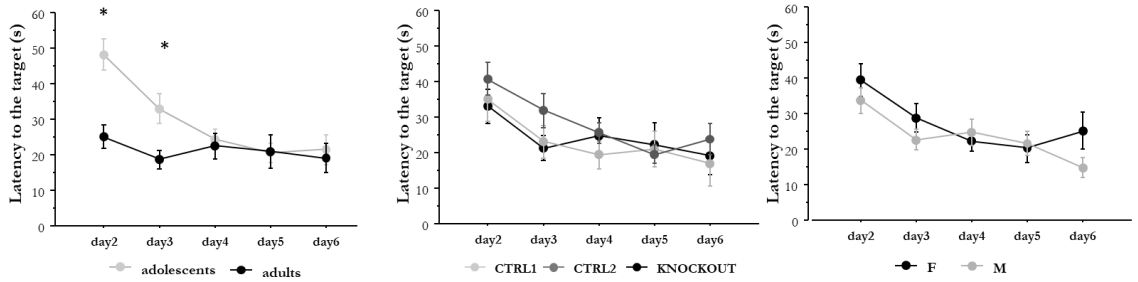

Figure 14. Time to reach the target area expressed in seconds (mean ± standard error) during the 5 days of acquisition stage, between age **a** (adolescence n 55, adults n 55), groups **b** (ctrl1 n 32, ctrl2 n 43, knockout n 35), and sexes **c** (females n 58, males: 52); ***** p<0.05 in the comparison between adolescents and adults the first and the second day of acquisition.

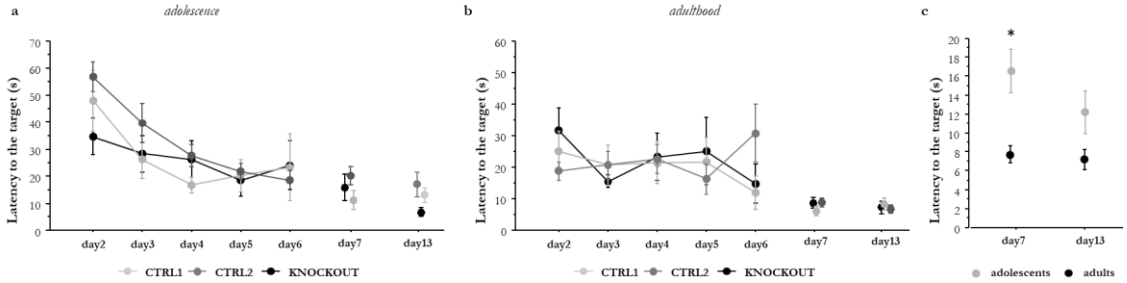

Figure 15. Latency to arrive at the target area in seconds (mean ± standard error) during the spatial learning stage (day1-5) and spatial memory analyses (short-term day6, long-term day13) in

adolescence **a**, and adulthood **b**; latency to the target (mean ± standard error), ***** p<0.05.in the comparison between the two ages.

T-maze

Data from the T-maze test suggested that, while age did not influence the number of alternations in the T-maze test, the sex ($F_{1,117}$=6.69; p=0.009, fig. **16a**) and the knockout did ($F_{2,117}$=15.88; p<0.001, fig. **16b**). Specifically, when pooling data from adolescent and adult mice, males alternated more than female, and knockout subjects had a lower percentage of spontaneous alternations compared to ctrl1 and to ctrl2 groups (knockout vs ctrl1, p<0.05 in the post-hoc test; knockout vs ctrl2, p<0.05 in the post-hoc test).

During adolescence, the knockout group showed a lower percentage of alternations than both controls ($F_{2,66}$=9.344; p=0.0003, p<0.05 in the post-hoc test, fig. **16b**). During adulthood, knockout subjects performed fewer alternations compared to ctrl2 mice (group: $F_{2,51}$=7.21; p=0.0017, p<0.05 in the post-hoc test, fig. **16b**). Moreover, in agreement with these data, while control mice exhibited an intact spontaneous alternation, with values significantly higher than 50%, knockout mice had remarkable impairments in this task (adolescence CI: ctrl1 [58.5-77.3]%, ctrl2 [70.1-78.5]%, knockout [49.5-64.3]%; adulthood CI: ctrl1 [55.5-71.2]%, ctrl2 [65.9-80.2]%, knockout [49-61]%). Specifically, neither adolescent nor adult knockouts exhibited a significant alternation compared to the 50% chance level (fig. **16b**).

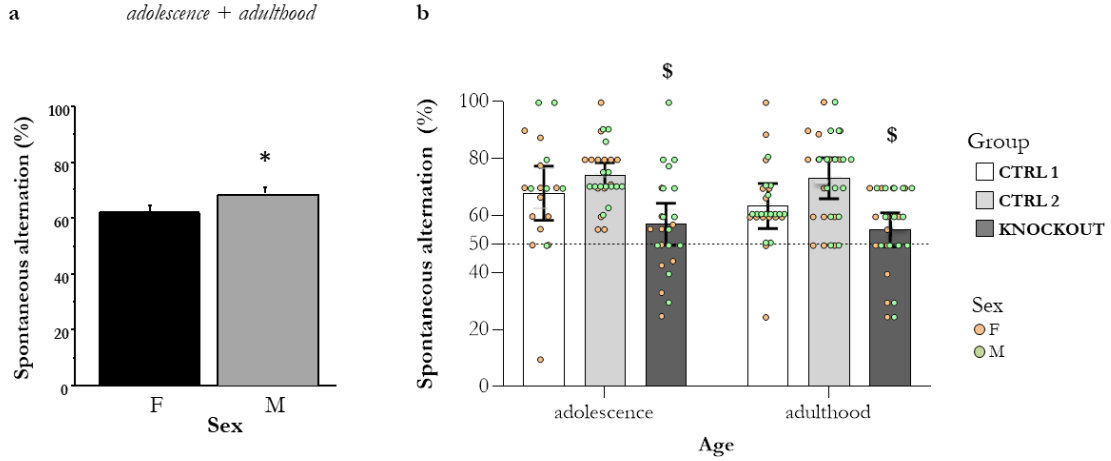

Figure 16. Percentage of spontaneous alternation expressed as the number of alternations divided by the total number of entries in the arms x 100 (mean + standard error), **a *** p<0.05 between females and males pooling ages and groups; **b** percentage of spontaneous alternation with the chance level represented by the dotted line (mean ± 95% confidence intervals) during adolescence and adulthood, **$** knockout's (adolescents n 24, adults n 20) confidence interval intersects the line and indicates that preference index was not statistically different from chance.

Open-field

When placed in a novel and unfamiliar environment, adolescents exhibited an increased locomotor activity compared to adult subjects (age: $F_{1,116}$=16.68; p<0.0001, fig. **16**). However, treatment (group: $F_{2,116}$=0.51; p=0.60) and sex ($F_{1,116}$=0.06; p= 0.81) did not influence the distance travelled. In the open-field test, a lower percentage of time spent in the centre of the arena (as an exposed and unprotected environment for mice) is considered an indicator of heightened anxiety-related state. Data suggested that adult subjects spent significantly more time in the centre of the arena (age: $F_{1,116}$=71.77; p<0.0001) compared to adolescent mice (fig. **17**). Furthermore, I observed a significant effect of the genotype in both ages (adolescence: $F_{2,66}$=8.03; p=0.0008, adulthood: $F_{2,50}$=9.04; p=0.0004). Specifically, the knockout group spent less time in the centre of the arena than ctrl2 subjects (p<0.05 in the post-hoc test, fig. **17**).

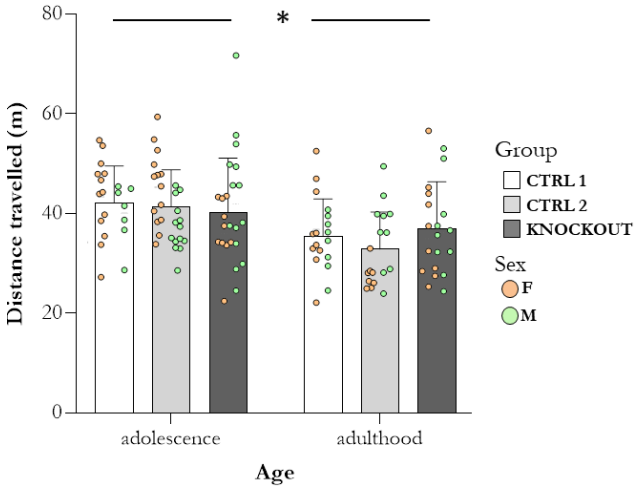

Figure 16. Total distance travelled in the open field arena expressed in meters (mean + standard error); ***** p<0.005 in the comparison between adults (n 56) and adolescents (n 72); subjects were free to explore the area for 10 minutes.

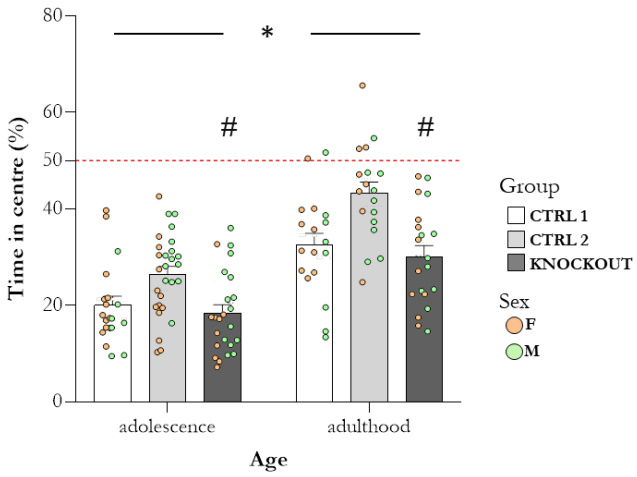

Figure 17. Percentage of time spent in the centre of the arena (mean + standard error); ***** $p<0.05$ between adults (n 56) and adolescents (n 72), **#** $p<0.05$ between knockout (n 24) and ctrl1 (n 28) during adolescence, **#** $p<0.05$ between knockout (n 20) and ctrl2 (n 18) during adulthood, dotted line represents the chance level, the test lasted 10 minutes.

Novel object recognition test

Short- and long-term recognition memory was evaluated through the preference for a novel object over a familiar one. Since the percentage of interaction with the novel object was never significantly different from the chance level (50% exploration), we assumed that all groups failed to exhibit the expected preference (fig. **18**), as also indicated by the CI (adolescence: ctrl1 [43.5-53.2] %, ctrl2 [47.9-55.8] %, knockout [44.9-55.2] %; adulthood: ctrl1 [43.3-57.6] %, ctrl2 [42-57.2] %, knockout [40.4-55,1]%). Such absence of phenotype was exhibited by all experimental groups in the assessment of the short-term memory (fig. **18a-b**), regardless of age ($F_{1,112}=0.17$; $p=0.68$), genotype ($F_{2,112}=0.24$; $p=0.79$) or sex ($F_{1,112}=0.05$; $p=0.82$). Just as for the short-term memory, so also for the long-term memory (fig. **18c-d**), such preference was not expressed (age: $F_{1,115}=0.16$; $p=0.90$, group: $F_{2,115}=0.34$; $p=0.71$, sex: $F_{1,115}=0.23$; $p=0.63$).

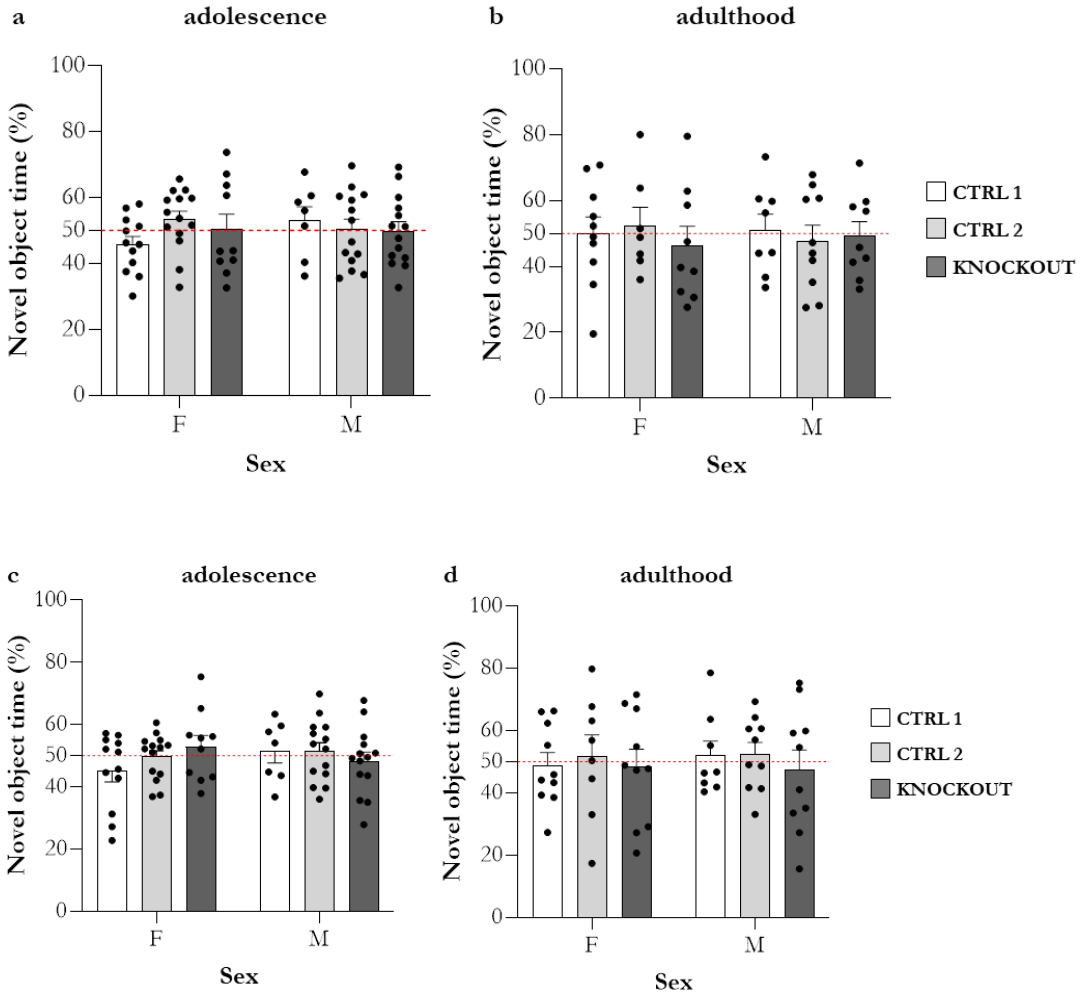

Figure 18. Novel object preference (mean + standard error) during a-b short-term memory assessment (1 hour after the acquisition) and c-d long-term assessment (24 hours after the acquisition); adolescents: knockout (n 24), ctrl1 (n 19), ctrl2 (n 28); adults: knockout (n 20), ctrl1 (n 18), ctrl2 (n 18); dotted line represents the chance level.

Elevated zero-maze

The behavioural phenotype observed in the elevated zero-maze suggests that, compared to adults, adolescents spent more time in the open sectors of the apparatus ($F_{1,107}$=7.55; p=0.007, fig. **19**). Moreover, the knockout reduced the time in the open sectors. Specifically, in both ages, knockouts spent less time in the open sector compared to ctrl2 mice (adolescents: $F_{2,59}$=8.045; p=0.0008; p<0.05 in the post-hoc test; adults: $F_{2,48}$=7.279; p=0.0017; p<0.05 in the post-hoc test, fig. **19**).

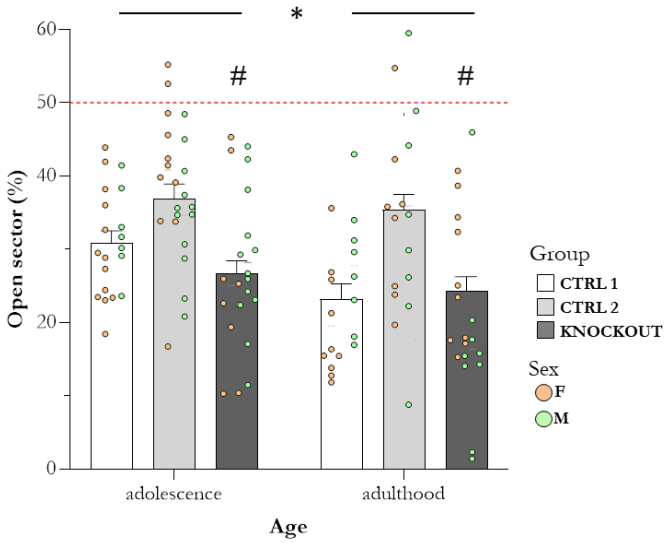

Figure 19. Percentage of time in the open sectors (mean + standard error) expressed as seconds in the open sector divided by the total time of the session (six minutes) *100; ***** p<0.05 between adolescents (n 65) and adults (n 54); **#** p<0.05 between knockout (n 22) and ctrl2 (n 23) during adolescence; **#** p<0.05 between adult knockout (n 19) and adult ctrl2 (n 17); dotted line represents the chance level.

Attentional set-shifting task

The age of the subjects ($F_{1,79}$=0.001; p=0.97), the sex ($F_{1,79}$=3.54; p=0.06), and the absence of the gene ($F_{2,79}$=1.57; p=0.21) did not influenced the learning stage of the ASST (SD, fig. **20a-b**).

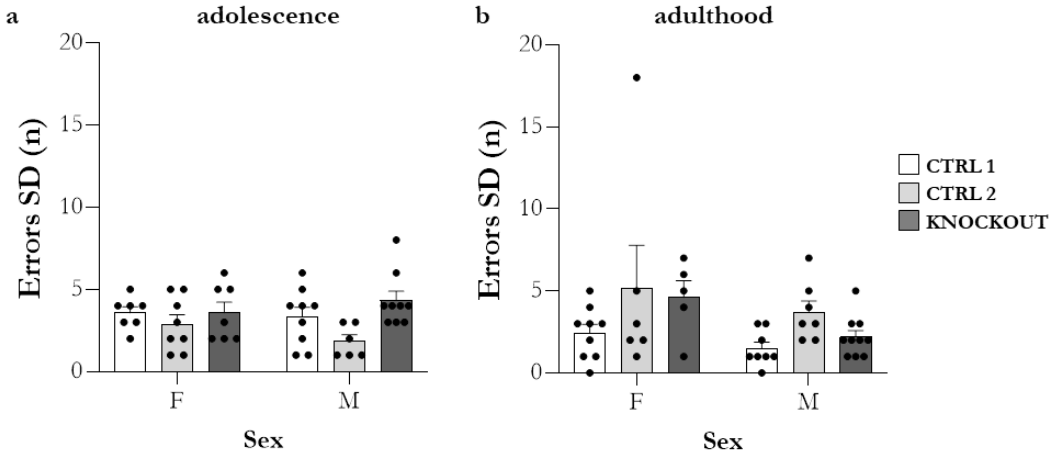

Figure 20. Number of errors committed in the SD stage (mean + standard error); **a** adolescents: ctrl1 F (n 7), ctrl2 F (n 8), knockout F (n 7); ctrl1 M (n 9), ctrl2 M (n 6), knockout M (n 9); **b** adults: ctrl1 F (n 9), ctrl2 F (n 6), knockout F (n 5); ctrl1 M (n 8), ctrl2 M (n 7), knockout M (n 10).

Each stage of the ASST maps on a specific cognitive domain. The number of errors for each stage was consistent with the difficulty to achieve it (stage: $F_{3,237}$=51.75; p<0.001). In particular, the highest number of errors was committed in the CDR stage which assesses the reversal learning capabilities (fig. **21a**). Moreover, the sex influenced the attentional capabilities. Specifically, female mice committed more errors than male mice in the CDR stage (p<0.05 in in the post-hoc test on stage * sex= $F_{3,237}$=2.67; p=0.04, fig. **21b**).

Differently from the acquisition phase (SD), the knockout affected the number of errors committed during each stage. Specifically, knockout adolescents committed more errors to achieve the CDR stage compared to both control groups (group x stage: $F_{6,120}$=2.70; p=0.017, fig. **22a**), while knockout adults only differed from ctrl2 (group x stage: $F_{6,117}$=2.54; p=0.02, fig. **22b**).

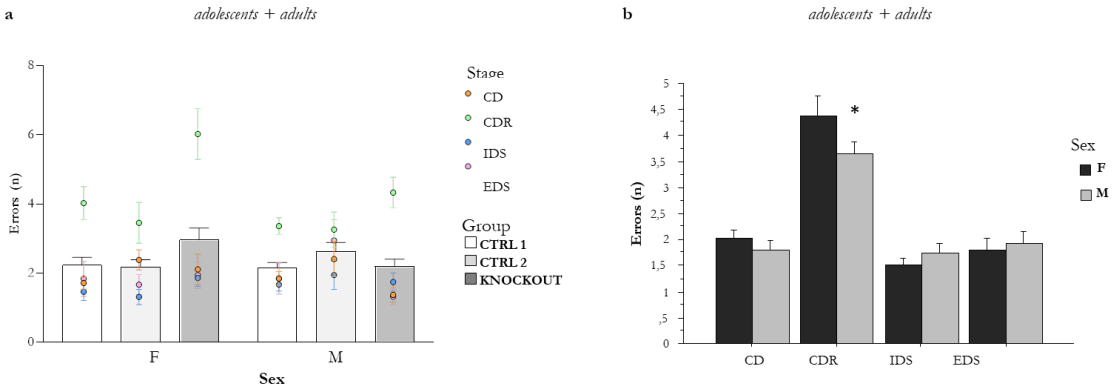

Figure 21. Number of errors committed pooling all the stages of the test (mean + standard error); dots represent the average of errors (± standard error) for each stage pooling adolescents and adults; **a** as showed by the green dot, the CDR required more errors than the other stages being consistent with the difficulty to achieve it; **b *** p<0.05 between females and males in the CDR; CD-compound discrimination, CDR-compound discrimination reversal, IDS-intra dimensional shift, EDS-extra dimensional shift.

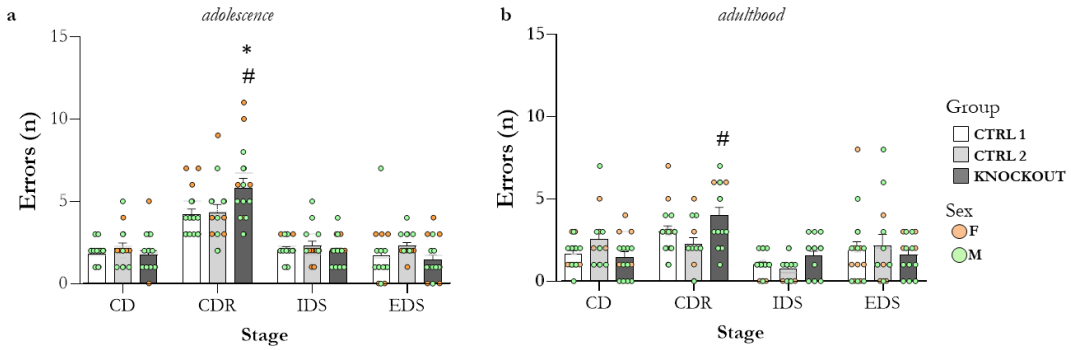

Figure 22. Number of errors for each stage of the test (mean + standard error); **a** adolescents: ***** $p<0.05$ in the comparison between knockout (n 16) and of ctrl1 (n 16), **#** $p<0.05$ in the comparison between knockout and ctrl2 (n 14), during the CDR stage; **b** adults: **#** $p<0.05$ between knockout (n 15) and ctrl2 (n 13) during the CDR.

Marble burying test

In the marble burying test, adult subjects buried significantly more marbles than adolescents ($F_{1,115}=13.43$; $p=0.0004$, fig. **23**). Moreover, while knockout adolescent mice buried fewer marbles than ctrl2 ($F_{2,65}=4.19$; $p= 0.02$; $p<0.05$ in the post-hoc test, fig. **23**), knockout adults did not differ from control groups ($F_{2,51}=0.47$; $p= 0.62$, fig. **23**).

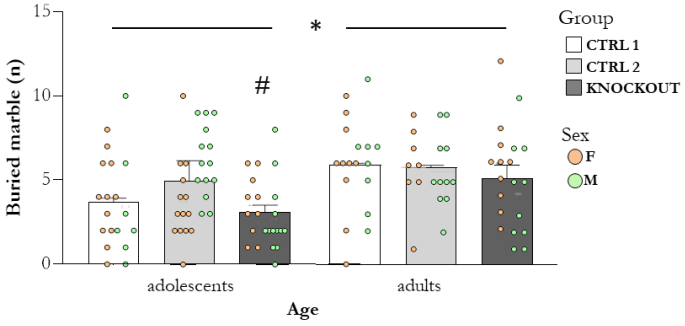

Figure 23. Number of buried marbles (mean + standard error); ***** $p<0.05$ between adolescents (n 70) and adult (n 57); **#** $p<0.05$ between adolescent knockout (n 23) and adolescent ctrl2 (n 28).

Discussion

Insulin signalling is a crucial pathway that affects both peripheral metabolism and cognitive function [36]. Research has shed light on the role of KCNQ1 as a significant player in this interplay [37,38]. In particular, in experimental animal models, the whole-body deletion of *kcnq1* has been shown to induce both metabolic [33] (i.e. altered insulin sensitivity) and cognitive (i.e. behavioural inflexibility and compulsivity disorders) dysfunctions. This indicates that KCNQ1 plays a pivotal role in the

regulation of insulin signalling pathways that are critical for maintaining metabolic homeostasis and cognitive health. However, the challenge arises in delineating the specific contributions of KCNQ1 to central (brain-related) versus peripheral (body-wide) insulin signalling effects. To address this, we selectively deleted *kcnq1* in the CNS while preserving its expression in peripheral tissues. Moreover, age and sex-related differences have been investigated. Three experimental groups were used and tested during adolescence and adulthood: the knockout (expressing Cre, receiving Tamoxifen), ctrl1 (expressing Cre, receiving vehicle), and ctrl2 (not expressing Cre, receiving Tamoxifen). For the results to be valid and clearly interpretable, it is expected that: (i) the KO group should be significantly different from ctrl1, to rule out the possibility that the observed effects are due to tamoxifen rather than the absence of the gene; (ii) the knockout group should also differ from ctrl2, to ensure that the observed effects are not related to the Cre construct, (iii) ctrl1 and ctrl2 should not show significant differences between them, to confirm that any alterations in behaviour or metabolism in the knockout group can be attributed to the deletion of the gene, and not to side effects or methodological factors. Thus, the validity of the results depends on these experimental conditions being met. If this were not the case, it would not be possible to make consistent considerations regarding the *kcnq1* knockout.

Our results reveal clear distinctions between adulthood and adolescence in both metabolic and cognitive functions. Sex-related effects were noted not only in predictable areas, such as body weight and metabolism, but also in the perseverative behaviour and executive functions. However, both age and sex- related effects were independent of *kcnq1* deficiency in the brain. Notably, the metabolic effects of *kcnq1* absence were mainly restricted to adolescence. Specifically, in adolescent knockouts, energy metabolism shifts towards lipids rather than carbohydrates, with lower locomotor activity compared to controls. Regarding cognition, perseverative behaviours and attention deficits were observed in adolescent knockouts, whereas in adults, only compulsivity traits were present (fig. **24**).

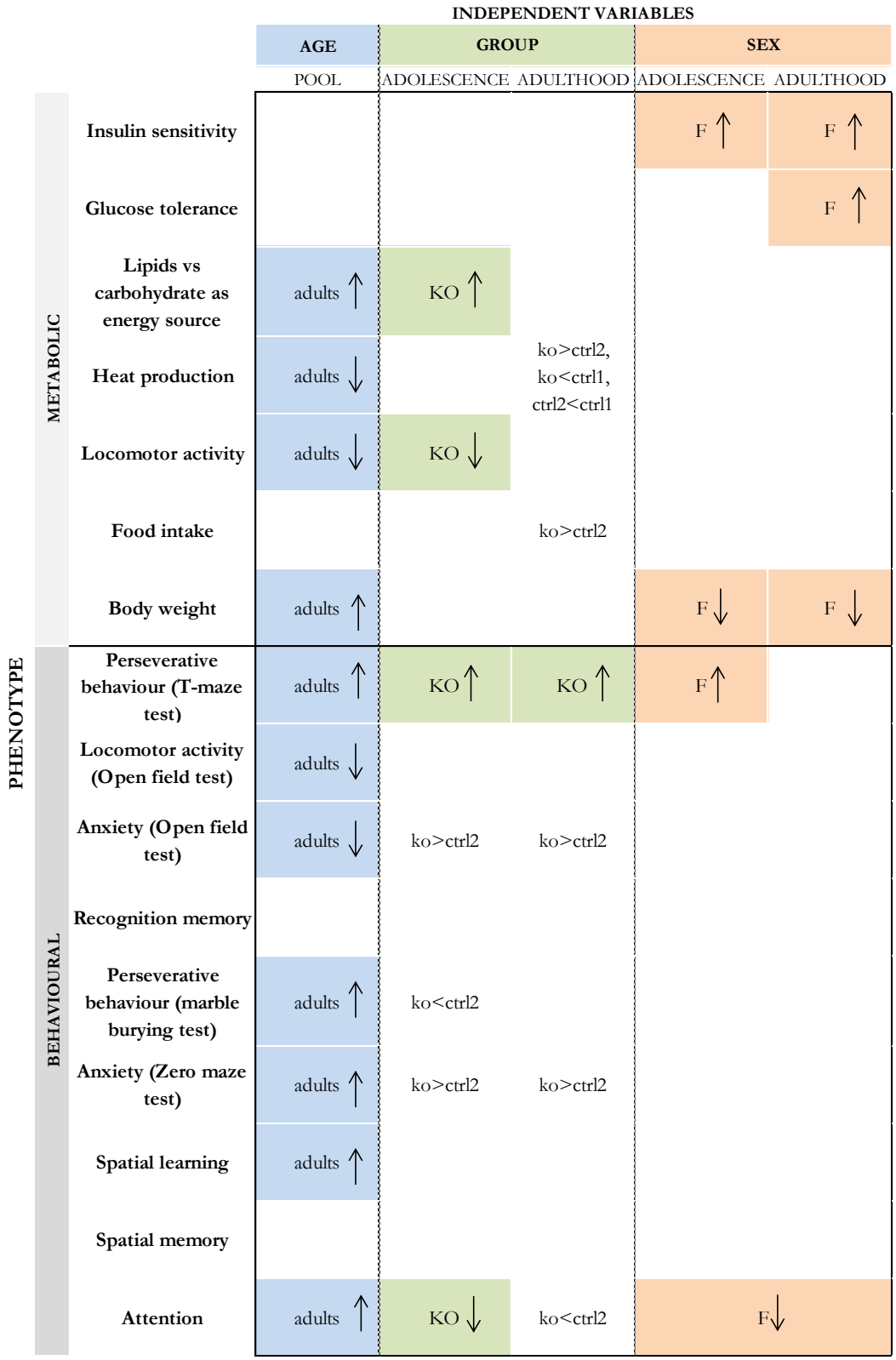

Figure 24. Summary of results: age-, sex-, and treatment- related effects for each phenotype. All the results are showed but only painted cells reflect the real effect of the knockout (in the light of the above described double dissociations). Arrows indicate the directionality of the effect observed.

As we expected, the alteration of *kcnq1* expression in the brain induced attention deficits assessed through the ASST test. Attentional set shifting tasks have been used as a measure of executive functions across species [39,40]. The major contribution these tasks have made has been the quantification of cognitive deficits associated with human pathologies such as schizophrenia, attention deficit/hyperactivity disorder and dementia [41–43]. Specifically, the reversal learning paradigm (assessed here with the CDR stage) is among the most widely tests used for cognitive flexibility [44]. It is the ability to rapidly change behaviour in the face of changing circumstances and it is disrupted in many psychiatric and neurological disorders [44]. Neuroimaging studies report increased activity in orbitofrontal cortex and medial prefrontal cortex in human subjects performing reversal learning tasks [45]. Being expressed in the cortical regions, *KCNQ1* [46] plays a critical role in the maturation of neural circuits involved in cognitive control and attention. We can assume that the downregulation of *kcnq1* in the knockout group may have disrupted prefrontal cortex circuits, potentially resulting in attentional deficits.

We have not found the same attentional deficit in the adult knockout group. While adolescent brain was particularly vulnerable to *kcnq1* absence, in adults, compensatory mechanisms or the stabilization of neural circuits could have mitigated some of the cognitive impairments [47].

The observation that subjects with early-onset diabetes perform more poorly in cognitive tasks than those with later-onset diabetes or nondiabetic controls [48], highlights the critical impact of early-life metabolic disturbances on cognitive function. Early-onset diabetes, particularly during developmental stages, interferes with neurodevelopmental processes, potentially affecting brain regions involved in learning, memory, and cognitive flexibility. This mirrors the findings in our study, where adolescent mice showed greater cognitive deficits compared to adults following gene knockout. Being involved in the central-insulin signalling, the earlier is the kcnq1 disruption the more pronounced are the cognitive impairments.

Perseverative behaviour refers to the inability to shift actions or thoughts in response to changing situations, often linked to impaired cognitive flexibility [49]. T-maze test has been widely applied to assess repetitive/persistent behaviour relevant to obsessive-compulsive disorder and autism spectrum disorders in rodent models [50,51]. Disruptions in potassium current, deriving from the *kcnq1* knockout, could have affected cognitive processes, particularly those involving response inhibition, flexible thinking, and adaptive behaviours, ultimately resulting in persistent behaviours, as showed by the percentage of spontaneous alternations in our knockout mice. Moreover, *kcnq1* deletion could have influenced the release of neurotransmitter involved in executive functions [52–54].

As often demonstrated, and as this project aims to highlight, brain health relies on body health. Together with a balanced diet, a physical activity is essential [55]. Cholinergic neurons are involved in both motor and cognitive functions. Instead of merely facilitating movement, these neurons appear to play a significant role in motor inhibition, particularly through their ascending connections with basal ganglia structures [56]. Additionally, cholinergic neurons support goal-directed locomotion and

reinforcement by signalling mismatches between expected and actual outcomes. This signalling enables the organism to adjust and effectively stopping specific actions and promoting new ones [57]. When cholinergic mechanisms fail, as seen in conditions like Parkinson's disease, the result is impaired sensorimotor integration and perseverative behaviours [58]. The cholinergic action at muscarinic receptors is also important for working memory. Muscarinic receptors modulate working memory performance and activity via KCNQ potassium channels in primate prefrontal cortex [59]. Our findings align with existing research, showing that alterations in kcnq1 channels led to reduced locomotor activity and working memory impairments and underscoring the essential nature of ion channel functionality in maintaining both cognitive flexibility and motor coordination.

Kcnq1 has the highest expression in the choroid plexus [60]. One of the roles of choroid plexus is in regulating cerebrospinal fluid (CSF) composition. Altered CSF composition could contribute to neuronal excitability disorders [61]. Moreover, the choroid plexus synthesizes many growth factors, including insulin-like growth factor 1 (IGF-1) [62]. IGF-1 plays a crucial role in growth and development and acts as a trophic factor in the central nervous system. It is an important signal during development, including brain growth, neuronal activity, hippocampal neurogenesis, or amyloid plaque clearance [62,63]. IGF-1 deficient animals show not only specific metabolic defects, but also a wide range of neurological complications [64,65]. An alteration in the CSF, due to *kcnq1* absence, could have led to both cognitive and metabolic deficits observed in adolescent knockout mice. Since the adult brain synthesizes very low levels of IGF-1 and only in very specific locations, most brain IGF-I in the adult is from peripheral origin [66]. This could suggest a critical dependence on peripherally-derived IGF-1 for maintaining cognitive function in adult. This reliance on peripheral IGF-1 may help explain the reduced cognitive deficits observed in knockout adult mice compared with adolescents. If these mice still retain the ability to utilize circulating IGF-1, they may partially compensate for the lack of local IGF-1 synthesis.

IGF-1 also plays a role in the storage of fat and has been shown to reduce adiposity [67]. This could explain the shift in energy metabolism towards lipid utilization observed in knockout adolescents. In this case, a reduction of the body weight (due to the knockout effect on IGF-1) would have been expected. However, in our study, the knockout body weight did not differ from controls. This observation aligns with previous research. Studies in IGF-1 deficient animals have demonstrated that these animals maintain normal body size, even in the absence of circulating IGF-1, suggesting that other factors compensate for the loss of IGF-1 [68].

Another important consideration is that *kcnq1* is largely expressed in the pituitary gland/hypophysis [46]. It is an endocrine gland protruding from of the hypothalamus [69]. Hormones secreted from the pituitary gland (like the thyroid-stimulating hormone, TSH) help control general metabolism. Thyroid hormones play a crucial role in regulating carbohydrate metabolism by increasing glucose uptake by cells and stimulating glycogenosis [70]. An imbalance in the pituitary gland that reduces TSH production can lead to hypothyroidism, resulting in a lower basal metabolic rate and a slowdown in

carbohydrate metabolism [70]. As a result, the body may burn less glucose and shift towards using other energy sources, such as lipids, as shown in our study. In this context, *kcnq1* knockout, disrupting the balance of THS, may have led to rapid glucose utilization by cells, depleting circulating glucose levels and reducing carbohydrate availability. Consequently, the body may have preferred fats as its primary energy source. A similar reduction in glucose availability may have occurred in the brain, potentially leading to neuronal disturbances and cognitive alterations.

In summary, our findings underscore the role of KCNQ1 in modulating cognition, especially executive functions. The selective knockout of *kcnq1* in the brain, leading to altered synaptic activity and neuronal excitability, shows the gene's neuro-specific functions related to compulsive traits, working memory, and attentional skills. Kcnq1 in the brain also has a role in modulating energy metabolism (particularly the balance between carbohydrate and lipid use) via central signalling and without directly affecting body weight or insulin signalling. In our study, peripheral insulin sensitivity and glucose tolerance were not altered in the knockout group. We could confirm that the effects of kcnq1 downregulation in the brain are limited to central insulin signalling. While the metabolic dysfunction observed in the whole-body knockout is predominantly driven by peripheral mechanisms, alterations in cognitive function associated with KCNQ1 are primarily linked to central dysregulation.

Moreover, the fact that both metabolic and cognitive effects are more evident during adolescence underlines the importance of a proper neurodevelopment during this critical period. Adolescence is a period of significant energy demand and brain restructuring [71,72], and the absence of KCNQ1 during this critical window may disrupt the development of neural structure essential for plasticity and cognition. In adulthood, these structures are typically fully developed and stabilized, making them less susceptible to perturbations such as a gene downregulation [73].

Other considerations

The primary aim of this study was to assess potential metabolic and cognitive differences (due to the absence of kcnq1) between adolescence and adulthood, as well as between males and females. However, our findings did not reveal any sex-dependent effects related to *kcnq1* deficiency. We can still draw important conclusions regarding the behavioural and metabolic differences observed across the two age groups. Adults depended more on lipids as an energy source and produced more heat, which aligns with their higher body weight. Furthermore, adults showed greater perseveration, spatial learning and memory, and attentiveness than adolescents. As showed by previous research, adults exhibit higher executive functions and cognitive control compared to adolescents [74,75]. It could be related to the maturation of brain circuits, particularly in the prefrontal cortex. During adolescence, this region is still developing, which often leads to greater impulsivity, risk-taking, and reduced

attentional capacity [72,76]. As expected, in our study, adults were less impulsive and thus more anxious than adolescents, as demonstrated by the time spent in the open sectors of the elevated zero-maze.

The convergence of results obtained using different methodologies strengthens the association between the expected effects and those observed. Heterogenized experimental designs tend to produce more stable and reliable outcomes, with fewer false positives compared to homogenized designs [77]. We tested the same anxiety-related phenotype with a different test (the open field test). Interestingly, we found the opposite result. This divergence is not unusual, as different behavioural tests, even when targeting the same phenotype, can capture distinct aspects of behaviour [78,79]. In the open field test, for instance, locomotor activity plays a significant role. While in the open field, animals are free to explore a large open space (40 cm L x 40 cm W x 30 cm H), during the zero-maze they are restricted in a 5 cm wide-section. These discrepancies could be mitigated considering not only the classical temporal and spatial parameters (such as the time spent in the most aversive parts of the apparatus) but also a series of "ethological" parameters, including rearing, sniffing, immobility and risk assessment behaviours (head-dipping and stretched attend posture), which can provide important information on the state of anxiety [80].

Similar to the prefrontal cortex, the hippocampus, which is essential for spatial memory, continues to mature throughout adolescence [81]. Spatial memory, refers to the ability to remember the locations of spatial cues, navigate environments, and orient oneself in space, undergoes significant changes across the lifespan, particularly between adolescence and adulthood [81]. These differences can be attributed to the development and maturation of brain structures, as well as cognitive and environmental factors. As we found in the Barnes maze test, adults generally perform better on spatial tasks that require integration of multiple types of spatial information [82], such as learning the position of the escape box in relation to spatial cues surrounding the apparatus. Moreover, while adolescents tend to use more egocentric strategies (self-to-space), adults are more likely to use allocentric patterns (object-to-space) [83]. This allows adults to navigate more efficiently and adapt better to changes in spatial layouts.

We also found interesting sex-related differences. From a behavioural perspective, females were more perseverative but less attentive than males, reflecting different cognitive processing or attention management strategies between the sexes [84].

Females were more insulin-sensitive and glucose-tolerant compared to males, a factor that can have been influenced by their generally lower body weight. We can also assume that physiological and hormonal factors could have influenced the results. For instance, estrogens, the primary female sex hormones, play a significant role in insulin sensitivity [84]. It improves glucose uptake in muscles and other tissues, increases insulin receptor expression, and enhances the action of insulin in peripheral tissues. This hormonal advantage contributes to better glucose regulation and reduced risk of insulin resistance in females. These factors together explain why females, especially before menopause, tend

to have higher insulin sensitivity than males, making them less prone to conditions like type 2 diabetes in earlier stages of life [84].

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GENERAL DISCUSSION AND CONCLUSIONS

The intricate relationship between metabolic diseases and cognitive decline is a growing area of research, revealing how conditions such as obesity, diabetes, and metabolic syndrome can lead to profound changes in brain structure and function [3,6,18]. These disorders not only affect a person emotionally, potentially leading to anxiety and depression, but also functionally, as many patients exhibit cognitive impairments such as difficulties with attention, memory, and executive functions.

Metabolic imbalances, influenced by biological, physiological, and environmental factors, are increasingly recognized as significant moderators of cognitive decline and neurological disorders like dementia, attentional deficit disorders, autism spectrum disorders and obsessive-compulsive disorders [19,52,156,157]. This highlights the deep interconnection between physical and mental health, a relation that I progressively realized over the course of my PhD, moving from theoretical exploration (Chapter I) to preclinical experimentation (Chapter II) and finally to clinical observations during my internship in Barcelona.

Firstly, in the early stages of my PhD, I focused on expanding my theoretical understanding of the link between metabolic and cognitive health through extensive literature review and systematic analysis. Specifically, I reviewed the association between hyperglycaemia - as a proxy of insulin-related metabolic dysfunctions - and impairments in different cognitive domains in laboratory rodents. In addition to its primary focus, my review offered a comprehensive overview of key elements in preclinical research, particularly emphasizing the validity of animal models. This includes an in-depth examination of both construct and face validity, ensuring a thorough understanding of their significance in research outcomes.

In the reviewed studies, hyperglycaemia was induced via various methodologies, including pharmacological interventions, environmental modulations, and genetic determinants which effectively capture the heterogeneity of metabolic imbalance's aetiology in humans (e.g. genetic predisposition, lifestyle, obesity, or specific drugs). There was not a single animal model recapitulating all the causative factors and associated phenotypic abnormalities observed in our species. When preclinical research involves complex biological processes, such as insulin signalling, high construct validity is critical. Construct validity ensures that these animal models accurately represent the disease or biological process and that the results are meaningful, relevant, and can be extrapolated to humans [158]. All the experimental approaches adopted in the review reflected the diverse factors contributing to metabolic dysfunctions, enabling a more comprehensive understanding of how hyperglycaemia impacts both systemic health and cognitive function.

I found that the relationship between mental and physical health is not limited to human clinical cases but is also reflected in animal models, and I confirmed that disruptions in brain metabolic function, such as those caused by hyperglycaemia lead to both cognitive and metabolic disturbances. Specifically, metabolic disruptions manifested in impairments in working and spatial memory, and attention, and revealed that the effects of metabolic imbalances extend to cognition even in animal

models. These cognitive domains align with findings from human studies that indicate a significant role of insulin signalling in neurological disorders such as dementia, ADHD, and ASDs [19,52,156]. Mirrored outcomes revealed high face validity in these animal models. In preclinical research, face validity refers to the degree to which an experimental model appears to exhibit the salient features of the condition being modelled [158]. It is an essential aspect of the research design as it helps determine whether the methods, outcomes, and models used are appropriate for addressing the research question. Many metabolic disorders, such as obesity and TD2M, coexist with cognitive impairments. Face validity helps clarify how these comorbidities interact and, together with high construct validity, enhance the credibility, applicability, and translational value of preclinical research, ultimately contributing to the development of effective therapies and interventions for human health conditions. Another critical finding in my review was the presence of a male-bias in preclinical studies, where research often does not include female subjects. This bias underscored the need for more diverse experimental populations, as biological processes can manifest differently in males and females. In the context of metabolic and cognitive disorders, sex differences can be significant, making it crucial to study both genders and to draw more accurate and applicable conclusions. A balanced approach in preclinical research can lead to more insightful findings and more inclusive treatment strategies.

Human populations exhibit significant variability in metabolism and cognitive function due to factors like age, sex, genetics, and existing health conditions. This variability can complicate the validity of findings from more homogeneous animal models, which may not account for the diversity seen in human populations.

Building on this theoretical foundation, I designed an experimental plan that accounted for both sexes and different life stages, focusing on the specific contributions of the *kcnq1* gene to insulin signalling and brain function. Specifically, I employed a conditional *kcnq1* knockout mouse model, with the gene downregulated in a time-specific (during adolescence and adulthood) and spatial-specific (only in the brain) manner. My focus on kcnq1 stemmed from prior research, from different groups in the PRIME consortium (within which my research project was embodied) [152], which indicated kcnq1's role in both metabolic and cognitive health. For instance, *KCNQ1* has been identified as a susceptibility gene for TD2M in several genome-wide association studies and it is a key molecule in the landscape of OCD. Moreover, mice with whole-body *kcnq1* deletion exhibit altered insulin sensitivity and release, along with cognitive changes like repetitive behaviours and cognitive rigidity. Thus, the hypothesis I formulated built on the collaborative efforts of previous experimental work. Science thrives on collaboration, and my work was no exception, relying on the expertise and findings of other researchers to refine my experimental approach. This collective effort underscores the importance of collaboration in advancing scientific understanding and developing effective treatment strategies for complex conditions like metabolic and cognitive disorders.

The results of my experiments provided significant insights into the link between metabolism and cognition. I found that the effects of *kcnq1* deletion were more pronounced during adolescence than adulthood. Specifically, the metabolic effects of *kcnq1* absence were mainly restricted to adolescence. Moreover, while adults showed primarily compulsivity traits, adolescent knockouts exhibited perseverative behaviours and attention deficits. This suggests that the adolescent brain is more vulnerable to biological disruptions, likely because of the ongoing development of neural circuits, whereas adult brains may have compensatory mechanisms that mitigate some of the cognitive impairments [159].

My findings highlight the particular susceptibility of the adolescent brain to metabolic and cognitive disturbances, also mirroring what I observed in my clinical experience which bridged the gap between the preclinical experimentation and clinical issue. During my internship in Barcelona, I encountered patients with both metabolic disorders (like obesity and anorexia) and mental health conditions (such as anxiety and depression). The majority of these patients were adolescents, underscoring the vulnerability and the critical nature of this developmental stage.

The brain continues to mature throughout adolescence, with significant changes occurring in areas responsible for higher-order brain functions. The prefrontal cortex, which is crucial for decision-making, impulse control, and planning, matures more slowly than the limbic system, which is associated with emotions and reward processing [160]. Adolescents tend to engage in more risk-taking behaviours compared to adults [161]. This is often attributed to the heightened activity in the reward system and underdeveloped prefrontal cortex, leading to a greater focus on potential rewards rather than consequences. Moreover, adolescents may struggle with working memory tasks that require complex manipulation of information [162]. Adults usually have more refined strategies for encoding and retrieving information [163]. Finally, while adolescents may show less cognitive flexibility, adults often adapt their thinking to changing circumstances more efficiently and exhibit greater cognitive flexibility [164]. Since the adolescent brain is still maturing, disruptions in mental or somatic equilibrium during this period can have profound and long-lasting effects.

Limitations but benefits, and future perspectives of my study (and beyond)

While my studies provided important findings, certain limitations should be acknowledged, which may nevertheless simultaneously stand as key advantages, contributing to the studies' robustness.

The risk of bias is the quality assessment of a preclinical study. It refers to the potential for systematic errors or distortions in the design, conduct, analysis, or reporting of studies involving animal models or other laboratory experiments. A low risk of bias avoids inaccurate or misleading conclusions and improves the reproducibility and reliability of research findings. As highlighted in my review, a cautionary note for our preclinical community arises from the review's overall risk of bias that reached a value of 52.09%. Although an unclear risk of bias does not necessarily indicate

poor study planning, execution, or reporting, it does limit the ability to fully comprehend critical details. The report of methodological aspects in animal studies should be significantly improved. Promoting high-quality standards for the registration and reporting of such studies should be a key objective in preclinical research.

Selecting the right control group is essential in preclinical research. It helps establish the validity of experimental findings, allows for meaningful comparisons, and reduces the risk of bias. The complexity of my experimental model may have required more than two control groups to assess each genomic construct (i.e., the floxed sequences, the kcnq1 gene in heterozygosis and in homozygosis). However, the control groups selected were sufficient to enable meaningful relative comparisons, while also adhering to the 3Rs principle (Replacement, Reduction, and Refinement).

Future research will provide a comprehensive understanding of insulin comorbidities in animal models, offering valuable insights for future clinical research on diagnosis, prognosis, and the development of appropriate therapies. Ultimately, identifying a medication that targets both metabolic disorders and their associated comorbidities is crucial for advancing human health, particularly in the prevention, treatment, and management of disease.

Beyond the objective scientific considerations, I would like to offer similar personal reflections of pursuing a PhD, emphasizing both its challenges and benefits. Challenges related to experimental workload, anxiety and stress are inherent to the PhD; however, the personal and professional growth that comes from this experience is invaluable. With regard to my personal future prospective, even if the path is still uncertain, my hope is to always engage in science.

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APPENDIX

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Insulin and disorders of behavioural flexibility

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ABSTRACT

Behavioural inflexibility is a symptom of neuropsychiatric and neurodegenerative disorders such as Obsessive-Compulsive Disorder, Autism Spectrum Disorder and Alzheimer's Disease, encompassing the maintenance of a behaviour even when no longer appropriate. Recent evidence suggests that insulin signalling has roles apart from its regulation of peripheral metabolism and mediates behaviourally-relevant central nervous system (CNS) functions including behavioural flexibility. Indeed, insulin resistance is reported to generate anxious, persever- ative phenotypes in animal models, with the Type 2 diabetes medication metformin proving to be beneficial for disorders including Alzheimer's Disease. Structural and functional neuroimaging studies of Type 2 diabetes patients have highlighted aberrant connectivity in regions governing salience detection, attention, inhibition and memory. As currently available therapeutic strategies feature high rates of resistance, there is an urgent need to better understand the complex aetiology of behaviour and develop improved therapeutics. In this review, we explore the circuitry underlying behavioural flexibility, changes in Type 2 diabetes, the role of insulin in CNS outcomes and mechanisms of insulin involvement across disorders of behavioural inflexibility.

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Introduction

Behavioural processes are key to an organism's response to the environment and to each other. These behavioural processes are often conditioned either by reward or punishment and involve changes to learning, memory, attention and behavioural flexibility. Behavioural flexibility, or the adaptive change of behaviour in response to changing environmental contingencies, is compromised in numerous disorders, including those that emerge in early life such as Autism spectrum dis- order (ASD), attention-deficit/hyperactivity disorder (ADHD) and Tourette's Syndrome; those that emerge in adolescence, including schizophrenia and mood disorders; and those in later life including de- mentias. While many of these conditions share flexibility deficits, the treatment of flexibility as a discrete feature in its own right is a chal- lenge, owing to the heterogeneous nature, severity and patterns of co- morbid symptoms. Treatment options rely heavily on non-pharmacological therapies such as Cognitive-Behavioural Therapy (CBT), which aims to attenuate symptoms by altering the thoughts and responses to triggers. Pharmacotherapy includes Selective Serotonin Reuptake Inhibitors (SSRIs) but up to half of all patients fail to respond requiring new treatment approaches. Furthermore, the long lag time of three to six weeks before a behavioural change is elicited with SSRIs leads to poor rates of treatment

maintenance and low adherence. The aetiology of behavioural inflexibility is poorly understood, with factors including genetics, environment and specific life stressors all involved. Diagnostic criteria are based upon presentation. Diagnostic strategies differ drastically across disorders of behavioural inflexibility, owing to its various presentations. For example, the main diagnostic strategies for ASD are the Autism Diagnostic Interview-Revised (ADI-R) and the Autism Observational Diagnostic Schedule (ADOS), which capture a child's early developmental history, social and communicative func- tioning. On the other hand, the Yale-Brown Obsessive-Compulsive Scale (YBOCS) is a semi-structured interview that is commonly utilised for OCD diagnosis and instead aims to capture the severity of intrusive, obsessive thought patterns and compulsions. Behavioural changes in animals can be identified using tasks such as reversal learning, attention set shifting, and radial arm mazes. In humans, the CANTAB (Cambridge Neurophysiological Test Automated Battery) tests, a standard in cogni- tive research can also assess behavioural flexibility using tasks incor- porating discrimination reversal (switching reward-response associations) and extra-intra dimensional set shifting (attention and cognitive flexibility). Learning and memory impairments utilising the CANTAB have been observed in patients with ASD (Spatial Span (vi- suospatial working memory), Spatial Working Memory, Stockings of Cambridge (spatial planning and working memory), and Intradimen- sional/Extradimensional Shift Test) (Chen et al., 2016), in addition to reduced sensitivity to outcome devaluation in OCD (Pattern Recognition Memory (matching information input to already-stored memory), Paired Associates Learning (inter-stimuli associations) and Intra-Extra Dimensional Set Shift (attention shifting)) (Gottwald et al., 2018).

The generation of reliable rodent models to encompass inflexibility disorders remains a challenge due to the relative heterogeneity of symptom presentation. As such, not all 'compulsive' or 'behaviourally rigid' animal models have the same phenotypic type of behavioural inflexibility, and it is unknown whether the same molecular mechanisms subserve these different types of behavioural inflexibility. Genetic knockout rodent models aim to encompass specific symptom types, for example *Integrin beta3* knockout mice show an autistic phenotype with reduced preference for social novelty and increased grooming behaviour (Crawley, 2012), while both the serotonin transporter *5-HT2C* knockout and SLITRK5 knockout mouse models of OCD show increased repetitive, habitual behaviours (Chou-Green et al., 2003). Pharmacological in- duction of behavioural inflexibility can be initiated prenatally by both valproic acid and the immunostimulant polyinosinic:polycytidylic acid (poly IC), while administration of the dopamine receptor D2/D3 agonist quinpirole in adulthood incudes compulsive checking behaviour(Szechtman et al., 1998).

Both structural and functional MRI studies have shed light on par-allel, partially segregated cortico-striatal-thalamo-cortical (CSTC) loops (Parmar and Sarkar, 2016; Vahabzadeh and McDougle, 2014)) which mediate different behavioural, cognitive and sensorimotor processes, the dysregulation of which mediates abnormal cortical excitation pat- terns and compulsive behavioural phenotypes (Stein et al., 2019). Table 1 below illustrates structural and functional changes in CSTC circuit regions in both ASD and OCD. Beyond these CSTC loops, both the hippocampus and the cerebellum have a role in behavioural inflexi- bility. The hippocampus has roles in episodic memory, can denote emotional contexts to memory and enable approach-avoidance conflict, encompassing the weighing up of pros and cons of a decision. Indeed, this region has connections to CSTC regions including the nucleus accumbens (reward and addiction behaviours) orbital area (valuation and decision-making) and anterior cingulate cortex (adaptive switch- ing). The cerebellum is a region that has gained attention for its roles in compulsive behaviour in recent years (Xing et al., 2020) and is impli- cated in learning (Fullana et al., 2018) and habit formation (Miquel et al., 2019). Neuroimaging studies reflect this CSTC involvement across OCD (Rasgon et al., 2017), (Xu et al., 2019; Zhang et al., 2019a), ASD (He et al., 2021), Depression and Parkinson's Disease (which commonly occurs with comorbid addiction/reward-related impairments) (Vriend et al., 2014). Additionally, this circuitry regulates synaptic plasticity changes (Song et al., 2017) and neurotransmitter imbalance, such as reward and motivation-related dopamine (Iino et al., 2020). Addition- ally, anxiety plays a role in the generation of compulsive symptoms. In OCD, anxiety is deemed the driving force behind the need to complete various repetitive compulsion behaviours, while in ASD, deviation from routine may generate anxiety in the individual, warranting inflexible behavioural patterns. Tourette's Syndrome, while typically presenting in the form of physical tics and repetitive movements, may also be aggravated when the individual is anxious and is often comorbid with OCD. Studies of brain regions involving anxiety predominantly encom- pass the hypothalamus, hypothalamic-pituitary-adrenal axis, amygdala, with reciprocal projections with the anterior cingulate cortex and hip- pocampus, governing fear-memory links (Anon, 2021) and regions of the mPFC (Calhoon and Tye, 2015).

Recently, aberrant insulin signalling has been proposed as a key mechanism behind behavioural inflexibility (van de Vondervoort et al., 2016, 2019). Insulin is a hormone synthesised in pancreatic beta cells, and regulates metabolic processes including new lipid production and glucose uptake. Insulin resistance is a feature of Type II Diabetes and involves an insensitivity of target receptors to insulin. Networks resulting from genome-wide association study (GWAS) examining OCD have identified insulin signalling as the most enriched network under- lying OCD symptomology, with many associated genes implicated in neuronal dendritic spine formation (van de Vondervoort et al., 2016). According to In situ hybridisation data on the Human protein Atlas, most brain regions express the insulin receptor (INSR) and Insulin-like growth factor-1 receptor (IGF1R). Insulin reportedly can regulate brain function and shows links with cognitive dysfunction in Alzheimer's Disease, coined "Diabetes Type 3". Interestingly, insulin was utilised as an early therapeutic for psychiatric disease in the early 20th century (James, 1992). Insulin is capable of crossing the blood-brain barrier. Addition- ally, evidence suggests that insulin is also locally secreted in brain (Gray et al., 2014), with mRNA reported in striatum, thalamus, frontal cortex, hippocampus and brainstem in mice of various ages (Mehran et al., 2012a). Insulin

resistance is reported to affect processes including syn- aptogenesis (Chiu et al., 2008; van de Vondervoort et al., 2016; Lee et al., 2011), myelination (Mozell and McMorris, 1991), aggregate accumulation (Han et al., 2016; Jolivalt et al., 2008)) and neurotrans- mitter homeostasis (Stouffer et al., 2015a). Insulin-Growth Factor 1 and 2 (IGF-1 and IGF-2), molecules of similar structure to insulin, also engage with similar processes. Indeed, the IGF-1 receptor and Insulin receptor are highly homologous, despite having varying tissue expression patterns. While insulin signalling primarily mediates meta- bolism, IGF-1 mediates growth and proliferation signalling (Cai et al., 2017). However, both insulin and IGF-1 can bind to each other's re- ceptors as agonists. Animal models of insulin resistance further support the importance of insulin in the brain, with the TALLYHO/JngJ mouse model of Type 2 Diabetes (T2D) demonstrating compulsive behaviours and increased anxiety (van de Vondervoort et al., 2019). Additionally, T2D patients reportedly demonstrate increased obsessive-compulsive symptomology (Kontoangelos et al., 2013).

This review will be structured into four main parts. The first will discuss current knowledge of the aetiology of behavioural rigidity. The second will discuss insulin signalling in brain and T2D-associated structural and functional changes. The third section will examine spe- cific brain processes governed by insulin. The final section will explore the input of insulin signalling into various disorders in which rigidity is a feature, i.e. Obsessive-Compulsive Disorder, Addiction, Anorexia nerv- osa, binge eating, Alzheimer's Disease and Parkinson's Disease.

Table 1
Summary of compulsivity-associated regions; their role, structural and functional changes in OCD and ASD.

Region	CSTC Circuit	Role	Changes in OCD, ASD	
			Structural	Functional
Orbitofrontal cortex	Ventral-motivational circuit (Stein et al., 2019).	Assesses the value of reward and motivational value of a stimulus (Graybiel and Rauch, 2000).	Smaller in OCD (Heuvel et al., 2022) Increased cortical thickness in ASD (Van Rooij et al., 2018)	Increased activation in OCD patients vs controls (Thorsen et al., 2018). Altered functional connectivity across ages (Long et al., 2016)
Dorsal Anterior Cingulate Cortex	Frontolimbic, dorsal-cognitive circuits	Value-based decision making (Fatahi et al., 2018). Roles in linking reward and error in decision making in reversal learning paradigms (Chudasama et al., 2013). Cognitive conflict, decision making and fear extinction (Milad and Rauch, 2012)	Smaller in OCD (McGovern and Sheth, 2017) . Increased cortical thickness in ASD (Jiao et al., 2010) (Van Rooij et al., 2018)	Increased activation in OCD patients (Thorsen et al., 2018) (Rotge et al., 2008) Reduced activation (Ameis and Szatmari, 2012) Reduced functional connectivity (Long et al., 2016)
Midcingulate Cortex	Frontolimbic, dorsal-cognitive circuits	Goal-directed action (Holroyd et al., 2018).	No data Increased cortical thickness in ASD (Hyde et al., 2010) (Van Rooij et al., 2018)	Decreased neuron size (Uppal et al., 2014) Reduced activation (Ameis and Szatmari, 2012)
Amygdala	Frontolimbic circuit (Stein et al., 2019)	Fear extinction (Sun et al., 2019)	Asymmetry across hemispheres (Szeszko et al., 2004) Reduced size (Szeszko et al., 1999) Smaller in ASD (Van Rooij et al., 2018)	Increased activation in OCD patients (Rotge et al., 2008) Altered connectivity (Heuvel et al., 2022) Reduced activation (Ameis and Szatmari, 2012)
Amygdala	Frontolimbic circuit (Stein et al., 2019)	Fear extinction (Sun et al., 2019)	Asymmetry across hemispheres (Szeszko et al., 2004) Reduced size (Szeszko et al., 1999) Smaller in ASD (Van Rooij et al., 2018)	Increased activation in OCD patients (Rotge et al., 2008) Altered connectivity (Heuvel et al., 2022) Reduced activation (Ameis and Szatmari, 2012)
Nucleus Accumbens	Ventral-motivational circuit (Stein et al., 2019).	Forms stimulus-reward relationships (Day and Carelli, 2007).	Larger in OCD (Heuvel et al., 2022) Smaller in ASD (Van Rooij et al., 2018)	Reduced activity (Wu et al., 2021) Reduced functional connectivity in ASD to regions including cingulate cortex, dorsal striatum and thalamus (Polk and Ikuta, 2022)
Dorsomedial striatum (encompassing caudate and putamen	Dorsal cognitive and ventral cognitive circuits	Attention (Rubia et al., 2011) Fear, reward conditioned associations (Strata, 2015)	Altered volume in OCD patients (Heuvel et al., 2022) Smaller in ASD (Van Rooij et al., 2018)	Reduced activity (Lipton et al., 2019) Reduced activation during socialisation (Delmonte et al., 2012)
Hippocampus	Extra-CSTC	Role in formation of episodic memory. Connections with amygdala facilitate emotional context to memory. Memory formation Spatial memory Approach-avoidance conflict	Smaller in OCD (Heuvel et al., 2022) Volume changes associated with symptom severity (Reess et al., 2018) (Boedhoe et al., 2017a) Asymmetrical in children with low-functioning ASD (Schumann et al., 2004), reduced size associated with symptom severity (Van Rooij et al., 2018)	Increased activation in OCD patients (Rotge et al., 2008). Reduced connectivity between hippocampus ad fronto-parietal network in memory retrieval (Cooper et al., 2017)
Cerebellum	Extra-CSTC	Cerebellum in inhibition, lesions in cerebellar vermis cause dysregulated executive function (Miquel et al., 2019)	Larger in OCD (Heuvel et al., 2022) Larger in ASD (Sparks et al., 2002)	Altered connectivity to cerebellum and cingulate in OCD (Zhang et al., 2019b) Reduced connectivity to frontal, motor cortex and striatum (Crippa et al., 2016)

1. Circuitry in rigidity disorders

1.1. Cognitive-affective dysfunction

Behavioural flexibility falls under the broader umbrella of executive functions, which are processes needed to regulate goal-directed behav- iour. These processes include appropriate valuation of a stimulus, inhi- bition of nonrewarding responses, switching behavioural strategy, appropriate allocation of attention and working memory.

1.2. Valuation of a stimulus

Stimulus valuation is necessary to allocate an appropriate rewarding value to a stimulus or environment, with overvaluation a common feature of unhealthy or maladaptive behaviours. Previous human func- tional neuroimaging studies identified the midcingulo-insular network comprising the dorsal anterior cingulate cortex, orbital frontoinsular cortex and subcortical structures including the amygdala and thalamus (Seeley et al., 2007; Uddin et al., 2019). Kable and Glimcher (2007) studied neural correlates of immediate vs delayed reward (Kable and Glimcher, 2007). They identified activation of the ventral striatum, medial prefrontal cortex and posterior cingulate cortex across subjects, with the most impulsive participants showing a steep decline in acti- vation as delay time increases. This indicates that these regions govern both the subjective value placed on a reward, and also the time at which the reward will be received.

1.3. Inhibition of nonrewarding responses

Shifting responses to adapt to environment requires the inhibition of a previous response. There are conflicting reports regarding the deficit in inhibition amongst patients of compulsivity. Indeed, OCD patients appear to show medium effect size (Shin et al., 2013), whereas a meta-analysis of studies in ASD patients showed more concrete impairment in response inhibition (Hlavata´ et al., 2018). Alzheimer's Disease is associated with significant impairments in controlled inhibi- tion (Amieva et al., 2004). Consistent with the human imaging studies, impaired ability to inhibit responding to the previously-rewarded but no-longer-correct stimulus (perseverative errors) has been observed in a rodent model of ASD in which cerebellar dysfunction is present (Dickson et al., 2010).

1.4. Switch behavioural strategy

The ability to effectively switch between behaviours depending on changes in environmental reward-salience is essential for adaptability, and is captured in the reversal learning task. *In vivo* recordings of the orbitofrontal cortex (OFC), dorsal medial striatum (DMS) and dorsal

lateral striatum during shifting reveal altered neuronal firing rates in the caudate and orbitofrontal cortex between goal-directed and habit-based actions (Germal and Costa, 2013). Neuronal monitoring in head-fixed mice demonstrated that neurons in the mouse OFC respond saliently and transiently to rule switches during reversal learning, a task measuring the effectiveness at which mice can shift from a non- rewarding to rewarding response (Banerjee et al., 2020). The dorso- medial striatum is thought to dynamically interact with multiple prefrontal subregions that generate new strategies to facilitate behav- ioural flexibility, with inactivation impairing the ability of the mouse to maintain a new choice pattern (Ragozzino, 2007). The ventral striatum governs reward sensitivity, with reduced reversal learning ability following ablation of orbitofrontal-nucleus accumbens projections (Groman et al., 2019). Temporal lobe epileptic patients, with hippo- campal atrophy, demonstrate an impaired ability to anticipate stimuli in reversal learning compared to healthy controls (Vila`-Ballo´ et al., 2017). Patients with cerebellar lesions also exhibit normal acquisition of stimulus contingencies, however impaired reversal learning (Thoma et al., 2008).

1.5. Attention

Another necessary process for affective shifting between tasks is attention, a cognitive process in which the brain dedicates sensory re- sources to relevant stimuli. However, this process is context dependent, and is influenced by factors including memory, reward motivation and anxiety (Bissonette et al., 2013). Evidence suggests that compulsivity may result from dysfunctional selective attention. For example, children with ASD are more likely to be influenced by visual distractors (Poole et al., 2018) and OCD is characterised by an atypical allocation of attention to normally unattended stimuli (Levy, 2018). Those with ADHD are reported to be more likely to develop addiction later in life (Davis et al., 2015). Attention can be split into two distinct processes: top-down for goal-directed stimuli and bottom-up for unexpected stimuli. The former (dorsal attention network; DAN) employs use of the intraparietal cortex and superior frontal cortex, the latter (ventral attention network; VAN) involves the temporoparietal cortex and infe- rior frontal cortex (Corbetta and Shulman, 2002). Hence, attention is- sues may arise from a hypoactive top-down DAN or hyperactive bottom-up VAN, or a mix of both. The attentional set-shifting task in rodents, which measures the ability to transition between cognitive attentional sets, is reported to require activity of the medial prefrontal cortex (mPFC) (Bissonette et al., 2013), which receives projections from hippocampus, and projects to orbitofrontal

cortex, ACC, striatum, amygdala, mediodorsal thalamus and ventral tegmental area (Vertes, RP., 2006).

1.6. Working memory

Adapting behaviour is also dependent on working memory, both in our ability to update our current knowledge in a situational context and in our conditioned associations to reward-based or aversive stimuli. Working memory is a temporary, short-term memory system required for decision making and reasoning. The employment of previous infor- mation contributes to our ability to predict the future and make de- cisions. A task that reflects the ability to update information and simulates real-life decision making is the Iowa Gambling Task. This re- quires participants to select cards from four decks, with various weights of advantageousness and disadvantageousness, associated with small rewards and punishments respectively. Patients with hippocampal damage do not develop a preference for either set, suggesting that pa- tients maintain only a momentary response to the outcome, and do not maintain or update choice-outcome relations (Gutbrod et al., 2006). The hippocampus interacts with other regions implicated in flexible decision-making, including the prefrontal cortex (PFC) (Simons and Spiers, 2003) and amygdala (Pikkarainen et al., 1998). One study showed, via optogenetic stimulation, that the central amygdala (CeA) sends robust inhibitory projections to the lateral substantia nigra (SNL) that contribute to appetitive and aversive learning in mice (Steinberg et al., 2020). This is associated with habitual behaviours, in which motor responses are produced without regard for the outcome (Lingawi and Balleine, 2012). The basolateral amygdala (BLA) on the other hand projects to the nucleus accumbens (NAc) to implement instrumental behaviour for conditioned reinforcement, with deep brain stimulation in the NAc a successful treatment for disorders of compulsivity including OCD (Everitt et al., 1991). Information about pleasant and aversive stimuli converge at the amygdala from regions including the prefrontal cortex and hippocampus, potentially influencing amygdala response to information including memories and expectations (Belova et al., 2007). To summarise, behavioural flexibility in the face of a changing environment is dependent on the interaction of numerous processes; the appropriate assignment of value to a stimulus, the inhibition of a pre- viously, but no longer-rewarding behaviour, the ability to switch from one behaviour to another, the ability to focus and refocus attention, and finally the integrity of our short-term working memory. Dysregulation of these mechanisms may generate errors in decision making and maladaptive behavior.

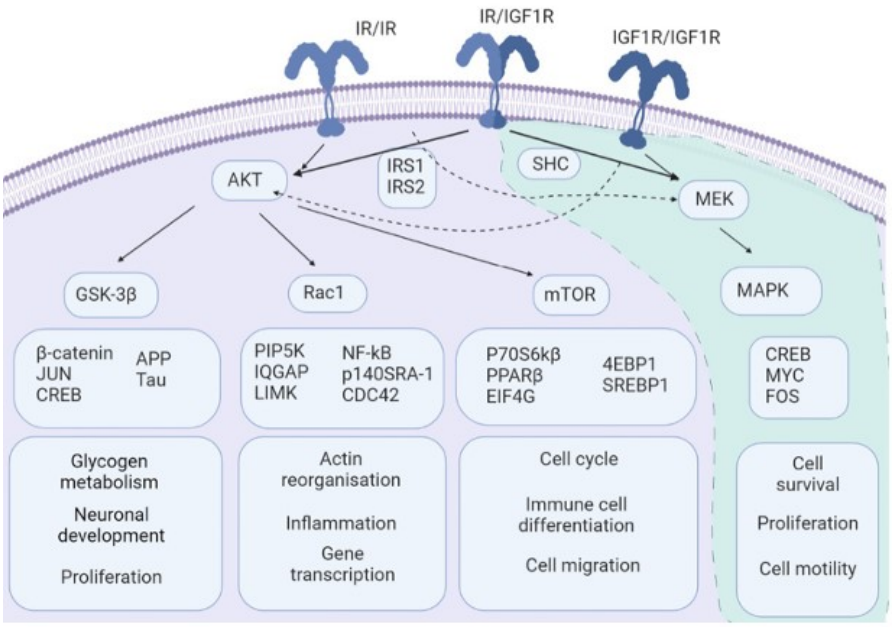

Fig. 1. Insulin/IGF1 signalling pathways. Insulin binds the insulin receptor homodimer (IR/IR) and IGF-1 binds the IGF-1 receptor homodimer (IGF1R/IGF1R), and both bind the IR/IGF1R heterodimer. Downstream signalling mechanisms govern metabolic processes including glycogen metabolism, inflammatory and immune processes and processes concerning cell survival and activity (GSK-3β: Glycogen synthase kinase 3-beta; mTOR: mammalian target of rapamycin; MAPK: mitogen activated protein kinase). Created with Biorender.com.

2. Insulin signalling in the brain

Diabetes mellitus is a disorder characterised either by impaired insulin synthesis (Type 1) or insulin resistance at the level of receptors (Type 2). First-line treatments involve insulin injection in the former, and medication to decrease insulin resistance, e.g. metformin, in the latter. Type 2 diabetes as a result of impaired insulin signalling is associated with impairments in working memory and cognitive func- tioning (Backestro¨m et al., 2021). Insulin is a peptide hormone syn- thesised in β cells of the pancreas in response to physiological triggers, predominantly peripheral glucose levels. Despite the decades-long assertion of the brain as an insulin-insensitive organ, both the insulin receptor and IGF-1 receptor are widely expressed across human brain regions including the cerebellum, frontal cortex, hippocampus and caudate (Anon, 2020a, 2020a). The

bulk of brain insulin is derived from the pancreas and transported across the blood-brain barrier (Banks et al., 2012), however insulin mRNA has also been identified in brain tissue indicating localised transcription takes place, including in hypothala- mus, hippocampus (Mehran et al., 2012b; Steen et al., 2005)), olfactory bulb (Kuwabara et al., 2011), striatum, thalamus and pyramidal neurons of the cortex (Dorn et al., 1982). Insulin appears to be derived in neurons but not in astrocytes, with no insulin gene expression reported in astrocyte cultures (Devaskarss et al., 1994). Depolarisation of neurons yields release of insulin from the synaptic terminal, suggesting that it may possess neurotransmitter-like properties (Clarke et al., 1986; Wei et al., 1990)). There is a scarcity of literature examining insulin pro- duction and secretion from microglia and oligodendrocytes, however activated microglia were found to secrete IGF-1 in the hippocampus of mouse models of Alzheimer's Disease (Myhre et al., 2019). Insulin sig- nalling in brain is also reported to regulate metabolism in peripheral tissues, with suppression of hepatic glucose production, lipolysis in ad- ipose tissue, hepatic catabolism of branched-chain amino acids and hepatic triglyceride secretion reported to occur independently from plasma insulin levels (Arnold et al., 2018).

The two predominant cascades induced by insulin are the AKT and MAPK pathways. The former is particularly relevant for T2D as this mediates the recruitment of the glucose transporter GLUT4 for glucose cellular uptake in muscle and adipose cells. The AKT pathway is also responsible for the phosphorylation of multiple downstream targets including mTOR and GSK-3 implicated in neural development, synapse formation, neurite branching (Takei and Nawa, 2014), axonal guidance, migration and tau accumulation (Salcedo-Tello et al., 2011). MAPK regulates synaptic plasticity (Giachello et al., 2010) and inflammation (Brown et al., 2015). Insulin growth factors are structural homologs of insulin and share common signalling cascades, with the key difference between the two in their different affinities for common receptor targets. IGF1 binds IGF1R and it may also bind the IR/IGF1R receptor hybrid. Insulin binds to the insulin receptor and also the IR/IGF1R hybrid. In order to successfully bind to their receptors, insulin and IGF interact with insulin growth factor binding proteins 1–7. These are shown to play roles in myelination and plasticity during crucial periods of neonatal brain development (Bunn et al., 2005). These pathways are summarised in Fig. 1. The kinase protein AMPK is important in the regulation of insulin signalling, and functions to suppress hepatic glucose synthesis and increases insulin sensitivity in low adenosine diphosphate (ADP) conditions. The T2D drug metformin activates AMPK activity, and by yet not fully defined mechanisms, optimises insulin use by cells (Ruderman et al., 2013).

2.1. Type 2 diabetes and the brain

2.1.1. Structural changes

MRI studies of adolescents with T2D have identified reduced grey matter volume in the hippocampus, dorsal striatum, amygdala and thalamus, left insular lobe, left nucleus accumbens area, right hippo- campus, putamen and amygdala compared to age matched controls (Nouwen et al., 2017; Chen et al., 2013b)). In middle age, glycaemic control is an important factor that has been linked to the degree of at- rophy in brain (Gold et al., 2007). This multivariate regression analysis found that HbA1c, a measurement of glycosylated haemoglobin, was the only significant predictor of hippocampal atrophy in individuals with T2D. Interestingly obesity, a common feature of T2D, was a significant predictor of smaller volume of the hippocampus, anterior cingulate gyrus, frontal lobes and thalamus (Raji et al., 2010), possibly due to input from associated low-grade inflammation (Miller and Spencer, 2014). Patients also demonstrate increased neuroinflammation (Rom et al., 2019), in addition to vascular changes (Kooistra et al., 2013; Zeng et al., 2016)). Comparison of white matter tracts revealed an increase in mean diffusivity in patients with T2D which, in turn, was related to worse memory performance and slower information processing speed (Reijmer et al., 2013). Overall, T2D is implicated with significant re- ductions in the cerebral grey:white matter volume, detrimental to cognition.

2.1.2. Functional changes

An rs-fMRI study of T2D patients showed reduced functional con- nectivity between the hippocampus and numerous regions including the anterior cingulate cortex, inferior parietal and medial temporal lobes (Zhou et al., 2010). Diffusion tenor image (DTI) analysis showed that the right inferior frontal gyrus presented increased nodal degree; the left post central gyrus presented decreased local efficiency; the right hip- pocampus and the superior pole of the right temporal lobe exhibited decreased global and local efficiency respectively; the left pallidum and the right amygdala presented decreased global efficiency (Zhang et al., 2019c). These represent regions implicated in memory and emotion-memory associations. Experiments by the Utrecht Diabetic Encephalopathy Study investigated associations between T2D and total cerebral blood flow (CBF) in a cross-sectional study of 98 patients and 47 control participants. In this sample, total CBF – as measured by blood flow within the internal carotid and basilar arteries – was significantly diminished in patients (Tiehuis et al., 2008). However, when the values were corrected for brain volume there was no significant difference in cerebral blood flow between patients and controls. Across all partici- pants, lower CBF was associated with poorer performance on cognitive tests (independent of white matter pathology and infarcts), but this as- sociation was independent of diabetes status. Based on these findings, the authors concluded that total CBF was likely not the underlying cause of cognitive impairment in patients but may be a contributing factor. Cerebral blood flow is also positively associated with impaired glucose tolerance in regions included orbitofrontal cortex, superior temporal gyrus and the inferior parietal lobule (Thambisetty et al., 2013). In addition to cerebrovascular changes, in patients with prediabetes or T2D, higher insulin resistance was associated with reductions in glucose metabolism in regions including posterior cingulate cortex, precuneus, temporal lobes and regions of prefrontal cortex

Table 2
Comparison of functional changes in compulsivity-associated brain regions in Obsessive-Compulsive Disorder (OCD), Autism Spectrum Disorder (ASD) and Type 2 diabetes.

Region	Functional changes		
	OCD	Autism	Type 2 diabetes
Orbitofrontal cortex	Increased activation in OCD patients vs controls (Thorsen et al., 2018).	Altered functional connectivity across ages (Long et al., 2016)	Reduced activation (Sun et al., 2017)
Dorsal Anterior Cingulate Cortex	Increased activation in OCD patients (Thorsen et al., 2018) (Rotge et al., 2008)	Reduced activation (Ameis and Szatmari, 2012) Reduced functional connectivity (Long et al., 2016)	Reduced activation (Sun et al., 2017)
Midcingulate Cortex	Decreased neuron size (Uppal et al., 2014)	Reduced activation (Ameis and Szatmari, 2012)	No data
Amygdala	Increased activation in OCD patients (Rotge et al., 2008) Altered connectivity (Heuvel et al., 2022)	Reduced activation (Ameis and Szatmari, 2012)	Right amygdala shows decreased global efficiency (Zhang et al., 2019c)
Nucleus Accumbens	Reduced activity (Wu et al., 2021)	Reduced functional connectivity in ASD to regions including cingulate cortex, dorsal striatum and thalamus (Polk and Ikuta, 2022)	No data
Dorsomedial striatum (encompassing caudate and putamen	Reduced activity (Lipton et al., 2019)	Reduced activation during socialisation (Delmonte et al., 2012)	Decreased activation (Antal et al., 2022)
Hippocampus	Increased activation in OCD patients (Rotge et al., 2008).	Reduced connectivity between hippocampus ad fronto-parietal network in memory retrieval (Cooper et al., 2017)	Reduced connectivity with regions including anterior cingulate cortex, inferior parietal and medial temporal lobes (Zhou et al., 2010)
Cerebellum	Altered connectivity to cerebellum and cingulate in OCD (Zhang et al., 2019b)	Reduced connectivity to frontal, motor cortex and striatum (Crippa et al., 2016)	Reduced frontal cortex – cerebellum connectivity (Zhang et al., 2020)

(Baker et al., 2011). This study also conducted a separate scan as participants completed a memory-encoding task. Healthy participants displayed the predicted increase in activation in regions associated with memory encoding, including medial cingulate, frontal and temporal cortices. Qualitatively, those participants with prediabetes/T2D had a more widespread pattern of activation extending into putamen, cerebellum and thalamic regions. Based on this pattern of diffuse activation, the authors draw parallels to changes in cerebral metabolism that are commonly seen in AD, involving frontal, temporal-parietal and cingulate regions. In summary, connectivity and activation states across memory and emotion-associated brain regions are impacted by T2D.

2.1.3. Association with cognitive dysfunction

While insulin receptors are expressed throughout the brain including the hippocampus, cerebral cortex and cerebellum, evidence of insulin synthesis in brain is patchy and while regulation of insulin secretion and action in the periphery is well documented, whether the same mecha- nisms are also involved in any local brain regulation of insulin action is not known. The molecular landscape by van de Vondervoort et al. (2016) reported enrichment of the insulin signalling pathway amongst OCD-associated genes and also highlighted the voltage-gated potassium channel KCNQ1, with a role in insulin secretion regulation in pancreas, as a significantly associated gene. A recent study by Bralten et al. (2020) utilised GWAS, polygenic-risk score-based analysis and gene-set analysis to examine genetic overlap between T2D and OCD. This showed shared genetic etiology between the insulin-linked gene set and symme- try/ordering/counting behaviour, with gene-set analyses reporting in- fluence of genes including brain-derived neurotrophic factor (BDNF), associated with neuroplasticity, DCC for axonal growth and SLIT3 in axonal guidance, however no single gene from the total gene set reached significance. Research suggests a link between T2D and the development of cognitive changes, and is associated with disorders including ADHD (Chen et al., 2013a), Anorexia Nervosa (Watson et al., 2019) and ASD (Stern, 2011) in addition to cognitive decline, with many patients re- ported to show impaired information processing speeds, attention and executive functioning over time (Van Den Berg et al., 2010). A system- atic review of these studies examined cross-sectional and longitudinal effects of T2D on cognition (Van den Berg et al., 2009). Across studies, the most common finding was impairment in processing speed (63% of studies), followed by attention (50%), memory (44%), and cognitive flexibility (38%), especially with older age. Interestingly, blood levels of glycosylated hemoglobin (HbA1c), a proxy for diabetes severity, corre- lates positively with OCD symptoms (Kontoangelos et al., 2013). Indeed, patients are reported to exhibit brain changes to varying extents, including reduced cerebral volume and atrophy (Roberts et al., 2014) and white matter abnormalities (Xiong et al., 2016), oxidative stress and mitochondrial dysfunction (Kleinridders et al., 2015), neuro- inflammation (Rom et al., 2019), neurotransmitter imbalance (Datusalia and Sharma, 2014), aggregate accumulation (Platt et al., 2016) and vascular damage (Kooistra et al., 2013; Zeng et al., 2016). Previous in- vestigations into the cognitive phenotype of the TALLYHO/JngJ model of T2D show decreased spontaneous alternation and repetitive entries into arms of a maze compared to control counterparts (van de Vonder-voort et al., 2019). A study by Kleinridders (2015) established a mouse model of brain-specific insulin resistance, which exhibited increased anxious and depressive behaviours compared to controls, in addition to abnormal mitochondrial activity, aberrant dopamine balance and evi- dence of protein aggregate accumulation (Kleinridders et al., 2015). Overall, evidence from recent years is mounting to indicate that pe- riphery or brain-derived insulin impacts neurophysiological processes, with associations between T2D and a variety of cognitive, neurodevelopmental and neurodegenerative disorders.

Metformin and cognitive function

Identifying the physiological aspects of T2D that contribute to cognitive decline is essential in determining a therapeutic strategy. This could be a range of possible factors, including obesity and lipid levels, hyperglycaemia, or insulin resistance. The T2D drug metformin is capable of crossing the BBB (Gantois et al., 2019) and is known for its ability to normalise blood glucose, which it achieves via mechanisms including inhibiting hepatic gluconeogenesis, decreasing intestinal glucose absorption, increasing peripheral glucose uptake and improving insulin sensitivity (DeFronzo and Goodman, 1995). Metformin is re- ported to reduce insulin-resistance-associated increases in reactive ox- ygen species (ROS)elicited through mitochondrial dysfunction, implicating it in an anti-inflammatory role (Ruegsegger et al., 2019). Metformin also inhibits NF-KB phosphorylation and reduces C-reactive protein (CRP; an inflammatory marker) levels in serum (Song-Nan and Wang, 2009). When administered to 18-month old healthy mice, met- formin elicited reduced microglial activation, enhanced autophagy in the hippocampus and reduced proinflammatory cytokines, in addition to improved cognition (Kodali et al., 2021). Metformin is capable of restoring normal endothelial function in high fat-fed rodents (Sena et al., 2011), and promotes nitric oxide (NO) synthesis via AMPK (Davis et al., 2006), mediating vasodilation. This is an important process in trans- cytosis of compounds from blood, including insulin. Metformin is also reported to prevent amyloid deposition in APP/PS1 mice, reduced hip- pocampal neuronal loss and improved spatial memory (Ou et al., 2018). Whether or not it does this via an insulin-mediated change is undeter- mined in this study, however metformin has separately been found to induce Insulin-degrading enzyme expression, a key factor in amyloid beta breakdown (Lu et al., 2020). Additionally, metformin is reportedly capable of inducing alternative splicing in a selection of genes, including exon 11 of the insulin receptor INSR, causing greater inclusion and a possible therapeutic mechanism in the insulin resistance-associated disorder Myotonic Dystrophy which also demonstrates aspects of behavioural inflexibility (Laustriat et al., 2015). Metformin may have benefits not only on T2D but also associated cognitive dysfunction via multiple mechanisms including its ability to alter splice variation of insulin target receptors and action on AMPK signalling.

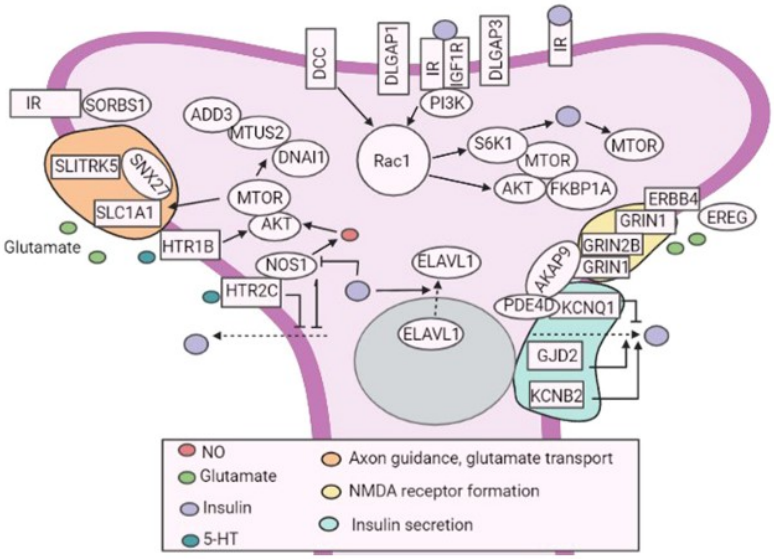

Fig. 2. Dendrite-associated cascades. Insulin is implicated predominantly in cytoskeleton formation and plasticity. Insulin also has roles in axon guidance, glutamate transport and NMDA receptor formation, with associated proteins and signalling shown (IR: Insulin receptor; IGF1R: Insulin-like growth factor 1 receptor). Created with Biorender.com.

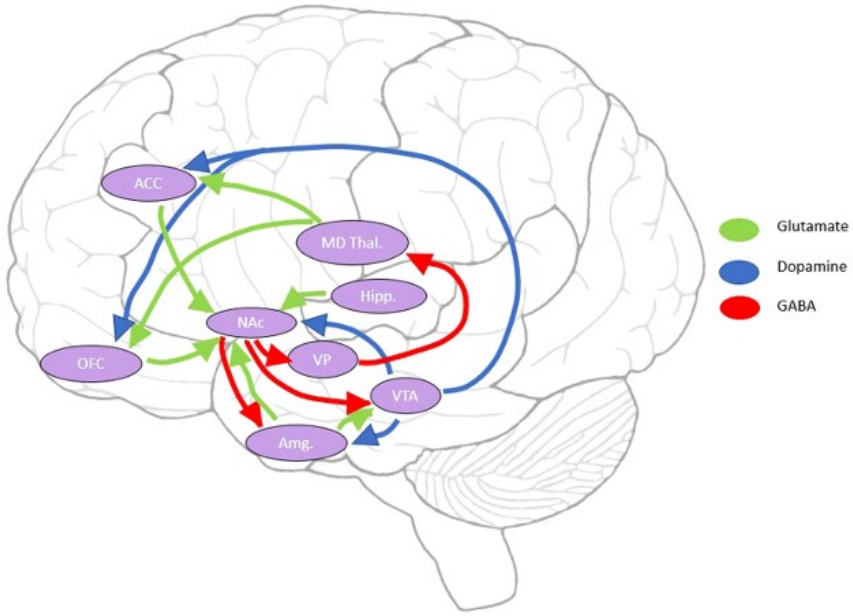

Fig. 3. Key projections and neurotransmitters implicated in reward and addictive/compulsive behaviours. ACC, Anterior cingulate cortex; OFC, Orbitofrontal cortex; NAc, Nucleus accumbens; VP, Ventral Pallidum; Amg, Amygdala; VTA, Ventral Tegmental Area; Hipp., Hippocampus; MD Thal., Mediodorsal Thalamus.

3. Insulin regulation of neurophysiological mechanisms

3.1. Glucose uptake and energy

Glucose is utilised for adenosine triphosphate (ATP) production via the tricarboxylic acid (TCA) cycle, with impaired ATP synthesis in pa- tients suffering with cognitive dysfunction in Alzheimer's Disease (Butterfield and Halliwell, 2019), ASD (Rossignol and Frye, 2012) and Depression (Bansal and Kuhad, 2016). Glucose is transported across the cell membrane via glucose transporters, some of which are insulin-independent (GLUT1, 2, 3, 5) and insulin-dependent (GLUT4, 8). GLUT4 is the principal insulin-regulated glucose transporter located mainly in the neuronal cell bodies and proximal dendrites at the syn- aptic level in the cortex, amygdala, hippocampus, hypothalamus, and cerebellum (Jurcovicova, 2014). Some GLUT4 immunoreactivity has been also observed in endothelial cells of microvessels. It is intracellu- larly present on the membranes of transport vesicles, Golgi apparatus, and rough endoplasmic reticulum. Grillo et al. (2009) demonstrated GLUT4 translocation is dependent upon PI3K/AKT signalling and could be inhibited by PI3 kinase inhibitor. GLUT4 shows the heaviest immu- noreactivity in the hippocampus, with chronic GLUT4 blockade impairing long-term memory, and reducing BDNF expression (Pear- son-Leary and McNay, 2016). An interesting study described that, dur- ing upregulated neuronal activity, GLUT4 was translocated to the synapse, providing glycolytic support in response to activation of AMPK (Ashrafi et al., 2017). In diabetic rats, GLUT4 translocation was reduced in the hippocampus, hypothesised as a possible contributor to memory impairments (Winocur et al., 2005). Brain-specific GLUT4 knockout mice show reduced glucose uptake in the hypothalamus, hippocampus, cerebellum, nucleus tractus solitarus and cortex (Reno et al., 2017). GLUT8 is expressed in several brain areas, predominantly hippocampus and amygdala, with expression also in cortex and hypothalamus. The expression of GLUT8 is principally neuronal (Reagan et al., 2002). It is hypothesised that GLUT8 may catalyze the transport of glucose after the glycolysation process of proteins from rough endoplasmic reticulum to cytosol for reuse (Piroli et al., 2002). In this way glucose can be recycled to provide cells with energy. Diabetic rats with hyperglycaemia and hypoinsulinemia showed reduced GLUT8, an effect that is exacerbated in the presence of stress (Piroli et al., 2004; Piroli et al., 2002)), sug- gesting that insulin might regulate glucose recycling. More recently, GLUT8 knockout mice showed ADHD-like hyperactivity (Schmidt et al., 2008). In summary, insulin regulates GLUT4 and GLUT8-mediated glucose uptake for metabolic support, which may influence cognitive processes and neuronal growth.

3.2. Atrophy

Insulin signalling promotes cell viability (Schubert et al., 2004), with application to neurons undergoing oxygen or glucose deprivation shown to elicit neuroprotective effects (Mielke and Wang, 2005). This is pro- posed to occur via PI3-K signalling, indeed the neuroprotective effect of insulin following serum-deprivation-induced apoptosis of cells reversed following addition of the selective PI3K inhibitors wortmannin or LY294002 (Ryu and Ko, 1999). Insulin addition to 1–Methyl-4–phenyl pyridinium (MPP^+) Parkinson's Disease model cells induced pro-survival PI3K/Akt/GSK-3 signalling (Ramalingam and Kim, 2016). Insulin also inhibits cytochrome c-mediated apoptosis (Sanderson et al., 2013; Jiang and Wang, 2004)). Insulin also promotes NO-mediated vasodilation, hence promoting oxygen and glucose perfusion of tis- sues, with impairments contributing to atrophy (Arnold et al., 2018). Indeed, experiments by the Utrecht Diabetic Encephalopathy Study identified reduced cerebral blood flow in T2D patients that corre- sponded with reduced cerebral volume (Tiehuis et al., 2008). Such volumetric changes are characteristic across psychiatric and cognitive disorders. A recent study by Cauda et al. (2018) generated a co-atrophy network based on grey matter decreases across ASD, OCD and schizo- phrenia. Here, they identified alterations predominantly in insulo-frontal, insulo-insular, insulo-hippocampal and frontoparietal nodes. Atrophy in Alzheimer's Disease involves a number of unique networks; medial-temporal atrophy with a dramatic effect on memory and language function, parieto-occipital atrophy with poor executive function and attention and diffuse cortical atrophy with a decline in visuospatial functioning (Ten Kate et al., 2018). Interestingly, this at- rophy appeared before the full onset of symptoms, and were predictive of the trajectory of symptoms. Depression is characterised by inflexi- bility in switching between emotions, and is characterised by signifi- cantly reduced volume in the putamen, pallidum, thalamus and hippocampus, while amygdala volume in those with comorbid anxiety was increased (Espinoza Oyarce et al., 2020). Taken together, insulin promotes cell viability via mechanisms including inhibition of mitochondrial-mediated apoptosis and oxygen perfusion of tissue, with atrophy a signature feature of some cognitive and mood disorders.

3.3. Synaptogenesis

Insulin and IGF1 receptors are present on presynaptic axon terminals and on postsynaptic densities of dendrites (Arnold et al., 2018), in addition to soma of Purkinje cells (Garcia-Segura et al., 1997), and are essential for normal dendritic growth (Cheng et al., 2003), synapto- genesis (Chiu et al., 2008; Popken et al., 2004) and for the regulation of synaptic plasticity (Van Der Heide et al., 2005; Sherrard and Bower, 2003). This is mediated via various downstream targets including mTORC1, GSK3β, and the FoxO family of transcription factors. mTORC1-mediated protein synthesis is important for synaptic plasticity (Stoica et al., 2011). Blockade of insulin signalling, specifically the PI3K-AKT-mTOR cascade yielded both a significant reduction in den- dritic spines and diminished excitatory postsynaptic currents

(Lee et al., 2011; Lee et al., 2005)). GSK3β regulates multiple aspects of neuronal functioning, including neural progenitor cell proliferation, neuronal polarity, and neuroplasticity (Salcedo-Tello et al., 2011). Insulin is hypothesised to mediate Long-term potentiation: Long-term depression balance via regulating GSK-3β, a protein previously associated with many neurological disorders, via PI3K/AKT signalling (Van Der Heide et al., 2005; Phane Peineau et al., 2007). GSK3β can also phosphorylate tau protein, a process involved in the pathogenesis of Alzheimer disease (AD). Insulin stimulates phosphorylation of GSK3β, and this reduces its enzymatic activity. Brain-specific knockout of IRS-2 results in decreased GSK3β activity and increased tau phosphorylation (Schubert et al., 2003) . FoxOs mediate the inhibitory action of insulin or insulin-like growth factor on key functions involved in cell metabolism, growth, differentiation, oxidative stress, senescence, autophagy and aging (Lee and Dong, 2017), with FOXO1 regulating mTOR signalling required for synaptogenesis (Southgate et al., 2007). FOXOs also regulate microtu- bule stability in neurons (Nechipurenko and Broihier, 2012). Insulin/IGF-1 signalling also activates the MAPK/ERK signalling, which phosphorylates synapsin protein required for appropriate synaptic vesicle release during neurotransmission (Giachello et al., 2010). The KCNQ1 voltage-gated potassium channel has roles in the regulation of insulin release in pancreas and interacts with the ASD-associated anchoring protein AKAP9 (Yamagata et al., 2011). The molecular landscape of OCD reports enrichment for signalling cascades implicated in postsynaptic dendritic formation, a process central to synaptogenesis and plasticity (van de Vondervoort et al., 2016), with mutations in associated genes identified in OCD mouse models and patients (Song et al., 2017). Synaptic dysfunction is hypothesised as a major mecha- nism of ASD, with multiple studies having revealed that mutations in genes like *NRXN, NLGN*, *SHANK*, *TSC1/2*, *FMR1*, and *MECP2* converge on common cellular pathways that intersect at synapses (Guang et al., 2018). These genes encode cell adhesion molecules, scaffolding proteins and proteins involved in synaptic transcription, protein synthesis and degradation, affecting various aspects of synapses including synapse formation and elimination, synaptic transmission and plasticity. In addition to dendritic expression, components of insulin receptor sig- nalling were found at high concentrations at the postsynaptic membrane in the hippocampus, cerebral cortex and cerebellum of cultured rodent neurons, suggesting a role in long-term potentiation and depression (Abbott et al., 1999; Van Der Heide et al., 2005)). The higher rate of cognitive decline amongst T2D patients, in addition to reported reduced brain volume support this hypothesis of insulin involvement in brain connectivity and neuron growth (Roy et al., 2020; Roberts et al., 2014). Insulin-associated signalling in synaptogenesis is shown in Fig. 2 (adapted from van de Vondervoort et al. (2016).

3.4. Neurotransmitter balance

3.4.1. Dopamine

Dopamine is a key neurotransmitter previously associated with motivational behaviour and Pavlovian conditioning (Saunders et al., 2018). In particular, the mesolimbic projection from the ventral-tegmental area (VTA) of the midbrain to the nucleus accumbens (NAc) is significant in disorders including depression and addiction (Xuet al., 2020). Dopamine binds to dopaminergic receptors D_{1-5}. Its sig- nificance in OCD symptomology is clear in clinical studies of deep brain stimulation of the nucleus accumbens (a dopamine terminal region) which offers symptomatic relief and the development of the dopamine based subchronic quinpirole mouse model of compulsive behaviour, following repeated administration of the D2/D3 agonist quinpirole (D_2 IC_{50} = 0.15; D_3 IC_{50} = 5.6) (Zaworski et al., 1999; Haluk and Floresco, 2009). The D_2 receptor family is the main target receptor of antipsy- chotic drug action, has the highest affinity for dopamine and is expressed in nucleus accumbens, cerebellum, cerebral cortex, hippo- campus and caudate to mediate spine enlargement and plasticity via downstream MAPK and AKT signalling, in addition to discrimination learning (Iino et al., 2020).

In a brain-specific insulin resistant mouse model, insulin was shown to negatively regulate expression of the monoamine oxidases MAO-A and MAO-B, responsible for dopamine degradation, in neuronal and glial cultures (Kleinridders et al., 2015). This study interestingly demonstrated elevated MAO-A/B expression and increased dopamine turnover in striatal regions, alongside increased anxious and depressive behaviour compared to controls. This was rescued by administration of the irreversible MAO-A/B inhibitor phenelzine. Insulin also promotes expression of the membrane-bound dopamine transporter DAT via PI3K signalling, increasing overall dopamine neuronal uptake (Carvelli et al., 2002). *Ex vivo* experiments showed that insulin indirectly promotes dopamine release in the striatum via binding to cholinergic neuron in- sulin receptors, yielding release of acetylcholine and activation of dopaminergic transmission via nAChRs (Stouffer et al., 2015a). Insulin receptors in astrocytes are also associated with exocytosis of ATP and dopamine release via purinergic signalling in the NAc (Cai et al., 2018). In addition to dopaminergic consequences of insulin receptor signalling, insulin resistance-associated elevated blood sugar is reported to induce hypoxia in brain, to which dopaminergic neurons are reported as highly sensitive (Mercuri et al., 1994). Indeed, T2D is a reported risk factor for the development of Parkinson's Disease, characterised by the deterio- ration of dopaminergic neurons (Pagano et al., 2018).

Research is still emerging about insulin's role in behavioural flexibility and the brain networks-associated with these functions. In- sulin is reported to play essential roles in the regulation of salience via its interaction with dopaminergic transmission, orchestrating motivational behaviour and classifying value to specific rewards (Daws et al., 2011). Diabetic rats were found to have dramatically reduced dopamine cellular uptake (~65%) (Owens et al., 2005a), with insulin adminis- tration proving beneficial in restoring dopaminergic homeostasis (Pat- terson et al., 1998a). Brain-specific insulin resistant mice demonstrate reduced dopamine signalling in dorsal striatum and nucleus accumbens in addition to increased anxious

and depressive behaviour (Kleinridders et al., 2015). Insulin's links to memory were described by an elegant experiment which induced a hippocampal-specific insulin receptor and IGF1 receptor knockout in mice (Soto et al., 2019), generating abnormal spatial learning and memory. Indeed, intranasal insulin delivery is reportedly beneficial in alleviating dementia symptoms (Avgerinos et al., 2018). No literature as of yet describes a precise link between insulin and attention, despite a reported link between T2D and attention deficit (Chen et al., 2013a).

3.4.2. Glutamate

Glutamate as the primary excitatory CNS neurotransmitter is essen- tial in processes including learning, plasticity and memory, however excess levels yield immediate or delayed neurotoxicity via increased Ca^{2+} influx into cells (Sanacora et al., 2008). Glutamate binds to both ionotropic and metabotropic receptors yielding immediate and slow modulation effects respectively, with excessive glutamate and excito- toxicity reported in fronto-striatal circuits of both ASD and OCD patients, particularly in frontal cortical regions and is correlated with symptom severity (Naaijen et al., 2017). Insulin, via downstream PI3K and MAPK signalling is reported to regulate glutamate levels and ameliorate excitotoxicity in addition to associated ATP and BDNF depletion (Krasil'nikova et al., 2019; Nampoothiri et al., 2014)). Insulin promotes internalization of glutamatergic AMPA receptors, hence impeding the proper functioning of mature glumatergic synapses (Renger et al., 2001). Additionally, insulin upregulates the expression of the membrane-bound excitatory amino acid transporters 1 and 2 (EAAT1,2) on astrocytes, which mediate glutamate uptake from the extracellular milieu (Han et al., 2016). Indeed, knockout of EAAT2 re- ported to yield synaptic hyperexcitability and increased repetitive be- haviours in mice (Aida et al., 2015). Despite this, the robustness of this "glutamate hypothesis" is under question, with the EU FP7 project TACTICS (Translational Adolescent and Childhood Therapeutic Interventions in Compulsive syndromes) stating that preclinical studies offer insufficient evidence of changes in frontostriatal glutamate tone in animal models of compulsivity (Anon, 2020a) and failure of NMDA antagonists such as memantine to abolish behavioural inflexibility.

3.4.3. GABA

GABA accounts for the most important neurotransmitter in the inhibitory system, with three main receptors, $GABA_A$, $GABA_B$ and $GABA_C$, the former of which, the ligand-gated ion channel $GABA_A$, is the target of the anxiolytic medication benzodiazepine. Insulin is reported to potentiate $GABA_A$ receptor-mediated tonic inhibition via AKT sig- nalling (Wang et al., 2003), and is capable of reducing excitability in brain regions including the amygdala (Korol et al., 2018) and hippo- campus. Indeed, in vivo neuroimaging of panic disorder patients demonstrated significantly reduced $GABA_A$ receptor levels in fronto- cortical regions (Nikolaus et al., 2010). GABA is also a key neurotrans- mitter implicated in repetitive behaviours in ASD (Chao et al., 2010). Interestingly, 90–95% of all neurons in the nucleus accumbens, a region associated with mood and addiction, are GABAergic medium spiny neurons, with GABAergic projections to the ventral pallidum and amygdala important in regulating addiction-like behaviour (Xu et al., 2020). A computational model of synaptic plasticity of such neurons in the nucleus accumbens highlighted a possible seesaw-like effect of dopamine and glutamate, with dopamine innervation from the ventral-tegmental area promoting GABAergic potentiation and gluta- mate promoting synaptic depression (Qi et al., 2011).

3.4.4. 5-HT

Traditional medications for the treatment of compulsive disorders are based upon the hypothesis that reduced serotonin yields patholog- ical changes, with FDA approval of selective serotonin reuptake in- hibitors including fluoxetine and sertraline which aim to attenuate such low levels. 5-HT is synthesised in the dorsal raphe nucleus of the midbrain, with receptors 5-HT1–7 mediating the release of neuro- transmitters including GABA, dopamine and glutamate across brain re- gions. The $5HT_{2A}$ and $5\text{-}HT_{2\ C}$ receptors are of particular interest, with the mechanism of many anti-psychotic medications based upon their antagonism (Thorneloe, 2019; Adams et al., 2005). Serotonin is the basis of the majority of mainstream selective serotonin reuptake inhibitors (SSRI's) such as fluoxetine and sertraline, however surprisingly little evidence links its function to compulsive processes (Stein et al., 2019), with many patients unresponsive to SSRI medications.

Insulin is reported to downregulate $5HT_{2A}$ receptors, with insulin resistance hence yielding insufficient internalisation and excessive re- ceptor binding (Ohkura et al., 2005). Such increased receptor expression and binding has been previously found in OCD patients (Adams et al., 2005; Flaisher-Grinberg et al., 2008). Insulin is also reported to block $5HT_{2\ C}$ activity (Hurley et al., 2003). Interestingly, patients with T2D show increased rate of polymorphisms in $5HT_{2A}/_{2\ C}$ receptors (Kring et al., 2009). The influence of these receptors on behaviour can be better understood with the administration of the $5\text{-}HT_{2A}/_{2\ C}$ agonist 2,5-dime- thoxy-4-iodophenyl-2-aminopropane (DOI), with mice demonstrating elevated cortical glutamate levels, indicating excessive excitatory neurotransmission that was attenuated via the administration of a se- lective $5HT_{2A}$ antagonist (Scruggs et al., 2003). Interestingly, increased glutamatergic transmission has previously been associated with OCD tendencies, with symptom severity correlated to the extent of glutamate change (Naaijen et al., 2017). DOI also increases the firing rate of dopaminergic neurons in the mPFC and VTA, in addition to increasing dopamine release, an effect attenuated with the administration of a se- lective $5HT_{2A}$ antagonist (Bortolozzi et al., 2005). $5\text{-}HT_{2\ C}$ antagonism in the orbitofrontal cortex is

reported to be particularly beneficial in attenuating compulsive lever pressing activity (Flaisher-Grinberg et al., 2008).

3.5. Inflammation and dysregulated immunity

Evidence suggests that dysregulated inflammation is a link under- lying metabolic disorders including diabetes and obesity (Dandona et al., 2004), and disorders of behavioural rigidity including ASD (Sin- iscalco et al., 2018), OCD (Attwells et al., 2017) and Alzheimer's Disease (Akiyama et al., 2000). A common feature of these behavioural rigidity disorders is the elevation of proinflammatory cytokines including IL-1β, IL-6, IL-8 and IL-12p40 (Ashwood et al., 2011), increased microglia activation, elevated numbers of CD4 T cells and enhanced macrophage M1 polarization (Prata et al., 2017). Indeed, exposure of OCD patients to LPS elicited a higher monocyte proinflammatory cytokine response than in healthy controls administered LPS, including IL1beta, IL-6, IL-8 and TNFalpha (Rodríguez et al., 2017). Chronic inflammatory responses yield a constant supply of toxic ROS and NO species, damaging neurons and glia. The possible role of insulin resistance in mediating such effects is described in T2D patients, who demonstrate reduced BBB integrity, in addition to upregulation of inflammatory genes in brain including eNOS, TNFalpha, TGFbeta1 and several chemokines (Rom et al., 2019). Insulin is reported to exert an anti-inflammatory effect in vitro, with one *st*udy reporting its regulation of IL-6 and IL-8 secretion in microglia and as- trocytes (Spielman et al., 2015). Indeed, these cell types express the insulin receptor and IGF-1 receptor, in addition to downstream IRS-1 and IRS-2. Rats that underwent inflammation-inducing trauma were reported to show reduced inflammation following administration of insulin (Zhu et al., 2018). The precise mechanism through which insulin may exert its effects is unclear, however various downstream effectors show immune regulatory functions. PI3K exerts effects on immune function (Hawkins and Stephens, 2015), with knockout mice of the p110α isoform showing impaired T cell proliferation and function, and a reduced number of CD4 + T cells (Sasaki et al., 2002). Trem2, activated in part by PI3K/Akt signalling, elicits an anti-inflammatory effect via inhibiting microglia/macrophage activation, neutrophil infiltration and suppressed neuronal apoptosis, an effect that was dampened following administration of the PI3K inhibitor LY294002 (Chen et al., 2020). T2D patients also demonstrate increased activation of p38 MAPK, which promotes IL-6, IL-8 and MCP-1 expression (Brown et al., 2015). The p38 MAPK pathway regulates the expression of several MMPs involved in inflammation including MMP-2 and MMP-9, in addition to proin- flammatory cytokines including IL-6 and TNFalpha (Song et al., 2006; Underwood et al., 2000). Interestingly, there is evidence that instead of insulin resistance inducing inflammatory changes, inflammation may instead induce insulin resistance. Studies in the 1990 s showed that TNFalpha application to adipocytes yielded a loss of IRS-1 and Glut4 expression (Stephens et al., 1997). Indeed, application of aspirin to T2D patients improves glycemic control (Hundal et al., 2002). Disruption to the normal balance of myeloid and lymphoid cells exacerbate insulin resistance (Stefanovic-Racic et al., 2012; Ricardo-Gonzalez et al., 2010; Winer et al., 2009)). Indeed, an unexpected effect of the Covid-19 virus was the development of hyperglycaemia and insulin resistance over time (Hayden, 2020).

The specific association between these neuroinflammatory media- tors and neurodegeneration remains disputed however Witte et al. (2010) proposed that mitochondrial dysfunction and oxidative stress are central to this progression. They suggest that altered mitochondrial function leads to impaired energy metabolism and induces neuro- inflammation via NO and ROS production, which results in neuro- degeneration. Indeed, mitochondrial dysfunction is a feature of numerous neurodegenerative disorders including Alzheimer's Disease, Parkinson's Disease and Huntington's Disease (Lin and Beal, 2006). Insulin signalling plays into this function, with brain-specific insulin receptor knockouts demonstrating mitochondrial dysfunction with impaired oxidative activity, particularly in the dorsal striatum and NAc, increased levels of reactive oxygen species and elevated lipid and pro- tein oxidation in striatum and nucleus accumbens (Kleinridders et al., 2015). This study suggests that such insulin-mediated mitochondrial dysfunction is hypothesised to occur in at least three possible ways. The first concerns decreased expression of electron-transport chain proteins, the second concerns increased monoamine oxidase in the mitochondrial membrane, and finally the third concerns changes in the morphology and number of mitochondria in brain, with fission favoured in place of fusion, reducing metabolic capacity. Insulin is reported to directly affect the first two processes, however its influence over mitochondrial fission remains unclear. The T2D medication metformin elicits an anti-inflammatory effect, and interestingly improves mitochondrial ef- ficiency in ATP production, reduces oxidative stress and rescues reduced levels of citrate synthase and COX proteins in the mitochondrial mem- brane of insulin resistant brains (Ruegsegger et al., 2019). Hence, insulin may have important anti-inflammatory actions, which in turn may in- fluence mitochondrial dynamics.

3.6. Aggregates

T2D has been associated with onset of aggregate-associated diseases including Alzheimer's Disease (Rad et al., 2018) and Parkinson's Disease (De Pablo-Fernandez et al., 2018). Studies investigating the link be- tween repetitive behaviour and aggregate accumulation in brain have positively correlated repetitive negative thinking/rumination habits and Aβ and tau accumulation, in addition to a more rapid decline of global cognition and memory. Insulin may play a role in aggregate accumu- lation via what is called the "GSK3 hypothesis of Alzheimer's Disease". This encompasses the insulin-governed PI3K/Akt-GSK-3β cascade, which inhibits GSK3β activation. Overactivation of this kinase, as would occur in insulin

resistance, yields tau hyperphosphorylation, elevated Aβ production and local plaque-mediated microglial inflammation (Hooper et al., 2008). This was reflected in brain-specific insulin resistant mouse models which demonstrated hyperphosphorylation of the tau protein via GSK-3β activation, consistent with AD (Kleinridders et al., 2015). Aberrant autophagy, responsible for aggregate clearance is another hypothesised mechanism of neurodegeneration. Indeed, the insulin-regulated downstream PI3K/AKT/mTOR signalling cascade is a major regulator of autophagic flux, and is altered in Alzheimer's disease and Parkinson's Disease (Heras-Sandoval et al., 2014). In turn, accu- mulation is reported to impair insulin signalling by impairing auto- phosphorylation of the receptor and reducing receptor density on dendritic spines (Zhao et al., 2008), generating a detrimental feed-forward loop. Interestingly, metformin was also shown to decrease tau and amyloid accumulation in an obese mouse model of AD (Li et al., 2012). Such aggregation promotes apoptosis via mechanisms including the disruption of normal mitochondrial function and dynamics (Han et al., 2017) and cytoskeletal dysfunction (Gendron, 2009; Alonso et al., 1994), yielding neuronal decline and impairing axonal transport.

Recent literature suggests and interesting insulin-amyloid-beta crosstalk. Insulin resistance is shown to induce Aβ accumulation (Son et al., 2012), which in turn is reported to impair insulin signalling by impairing autophosphorylation of the receptor and reducing receptor density on dendritic spines (Zhao et al., 2008). Additionally, the insulin degrading enzyme, induced by insulin signalling and responsible for its breakdown in a negative feedback loop, is also capable of metabolising Aβ. Hence, hyperinsulinemia in T2D may increase susceptibility to Aβ accumulation, and likewise Alzheimer's-associated accumulation of AB may induce insulin resistance. Indeed, Type 2 diabetes has been asso- ciated with onset of Alzheimer's Disease in numerous studies (Rad et al., 2018). Brain-specific insulin resistant mouse models demonstrated hyperphosphorylation of the tau protein via GSK-3β activation, consis- tent with AD (Kleinridders et al., 2015; Gratuze et al., 2018; Schubert et al., 2004). In summary, insulin signalling is associated with changes in Aβ and tau accumulation, with the insulin-degrading enzyme also capable of metabolising both insulin and Aβ.

3.7. *Myelination*

Myelination involves oligodendrocyte synthesis of the lipid-rich myelin sheath, which coats axons to increase the speed of electrical impulse propagation, essential for the formation of connective networks between brain regions and hemispheres (Felts et al., 1997). White matter structural connectivity changes are reported across regions including the cerebellum, anterior cingulate gyrus, hippocampus and frontostriatal circuits in disorders including OCD (Ziegler et al., 2019; Gan et al., 2017)), ASD (Galvez-Contreras et al., 2020) and Alzheimer's (Nasrabady et al., 2018). Altered myelination patterns were also iden- tified in quinpirole mouse models of compulsive behaviour (Straathof et al., 2020). Insulin is reported to govern oligodendrocyte precursor proliferation and maturation into myelinating cells, with early studies of insulin and IGF-1 administration to cultures demonstrating increased proliferation of oligodendrocyte precursor cells, increased maturation of oligodendrocytes and elevated myelin levels compared to unadminis- tered controls (McMorris and Dubois-Dalcq, 1988; Mozell and McMor- ris, 1991). This occurs via IGF1-induced expression of Noggin and Smad6, which regulate oligodendrocyte differentiation and maturation via inhibiting BMP signalling (Hsieh et al., 2004). Indeed, IGF1 knockout mice demonstrate reduced volume of the corpus callosum and anterior commissure, in addition to reduced oligodendrocyte population (Beck et al., 1995). Patients with diabetes type 2 are reported to demonstrate white matter microstructure abnormalities while TALLY- HO/JngJ mouse models of T2D demonstrate impaired white matter connectivity in corpus callosum, dorsomedial striatum and superior cerebellar peduncle (van de Vondervoort et al., 2019; Xiong et al., 2016; Breteler et al., 2003).

4. Insulin in disorders of behavioural inflexibility

4.1. *OCD*

According to the *Diagnostic and Statistical Manual of Mental Disorders,Fifth Edition, Obsessive-Compulsive Disorder is diagnosed on the basis of the* presence of obsessions, compulsions, or both, in which the obsessions or compulsions are time-consuming (e.g., take more than 1 h per day) or cause clinically significant distress or impairment in social, occupa- tional, or other important areas of functioning, symptoms are not attributable to the physiological effects of a substance (e.g., a drug of abuse, a medication) or another medical condition or mental disorder (Anon, 2020b). Compulsions are repetitive behaviours or mental acts that the individual feels compelled to do in response to an obsession according to rigid rules, or to achieve a sense of 'completeness'. Com- mon sets of obsessions and compulsions in patients with OCD include concerns about contamination together with cleaning, intrusive aggressive or sexual thoughts together with mental rituals, concerns about self-harm or harm to others together with checking, and concerns about symmetry together with ordering or counting (Bloch et al., 2008). Neuroimaging and cognitive-affective studies in animal models have indicated that hyperactivation of the parallel, partly segregated cortico-striato-thalamo-cortical loops responsible for motivational, sensorimotor, affective and cognitive processes is responsible for obsessive-compulsive symptomology (Ahmari et al., 2013; Parmar and Sarkar, 2016; J et al., 2019)). Extra-CSTC regions such as the hippo- campus (Boedhoe et al., 2017b), governing learning and memory, and the cerebellum (Zhang et al., 2019a), regulating both motor control and impulsivity (Miquel et al., 2019) have also been associated with OCD

pathology due to their interactions with regions implicated in flexible decision-making including the striatum and cingulate areas (Zhang et al., 2019b) PFC (Zhang et al., 2019a) and amygdala in conditioned response (Pikkarainen et al., 1998). Dopaminergic signalling is signifi- cant across studies, with administration of the D2 agonist quinpirole influencing stereotypy behaviour in mice (Korff et al., 2008), while glutamatergic and GABAergic neurotransmitter signalling has been implicated with the benefit of drugs including memantine and various anti-convulsants in symptom attenuation (Marinova, Z., Chuang, D.M., Fineberg, N. Z, 2017). Due to a lack of clear diagnostic biomarkers and heavy reliance on symptom-based diagnosis, large sample sizes are crucial to underpin relevant functional and structural brain changes in patients. This was the aim of the imaging genetics ENIGMA consortium, an international collaborative effort across 15 countries to better define functional and structural alterations in brain disorders including OCD (Heuvel et al., 2022). This study showed that patients had a larger thalamus and pallidum and smaller hippocampus. Medicated adult OCD patients, in comparison to controls, had widespread abnormalities in cortical thickness, mainly in relation to frontal, temporal, parietal and occipital regions. Centrality, mainly of the cingulate and orbitofrontal areas, was associated with OCD disease duration, indicative of greater involvement of these regions with chronicity. White matter structural connectivity changes are reported in OCD patients, with a study by Ziegler et al. (2019) ; Gan et al. (2017) demonstrating reduced myeli- nation in the dorsomedial and dorsolateral frontal regions and ventral striatum, correlated with a compulsive phenotype. DTI studies across patients report decreased white matter integrity in the corpus callosum, cingulate bundle and internal capsule (Koch et al., 2014). Altered myelination patterns were also identified in quinpirole mouse models of compulsive behaviour (Straathof et al., 2020).

Insulin signalling is hypothesised to play a role in OCD based on a previous molecular landscape study (van de Vondervoort et al., 2016), in which results of GWAS studies of 1465 individuals affected with OCD, 5557 ancestry-matched controls and 400 complete trios were compiled into significant networks. Other literature supports this hypothesis, with a recent study correlating compulsivity in OCD with insulin-associated traits (T2D, HbA1C status of glycosylated hemoglobin, fasting insulin levels, fasting glucose levels and 2 h glucose levels) (Bralten et al., 2020). Indeed, animal models of T2D show increased compulsive-like behaviour, in addition to elevated anxiety, another feature of OCD (van de Vondervoort et al., 2019). Key mechanisms through which in- sulin elicits such effects include its regulation of synaptogenesis and dendritic spine density (van de Vondervoort et al., 2016). According to this molecular landscape study, insulin additionally regulates gluta- matergic and serotonergic signalling, both of which have been linked to OCD (Stein et al., 2019). Dysregulated immunity features in OCD (Marazziti et al., 2018), with insulin reported to exert an anti-inflammatory effect in vitro. One study reports a role of insulin in regulating the inflammatory status of microglia and astrocytes via the regulation of IL-6 and IL-8 secretion (Spielman et al., 2015) while the downstream effector PI3K regulates T cell proliferation and function (Hawkins and Stephens, 2015). Insulin antagonises the FOXO family of transcription factors, with FOXO1 in particular reported to regulate phagocytosis, chemotaxis and neutrophil recruitment in addition to macrophage proinflammatory signalling (Dong et al., 2017; Fan et al., 2010). Insulin is also hypothesised to yield an anti-inflammatory effect via its regulation of hyperglycaemia. High glucose levels may generate an inflammatory response due to "glucose toxicity", generating cell stress and ROS production (Kumar et al., 2014).

4.2. Addiction

Addiction is a neurophysiological disorder characterised by intense and persistent urges to engage in specific behaviours regardless of harm. Typical signs encompass a persistent, compulsive engagement with rewarding stimuli, and difficulty in inhibiting behaviours despite negative consequences, consequently yielding short-term benefits and long-term harms. Typical examples include alcoholism, drug addiction (eg. cannabis, cocaine, nicotine, opioid), video game addiction and gambling addiction. It is widely accepted that the means through which addictive behaviours achieve their effects is via stimulation of dopamine signalling in the mesolimbic circuit in regions including the nucleus accumbens, a centre for motivation control (Di Chiara and Imperato, 1988). Dopamine receptor 1 (D1R) and 2 (D2R) are the two key asso- ciated receptors expressed throughout the brain, with D1R associated with sensitisation to drug-related reward and D2R implicated with compulsive drug intake (Bertran-Gonzalez et al., 2008; Perez et al., 2011). An fMRI study examining the circuits implicated with vulnera- bility and resilience to developing drug addictions revealed significant hypoconnectivity in orbitofrontal and ventromedial prefrontal cortical-striatal circuits (implicated in goal-directed behaviour and behaviour switching) (Ersche et al., 2020). Insulin signalling and dopaminergic neurotransmission are arguably not independent, with studies suggesting that crosstalk exists. An in vivo study of dopaminergic uptake via the DAT transporter in rat brain showed that velocity of dopamine transport is significantly reduced in fasted rats with reduced circulating insulin; an effect that was rescued via insulin administration (Patterson et al., 1998b). Streptozocin administration to rats, depleting peripheral and central insulin levels, also reduced dopamine clearance in rat striatum by 65% (Owens et al., 2005b). DAT expression on neu- rons is also regulated via the insulin-Akt pathway in vitro (Garcia et al., 2005). A key paper by Kleinridders et al. (2015) shows that in mice with brain-specific insulin resistance, dopamine metabolism is increased, in addition to increased anxious behaviour. Insulin receptors are expressed on dopaminergic neurons within mesolimbic projections. For example, insulin administration to the VTA, the main source of dopaminergic neuronal projections in the mesocortical circuit, generated reduced somatodendritic concentrations of dopamine (Mebel et al., 2012). Interestingly, the T2D diabetes medication metformin, when injected into

nucleus accumbens, effectively attenuates cue-induced cocaine seeking in rats, hypothesised as due to its activation of adenosine monophosphate activated protein kinase (AMPK), functioning as a cellular energy sensor (Chan et al., 2022).

4.3. Anorexia nervosa

Anorexia nervosa (AN) is an eating disorder that normally manifests during late childhood and adolescence that has one of the highest mortality rates among psychiatric illnesses. As consequence of the associated starvation and increased catabolic metabolism (Misra and Klibanski, 2014), clinical characteristics include loss of body fat and lean mass, decrease of bone mass, amenorrhea, hypertension, bradycardia and hypothermia (Von Schwanenflug et al., 2019). Additionally, pa- tients present with increased cognitive and behavioural rigidity (expressed by traits such as self-demanding, perfectionistic, lack of spontaneous behaviour, poor cognitive flexibility and attention to de- tails) (Buzzichelli et al., 2018; Maria et al., 2020), which was even contemplated as endophenotype with a high inherited and neurobio- logical component (Holliday et al., 2005; Milton et al., 2021). Neuro- psychological studies report significant deficits in attention, memory and executive function (e.g. decision making, set-shifting and global processing) (Foldi et al., 2021), in addition to inflexible thought patterns and rigid behaviour in patients with AN, with excessive self-regulation of diet and exercise regimens, even in the face of a rapid decrease in body weight (Foldi et al., 2021; Miles et al., 2020), (Wierenga et al., 2014)). This inflexibility is further reflected in AN individuals, who make more perseverative errors on set-shifting tasks, including the Wisconsin Card Sorting Test (WCST), than control participants (Tchan- turia et al., 2012, 2004), persisting even after weight recovery (Miles et al., 2020). Reports suggest an increased insulin sensitivity state in AN, with the potential to impact reward-based learning (Fukushima et al., 1993; Berner et al., 2019)). Central insulin signalling plays an important role in maintaining optimal dopamine (DA) tone in ventral and dorsal striatum by tuning rates of synaptic DA release (Stouffer et al., 2015b) and clearance (Schoffelmeer et al., 2011; Williams et al., 2007), with impairments in central insulin signalling having a direct impact on brain DA systems (Kleinridders et al., 2015; Ko¨nner et al., 2011; Schoffelmeer et al., 2011; Sevak et al., 2007; Williams et al., 2007). Specifically, in- sulin receptors are expressed on dopaminergic neurons in the ventral tegmental area (VTA), a major hub in the mesolimbic circuitry gov- erning food seeking behaviours (Ilyas et al., 2019). Alterations in normal insulin balance may promote restrictive dietary and exercise practices and maintenance of the disorder.

4.4. Obesity and binge eating

Functional, molecular and genetic neuroimaging studies point to decreased basal metabolism in the prefrontal cortex and striatum, dopaminergic alterations and increased activation of reward brain areas in response to palatable food cues in people living with obesity. Elevated reward region responsivity may trigger food craving and predict future weight gain, whereas reduced activation of executive or inhibitory control pathways is associated with the development of obesity and weight regain after dieting. The direction of causality of these associa- tions remains underexplored by a paucity of longitudinal studies and the underlying mechanism for these associations is not known. Dopamine pathways in particular are thought to play an important role in the processing of food reward salience (De Araujo et al., 2012; Lindgren et al., 2018; Martel and Fantino, 1996; Schultz, 2016). Reward from natural (e.g. food and sex) and non-natural (e.g. drugs of addiction, which supplant natural rewards in valence, and have no beneficial evolutionary purpose) sources, both lead to increased dopamine release in the nucleus accumbens and the ventral striatum. Dopamine pathways appear to be particularly important in processing the hedonic appeal rather than appetitive drive for food, for example preference for sugary food as opposed to hunger for any type of food (Szczypka et al., 2001; Volkow et al., 2008). Volkow's study found that the dopamine receptor 2 was reduced only at a BMI of over 50 (Wang et al., 2014), suggesting that binge eating or food addiction aligns more with dopamine hypo- function and reduced DRD2 availability. Binge eating, and the purchase and consumption of highly palatable calorie dense food has been asso- ciated with steeper discounting of delayed future rewards (Appelhans et al., 2011; Davis et al., 2010; Nederkoorn et al., 2009, 2007). Bello et al., in their review of the role of dopamine in binge eating, suggest that sustained stimulation of the dopamine systems by bingeing, pro- moted by pre-existing conditions (e.g. genetic traits (D2 receptor poly- morphisms), dietary restraint, stress, etc.) results in progressive impairments of dopamine signalling (Bello and Hajnal, 2010), which perpetuate the behaviour. People living with obesity exhibit difficulty inhibiting automatic responses on inhibitory control tasks, tending to engage in habitual or overlearned behaviours associated with weakened connectivity in executive control networks and enhanced connectivity in salience network and default mode networks and show a preference for smaller, immediate rewards over larger, delayed rewards relative to normal weight individuals (Donofry et al., 2020).

Insulin signalling alterations may induce executive functioning def- icits in the brain, with T2D associated with cognitive decline (Ebady et al., 2008). Insulin receptors appear abundantly in the brain, with the IRS-2 receptor substrate particularly important in excessive eating be- haviours. Indeed, knockout mice for IRS-2 develop a diet-driven obese phenotype (Lin et al., 2004). This effect may be partially attributed to insulin receptors in specific hypothalamic subnuclei. Furthermore, central insulin signalling may exert peripheral changes, with insulin signalling in the hypothalamus necessary for controlling hepatic glucose production (Heni et al., 2015). Insulin signalling may play a direct role in food reward processing via regulation of synaptic dopamine balance (Stouffer et al., 2015b; Schoffelmeer et al.,

2011; Williams et al., 2007), thus altering the rewarding properties of food. In humans, the hedonic appeal for food is altered by intranasal insulin in women (Schneider et al., 2022); women who have already eaten a meal experienced reduced hedonic appeal toward a dessert (e.g. chocolate cookies).

4.5. *Alzheimer's disease*

Alzheimer's Disease (AD) is the most common neurodegenerative disorder and the most prevalent cause of dementia in aged people (>65 years) (Mattson, 2004; Qiu et al., 2009), with the incidence expected to

increase three-fold by 2050 (Livingston et al., 2020). Almost two-thirds of AD patients are women. AD is characterised by a slow and progressive cognitive decline affecting learning and memory, language, and execu- tive function, and the presence of amyloid plaques, neurofibrillary tangles, activated microglia, reactive astrocytes, synapse loss, and cortical atrophy (Deture and Dickson, 2019). These classical AD neuropathological signs are accompanied by compromised brain glucose uptake and insulin sensitivity (Baglietto-Vargas et al., 2016), and downregulation of insulin binding sites along the blood-brain barrier (Leclerc et al., 2023).

In AD animal models, the age of onset of progressive cognitive decline and amyloid beta deposition is rather variable, with memory impairments starting as early as 3 months and amyloidosis at 4–6 months (Platt et al., 1832). Glucose intolerance has been described in 3xTg-AD mouse model (Vandal et al., 2015) and APP/PS1 mice (Takeda et al., 2010). As memory loss is the primary AD symptom, behavioural testing in AD mouse models has predominantly focussed on (spatial) reference memory and assessing aspects of executive function is less common. However, several AD animal models have been shown to have deficits in set-shifting and reversal learning tasks, mostly in aged ani- mals (Granger et al., 2016; Guarino et al., 2019; Romberg et al., 2013; Rorabaugh et al., 2017; Shepherd et al., 2021), reviewed in (Webster et al., 2014). Using a touchscreen-based reversal task which more accurately reflects testing in a clinical setting, Van den Broeck et al. (2019). showed early reversal impairment in 6-month-old APPPS1–21 mice). In human studies, compared to healthy controls, AD cases tend to complete fewer categories and make more perseverative errors in the Wisconsin Card Sorting Task, suggesting impaired set-shifting capability (Chiu et al., 2014; Guarino et al., 2019; Nagahama et al., 2003; Paulsen et al., 1995; Redondo et al., 2016; Tei et al., 1997).

How insulin might modulate AD-related neuropathology has been reviewed above (see section "Aggregates"). T2D is one of the most common comorbidities of AD (Wang et al., 2018) and the two disorders share pathophysiological characteristics, including insulin resistance, which suggests common or related underlying processes (Bla´zquez et al., 2014), although a recent study found no genetic overlap between Type 2 diabetes mellitus (T2DM) and AD (Hardy et al., 2022). T2DM increases the risk of developing dementia by 50% (Zhang et al., 2017), and the risk is more pronounced the earlier the onset of T2DM (Barbiellini Amidei et al., 2021). Manipulations which induce insulin resistance in AD ani- mal models have been shown to exacerbate the amyloid beta plaque accumulation in the brain and worsen cognitive impairments (Kimura, 2016). Antidiabetic drugs, including intranasal insulin, have shown promise in ameliorating cognitive symptoms and some AD biomarkers in patients (Michailidis et al., 2022), but large-scale longitudinal studies are required to establish their efficacy.

4.6. *Parkinson's disease*

Parkinson's disease is a chronic neurodegenerative condition which manifests predominantly as motor abnormalities including rigidity, increased muscle tone and resistance. Cognitive deficits including ex- ecutive dysfunction are a common feature, incorporating deficits in planning, problem solving and shifting attention on tasks. Psychiatric manifestations of Parkinson's can include features of a premorbid per- sonality including emotional and attitudinal inflexibility, mental rigid- ity, anxiety and a tendency towards depressive symptoms including introversion, apathy, deficits on reward processing and novelty seeking (Rodriguez-Oroz et al., 2009).

Supraspinal and cortical mechanisms, in addition to the basal ganglia and motor thalamus, have been proposed in the pathophysiological or- igins of motor rigidity. GABA and cholinergic interneuronal regulation in cortico-nigral-palladial microcircuits are likely engaged. Depletion of nigrostriatal and overactivity of prefrontal dopamine are associated with executive dysfunction in early stages of disease (Baig et al., 2017). Other neurotransmitters including acetylcholine and noradrenaline are likely involved in cognitive manifestations of the disease (Solinas et al., 2019). Striatal dopaminergic deficits are strongly implicated in some of the psychiatric features of Parkinson's including novelty seeking, impulsive and addictive behaviours (Baig et al., 2017). Psychiatric fea- tures in Parkinson's such as punding, hobbyism, impulse control disor- ders, behavioural addictions such as gambling, compulsive eating, shopping and hypersexuality manifest as complications derived from dopaminergic treatment. Other symptoms such as apathy may improve with treatment and symptoms may vary associated with on-off drug fluctuations and altered dopaminergic states.

T2D is reported to increase risk of developing Parkinson's disease, with a faster progression and worsening of motor and cognitive symp- toms over time (Cheong et al., 2020). Neuroimaging has revealed that T2DM has an adverse effect on striatal dopamine transporter binding and cortical thickness, consistent with a decline in executive function (Cheong et al., 2020). Higher cerebrospinal fluid (CSF) Tau, a non-specific fluid biomarker in several neurodegenerative diseases are reported to

be higher in Parkinson's patients with T2DM compared to patients without T2DM (Pagano et al., 2018). Insulin receptors are expressed in areas of the brain implicated in Parkinson's disease including the basal ganglia, substantia nigra, cerebral cortex and hy- pothalamus. Systemic insulin resistance observed in metabolic condi- tions such as T2DM is reflected in the brain, with the failure of cells to respond appropriately to insulin signalling. As insulin sensitivity de- clines, regions in the brain including the frontal lobes and hippocampus are susceptible to cerebrovascular disease and neurodegeneration re- flected through infarcts, reduced grey and white matter volume from atrophy (Ryan et al., 2014). Coupled to underlying biochemical alter- ations these may contribute to motor, cognitive and psychiatric im- pairments in Parkinson's patients with T2DM. Insulin in turn may play a role in neuroprotection. A recent meta-analysis of antidiabetic agents for the treatment of Parkinson's disease suggests that treatment with the diabetes medication exenatide is associated with the alleviation of cognitive, motor and nonmotor symptoms (Aviles-Olmos et al., 2013). However, the insulin-Parkinson's link has yet to be concretely established, and long-term studies with a large sample size of patients with Parkinson's are required.

1. Conclusion

Accumulating evidence points towards the significance of insulin, not only in metabolic roles, but also as a major regulator of neuro- physiological functions. Insulin and IGF1 receptors are highly expressed across brain, with T2D associated with changes in white and grey matter volume and functional connectivity. Indeed, there is evidence that T2D medication such as metformin can be repurposed for the treatment of associated behavioural alterations. Next steps involve elucidation of which specific mechanisms generate the strongest downstream behav- ioural impact, and how these may be therapeutically targeted.

As the English comedian Jasper Carrott once said, "laughter is the best medicine… unless you're diabetic, then insulin comes pretty high up the list". Perhaps the same can be said for behavioural inflexibility.

Declaration of competing interest

The authors declare no conflict of interest.

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Hyperglycemia and cognitive impairments anticipate the onset of an overt type 2 diabetes-like phenotype in TALLYHO/JngJ mice

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ABSTRACT

Type 2 Diabetes mellitus (T2DM) is a metabolic disorder characterized by chronic hyperglycemia, 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, its role in the central nervous system (CNS) is emerging only recently. Brain insulin has been lately associated with brain disorders like Alzheimer's disease, obsessive compulsive disorder, and attention deficit hyperactivity disorder. Thus, understanding the role of insulin as a common risk factor for mental and somatic comorbidities may disclose novel preventative and therapeutic approaches. We evaluated 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, previously reported to constitute a valid experimental model of T2DM due to impaired insulin signaling). Adult TH mice have also been studied for alterations in gut microbiota diversity and composition. While TH mice exhibited profound deficits in cognitive flexibility and altered glucose metabolism, we observed that these alterations emerged either much earlier (males) or inde- pendent of (females) a comprehensive constellation of symptoms, isomorphic to an overt T2DM-like phenotype (insulin resistance, polydipsia, higher energy expenditure, and altered lipid metabolism). We also observed significant sex-dependent alterations in gut microbiota alpha diversity and taxonomy in adult TH mice. Deficits in insulin signaling may represent a common risk factor for both T2DM and CNS-related deficits, which may stem from (partly) independent mechanism

Introduction

The association between glucose metabolism and mental distur- bances is not new – dating back to 1919, (Kooy, 1919) reported glycosuria in psychiatric patients compared to healthy individuals. The subsequent discovery of insulin, a key regulator of glucose metabolism, disclosed relevant avenues to identify potential shared risk factors be- tween mental and somatic disturbances. While alterations in insulin signaling have been traditionally associated with somatic disturbances (e.g., type 1 diabetes; type 2 diabetes mellitus, T2DM; metabolic syn- drome, MS; and obesity (Bennett and Knowler, 2005; Saltiel and Kahn, 2001; Samson and Garber, 2014)), recent findings have implicated them in mental disorders (e.g., Alzheimer's disease, AD (Klimova et al., 2018); obsessive compulsive disorder, OCD (van de Vondervoort et al., 2016); and attention deficit hyperactivity disorder, ADHD (Landau and Pinhas-Hamiel, 2019)). Understanding the contribution of insulin as a common risk factor in the aforementioned comorbidities represents a much-needed knowledge leap in the management and treatment of such

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chronic highly-debilitating diseases.

The involvement of insulin in T2DM and MS (Brown and Walker, 2016; Huang, 2009; Samson and Garber, 2014) is associated with its role in glucose metabolism. 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 (De Fronzo, 2004). In T2DM, insulin function is defective due to two main anomalies: beta-cell dysfunction and/or insulin resistance (De Fronzo, 2004) (a reduced sensitivity to insulin of cells in target organs (Lebovitz, 2001)), which ultimately cause insulin deficiency and hy- perglycemia (Banday et al., 2020). Recently, the gut microbiota – the trillions of micro-organisms present in the gastrointestinal tract playing an important role in host metabolism – has also been implicated in T2DM pathophysiology (Wang et al., 2012), in the light of its role in inflammation, production of short-chain fatty acids and regulation of gut hormones (Herna´ndez et al., 2019).

Beside its role in metabolism, insulin, either from the periphery or via direct neuronal synthesis, acts at the level of the central nervous system (CNS), wherein its receptors (e.g., Insulin Receptor, IR, and In- sulin Growth Factor-1 receptor IGF-1R) are highly expressed (Banks et al., 2012; Clarke et al., 1986; Pomytkin et al., 2018) and wherein it contributes to several non-metabolic functions, such as learning, mem- ory, integration of sensory information, and modulation of synaptic plasticity (Chiu et al., 2008). Genetic (Fanelli et al., 2022), clinical (Creo et al., 2021), and preclinical (Biessels and Despa, 2018; Nistico` et al., 2012) studies have shown a correlation between T2DM and AD and have highlighted common pathophysiological characteristics (Bla´zquez et al., 2014; Bosco et al., 2011; Klimova et al., 2018). Additionally, T2DM patients are at increased risk of mild cognitive impairment (MCI) and diabetes-associated cognitive decrements (Biessels and Despa, 2018; Koekkoek et al., 2015; Monette et al., 2014; Palta et al., 2014). In concordance, several clinical investigations reported that anti-diabetic drugs have beneficial effects on cognitive impairments in AD, MCI, and other cognitive decrements (Muno˜z-Jim´enez et al., 2021; Ryan et al., 2006; Sinclair et al., 2000).

While T2DM has been proposed to represent a risk factor for cogni- tive impairments, other scholars reported that mild forms of cognitive decline may also occur during pre-diabetic stages and slowly worsen over time (Biessels et al., 2014). The seemingly asynchronous temporal association between insulin-related metabolic disturbances and cogni- tive impairments calls for additional studies to investigate whether the former cause the latter, whether the latter cause the former, or whether they share common risk factors acting via potentially independent mechanisms.

Within this broad framework, “Animal models […] are essential to uncover a pathological substrate for cognitive dysfunction and dementia in T2DM” (Biessels and Despa, 2018). Evidence in support of this proposition is accumulating. Nistico` and collaborators (Nistico` et al., 2012) showed that heterozygous mice, haploinsufficient for the beta subunit of the insulin receptor, exhibit impaired memory capabilities and altered synaptic plasticity in brain regions essential for cognition. Ramos-Rodriguez et al. (Ramos-Rodriguez et al., 2016) reported that a murine model of AD (APP/PS1), with impaired cognitive capabilities, was characterized by poor glycemic control. Finally, leveraging an inbred mouse strain (TALLYHO/JngJ, JAX stock #005314, hereafter TH), van de Vondervoort and collaborators (van de Vondervoort et al., 2019) reported that alterations in insulin signaling correlate with increased compulsivity and anxiety. TH mice resemble human T2DM in terms of etiology (construct validity (van der Staay, 2006; Zoratto et al., 2011)), symptomatology and developmental course, whereby, while hyperglycemia and alterations in pancreatic beta cells can be observed in young adults (Sung et al., 2005) (analogous to a pre-diabetic stage), an overt T2DM-like phenotype emerges later in development (face val- idity (van der Staay, 2006)). 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 hyperglycemic mice (Kim et al., 2001; Kim and Saxton, 2012). Due to a polygenic inheritance mode, TH male mice develop insulin resistance, hyperglycemia, hyperinsulinemia, and obesity, within 10–14 weeks of age (Ramasubramanian and Hemachandra Reddy, 2019). These phenotypes are associated with increased cholesterol concentrations and an enlargement of the islets of Langerhans (Kim et al., 2006, 2001; Kim and Saxton, 2012). Insulin resistance has been proposed to represent the primary defect of TH mice (Kim et al., 2006), this proposition resting upon the following obser- vations: Kim and Saxton (2012) observed that, while both male and female young mice exhibit intact glucose tolerance, post-pubertal male TH mice exhibited glucose intolerance starting at eight weeks of age. This alteration was accompanied by increased insulin secretion during the glucose tolerance test, which was interpreted as a compensatory hypersecretion mechanism dependent on an already existing insulin resistance (Kim et al., 2006; see also Kim and Saxton, 2012). Thus, TH mice resemble human diabetes also with respect to the sequela of im- pairments: insulin resistance, progressive hyperglycemia, a temporary hyperinsulinemia aimed at contrasting insulin resistance, impairments in beta-cells function ultimately responsible for hypoinsulinemia (De Fronzo et al., 2004). The phenotypic alterations observed in TH mice have been ascribed to specific QTLs, detected via genome-wide linkage analyses, and located on chromosomes 19 (TALLYHO-associated-non-insulin-dependent-diabetes-1, *Tanidd1*) and 13 (*Tanidd2*) (Kim et al., 2001). The relevance of these observations in diabetes is also supported by the fact that the mouse chromosome 19 contains numerous consensus regions modulating glucose and insulin concentrations and regulating glucose tolerance (Schmidt et al., 2008). Given this background, SWR/J mice are considered the best control mice for TH studies based on their similarity and the fact that they share 86.8 % of their genotype and 67.1 % of their haplotype (Yang et al., 2011).

Based on the association between altered insulin signaling, somatic and mental disturbances, and on the remarkable construct and face validity of TH mice in T2DM research, we hypothesized that TH mice may exhibit behavioral abnormalities isomorphic to mental disorders such as AD, ADHD, and OCD. To test this hypothesis in an ontogenetic framework, we investigated the developmental course of body weight, hyperglycemia, insulin resistance, energy expenditure, respiratory ex-change ratio, and polydipsia in adolescent (5–6–7 weeks), young adult (11–12–13 weeks), and adult (>23 weeks) male and female TH mice. Totest whether T2DM-like abnormalities were concomitant with behav- ioral abnormalities associated with CNS disturbances (AD, ADHD, and OCD), we evaluated behavioral rigidity, attentional set-shifting capa- bilities, short- and long-term spatial and recognition memory and gen- eral locomotion. Finally, to provide further insight into the potential mechanisms underlying the predicted comorbidity, we investigated gut microbiota diversity and composition in adult subjects.

Materials and methods

Ethics statement

Experimental protocols were approved by the Italian Ministry of Health (license 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.

Subjects

Twenty male and 40 female progenitors of 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). The reader is referred to https://www.jax.org/strain/005314 and (Yang et al., 2011) for details on the haplotype similarity between SW and TH mice. Mice were housed in same-gender, same-genotype pairs per cage, 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, see (Hauser et al., 2021)). Mice had access to *ad libitum* water and food pellets (Mucedola s.r.l., Settimo Milanese, Italy), except for the attentional set shifting task (ASST), which required food restriction (see “ASST” for details). After two weeks of acclimatization, males and females were mated for one week to generate the experi-

mental progeny in accordance with the breeding procedures discussed in (Hauser et al., 2021). Pregnant females were housed individually and provided with special food to support gestation and lactation (2019 Teklad global 19 %, Envigo, Indianapolis, IN, USA). Weaning occurred 21 ± 1 days after delivery (post-natal day, PND, 0). Pup body weight was measured, using a precision scale (Kern & Sohn GmbH, Balingen, Germany), at PND 1, 8, 15 and 22, and then weekly in adolescence, young adulthood and adulthood. 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 and kept at the same environmental conditions.

Experimental plan

The experimental plan entailed the execution of several different tasks in three different life stages: adolescence (5–6–7 weeks), young- adulthood (11–12–13 weeks), or adulthood (>23 weeks). To guarantee an adequate number of subjects per test while avoiding pseudo-
replications, test battery and litter effects, and minimizing suffering in each experimental individual, the study was conducted in three inde- pendent experimental cohorts (144 subjects per cohort) allocated to a single test battery (see Fig. 1a and Supplementary Information for de- tails). The test batteries (A,B, and C) entailed the following tests: (A) T-maze, open field, novel object recognition and elevated zero-maze; (B) Barnes maze test, intra-peritoneal glucose tolerance test (IPGTT) and insulin sensitivity test (IST); (C) metabolic cages and ASST. The order of testing of the experimental subjects was randomized within each test. The number of subjects to be used in each task has been estimated via *a priori* power analyses (see Supplementary Information).

Metabolic tests

Intra peritoneal glucose tolerance test
As detailed elsewhere (Pisa et al., 2021), 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 before (time 0), 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).

Insulin sensitivity test (IST)
Mice were tested after 5-h of food deprivation. Experimental subjects were injected (i.p.) human recombinant insulin (0.4 U/kg; Humulin R, Eli-Lilly, 100 U/mL, Indianapolis IN, USA) and sampled before (time 0), and then 15, 30, 60, 120, and 180 minutes after insulin administration to measure blood glucose concentration.

Metabolic cages
To assess 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 (*PhenoMaster*, TSE System GmbH, Berlin, Germany). The latter allow the automated and continuous measurement of the aforementioned parameters, in undisturbed conditions, using indirect calorimetry (for energy expenditure and lipid vs. carbohydrate metabolism), infrared sensors (for general locomotion), and precision scales (for food and water intake; see (Spiers et al., 2017) and Supplementary Information). Mice were placed in the system 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 acclimatized to the novel cage and no measurement was collected. Days 2–3 were considered habituation during which data have been collected but not analyzed, while days 4–5 were considered proper experimental days during which data have been collected and analyzed. All measurements were taken every 21 minutes throughout the entire session.

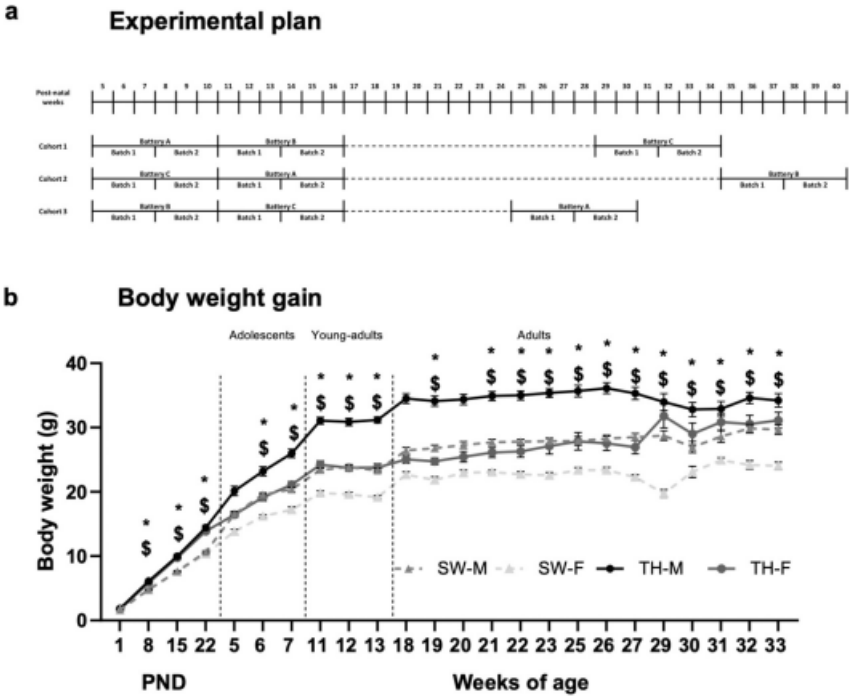

Fig. 1. Experimental plan (a) Body weight gain (b). (a) Allocation of experimental subjects to behavioral and metabolic testing. Test battery A entailed the T-maze test, the open field test, the novel object recognition test and the elevated zero-maze test; the test battery B included the Barnes maze 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. (b) Body weight (mean ± SEM) in SW and TH males (M) and females (F) at post-natal day (PND) 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 (>23 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.

Gut microbiota

Data acquisition, sequencing and data processing
The detailed collection, storage and wet-lab protocol is provided in the Supplementary Information. From the fecal samples of 55 animals (33 TH/22 SW), the V3-V4 region of the 16 S ribosomal RNA (rRNA) gene was amplified using primers 341 F and 785 R, and subsequently sequenced on the Illumina MiSeq platform (paired-end). Sequenced data were processed using the QIIME2 processing pipeline (v2021.8.0) using default settings (Bolyen et al., 2019), and Amplicon Sequence Variants (ASVs) were assigned taxonomic labels using a Naive Bayes classifier pre-trained on the SILVA reference database (v138) (Bokulich et al., 2018). Non-bacterial and unassigned ASVs were discarded, and samples with less than 10 % of all identified genera with non-zero values were removed. This resulted in the exclusion of one sample. Rarefaction curves were plotted for each sample, and showed that there was no as- sociation between read count and the number of ASVs (Fig. S1).

Alpha- and Beta-Diversity and Taxonomy
All microbiota analyses were performed in R (v4.2.1). Alpha- and beta-diversity analyses were performed on the ASV level without prev- alence filtering. Shannon diversity (microbiome::alpha and picante::pd functions in R) was used as a measure for alpha diversity. Aitchison distance (adonis::adonis2 function in R) was used as a measure of beta-diversity. For the taxonomic analyses the ASV table was aggregated to the genus level. Unclassified genera and genera with a prevalence lower than 10 % were discarded, and the 91 genera remaining after aggrega- tion and filtering were CLR-transformed (Galloway-Pena et al., 2017).

Behavioral tests

To assess different cognitive capabilities, we conducted the following tests: T-maze (spontaneous alternation), open field (spontaneous loco- motion and anxiety-like behavior), novel object recognition (recogni- tion memory), elevated zero-maze (anxiety-like behavior, see Supplementary Information), Barnes maze (spatial memory) and ASST (executive functions, e.g. attention and cognitive flexibility). Data from open field, novel object recognition, elevated zero-maze and Barnes maze tests were collected and scored automatically through the ANY-maze software 7.09 (Stoelting Europe, Terenure, Dublin, Ireland). All tests, except the Barnes maze, were conducted under dim light, 12 lux (Giorgio Bormac S.r.l., Carpi, MO, Italy).

T-maze test
This task was conducted on a T-shaped Plexiglass maze 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, and each session consisted of two consecutive trials: a trial started with the mouse positioned in the starting compartment and ended when it entered one arm. The animal was then relocated in the starting compartment, and allowed to perform a second-choice trial. If the subject entered the opposite arm, an alternation trial was scored. The percentage of spontaneous alternations was calculated (see Supplementary Information and (Hauser et al., 2021)).

Open field (OF) test
Spontaneous locomotion was evaluated during a 10-min test session conducted in an unfamiliar 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). Distance travelled and time spent in the center and in the periphery of the arena were measured.

Novel object recognition (NOR) test
The test consists of several phases: a *habituation* phase (the open field test) on day one, a 10-min *acquisition* phase on day two, and two 10-min *testing* phases, conducted one and 24 (short- and long-term recognition memory, respectively) hours after *acquisition*. The apparatus was the same described for the Open field. During *acquisition* (10 minutes), mice explored two identical objects (A). In both testing phases, one of the two objects was replaced with another (B or C). Time spent exploring both objects and novel object percent preference [(time spent exploring novel object/time spent exploring both objects) x 100] were measured (see Supplementary Information).

Barnes maze test
The test was performed as described in (Barnes, 1979). Mice exposed to a bright light (310 lux) on a circular grey arena (*Barnes Maze 40193*, Ugo Basile® S.r.l., Gemonio, VA, Italy, 100 cm diameter, elevated 60 cm above the floor) were required to locate a rectangular escape box (7 × 37 × 9 cm) located underneath one of 20 holes (target hole, diameter 5 cm). The experimental protocol entailed one day of habituation (two 1-min exploration trials), five days of acquisition, and two probe trials, conducted 24 hours and seven days after the last acquisition session. Probe trials consisted of a 90 s free exploration during which the escape box was removed. Memory was evaluated through the automated scoring of the latency to reach the target area and time spent in the target quadrant.

Attentional set-shifting task
In this test, mice kept at 85–90 % of their free feeding body weight, were required to learn a rule associated with a reward and, upon the achievement of the learning criterion, subsequently inhibit it in favor of a new one that would allow to achieve a new purpose (e.g. shifting from Intra- to Extra-Dimensional discrimination). Ultimately, owing to its consecutive stages, the ASST evaluates rule acquisition and rule updates as proxies for perseveration, learned irrelevance, and set formation (Garner et al., 2006; Hauser et al., 2021). The apparatus consisted of a custom-made PVC U-maze (45 cm L x 30 cm W x 25 cm H) subdivided into three sections: a start compartment (30 cm L x 30 cm W) con- nected, through a sliding door, to two identical choice compartments (15 cm L x 15 cm W), each containing a metal food cup (4 cm high, 7 cm diameter). The latter was baited with a 1/4 of cereal (30 mg; Honey Nut Loop, Kellogg, Battle Creek, MI, USA), covered with a layer of scented digging medium (20 mm, see Table S1). The reward was associated with either tactile (type of digging medium) or olfactory stimuli (scent of the digging medium), and the mouse was trained to obtain the food reward by digging in one of the two bowls. The learning criterion was eight correct responses out of eight-ten consecutive trials (see Supplementary Information). Using this test, we obtained information about: simple discrimination (SD); compound discrimination (CD); CD reversal (CDre); intra-dimensional shift (IDS); and extra-dimensional shift (EDS). We measured the number of trials and errors until criterion. Impor- tantly, in this task, individual motivation towards rewards is controlled for by allowing mice to execute as many trials per session as they are motivated to. Thus, the duration of the session is performance-based and not time-based. Specifically, it is interrupted upon the observation that a mouse is no longer motivated to execute any additional trial (e.g. a mouse digging without eating reward; a mouse not entering any of the choice compartments for 10 minutes). The session is then continued on the following day. Therefore, a potential difference in motivation would theoretically interfere with the length of the achievement of the learning criterion but not with the total number of trials or errors.

Statistical analysis

For all data except gut microbiome, statistical analyses were conducted using 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) (Faul et al., 2007). The experimental design entailed 2 ge- notype (TH vs. SW) x 2 sex (males vs. females) x 3 developmental stage (adolescence, young-adulthood, adulthood) x k repeated measure (var- iable depending on the specific test) and their interactions. Experimental data have been analyzed through repeated measure analysis of variance (ANOVA). Post-hoc comparisons were performed using Tukey test.
Significance level was set at $p<0.05$.
To assess the associations between genotype, blood glucose concentrations, and gut microbiota alpha- and beta-diversity and taxonomy we used R (version 4.2.1). The association with alpha diversity was evaluated with ANOVA (for genotype) or linear regression (for blood

glucose concentrations) along with permutations testing (1000 permu- tations). The model also accounted for sex and interactions between genotype/blood glucose and sex (alpha diversity ~ genotype/blood glucose + sex + genotype*sex). Additionally, PERMANOVA (vegan:: adonis2 in R, 1000 permutations) was used to evaluate the associations between beta-diversity (Aitchson) and genotype/blood glucose and sex (Aitchison ~ genotype/blood glucose + sex). For taxonomic analyses, randomized lasso was used to perform feature selection (using the monaLisa::randLassoStabSel function in R (Machlab et al., 2022), and all genera with a selection probability threshold ≥ 0.20 were selected for statistical testing. A detailed description of the randomized lasso is available in the Supplementary Information. Regression analysis along with permutation testing (1000 permutations) was used to examine the association between genotype (logistic regression) or blood glucose levels (linear regression) and the selected genera, sex and their interaction (i.e., genotype/blood glucose ~ genus + sex + genus*sex). A Bonferroni correction was applied to adjust for the total number of selected genera ($\alpha = 0.05$ / number of selected genera). If the parameter of interest showed a significant association, an additional model including body weight was created, to assess whether the observed associations were independent of body weight.

Results

Metabolic testing

Body weight gain

Predictably, TH male and female mice were heavier than SW controls throughout the entire course of the study (genotype: $F(1,26)=159.764$, $p<0.0001$, Fig. 1**b**). Male subjects of both genotypes were heavier than same-genotype females from around puberty onwards (sex: $F(1,26)= 129.131$, $p<0.0001$). $^{TH-F}$

Intra peritoneal glucose tolerance test

Glucose administration elicited the expected physiological response 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: $F(4,484)=315.198$, $p<0.0001$, Fig. 2a,b,c). Additionally, baseline glucose concentrations varied as a function of genotype and sex (genotype x sex: $F(1,121)=11.817$, $p=0.0008$). While, in males, TH mice exhibited base- line hyperglycemia compared to SW at all developmental stages ($p<0.05$ in post hoc tests), in females, baseline blood glucose concentrations never differed between SW and TH. Finally, different developmental stages where characterized 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), in females, such genotype-dependent difference only emerged in young adulthood (T30 and T60) to remain stable later in adulthood (T30, T60 and T120: $p<0.05$ in post hoc tests).

Insulin sensitivity test

During the test, many control subjects had to be removed due to severe hypoglycemia. Within SW mice, approximately 58 % of adoles- cents, 33 % of young adults and 67 % of adults had to be administered a glucose bolus to guarantee their survival. Therefore, we preliminarily analyzed survival rates through chi-square test, and observed that TH mice of all developmental stages, except young-adult females, were insulin resistant compared to controls ($p<0.05$). Blood glucose concentrations decreased consistently after insulin administration in all experimental groups (time bins: $F(5,400)=17.531$, $p<0.0001$, Fig. 2d,e, f), yet at a differential rate depending on genotype, sex (genotype x sex: $F(1,80)=5.582$, $p=0.0206$) and age (genotype x age: $F(2,80)=3.836$, $p=0.0257$).

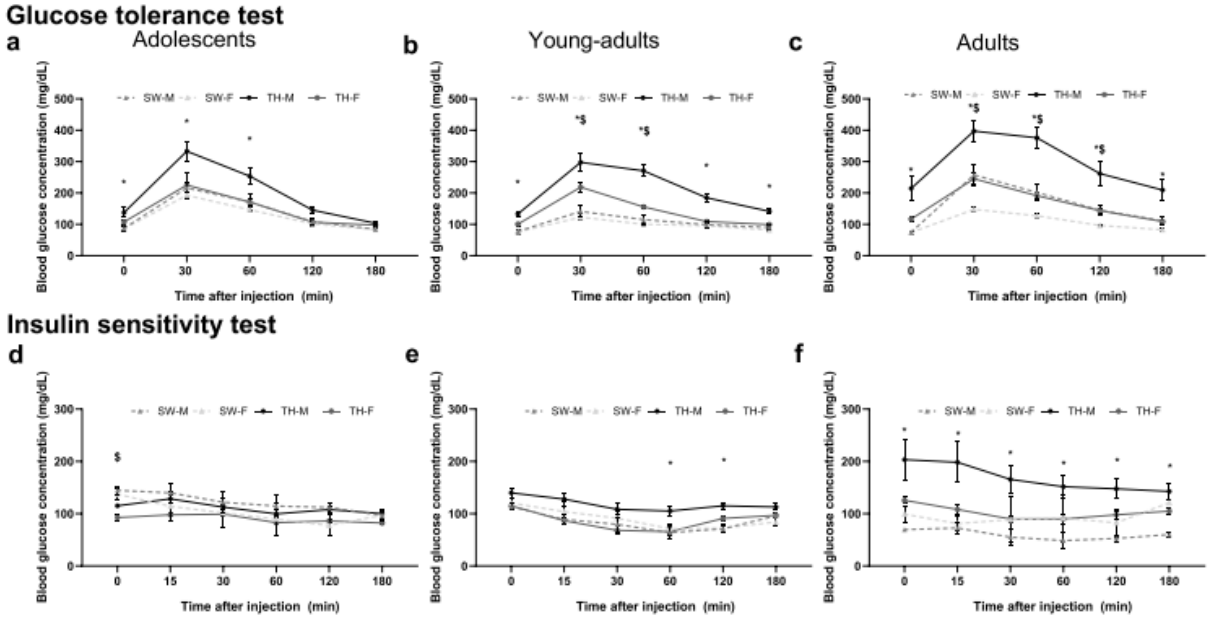

Fig. 2. Glucose tolerance and insulin sensitivity tests. Blood glucose concentration (mg/dl, mean ± SEM) in IPGTT (a,b,c) at (a) adolescence (SW males and females n=12, TH males and females n=6), (b) young adulthood (n=12) and (c) adulthood (SW males and females and TH females n=12, TH males n=13). Blood glucose concentration (mg/dl, mean ± SEM) in IST (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 due to severe hypoglycaemia), (e) young adulthood (n=12, but 3 SW males, 5 SW females and 3 TH females were excluded from the analysis due to severe hypoglycaemia) 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 due to severe hypoglycaemia). * $p<0.05$ 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

In Fig. 3 we report the ontogenetic development of food and water intake, energy expenditure, and lipid metabolism over 24 hours. Adult TH male mice exhibited a full-blown diabetes-like phenotype whereby, compared to controls, they exhibited polydipsia (genotype x sex x age: $F(2,87)=10.682$, $p<0.0001$, Fig. 3a,b,c), higher food intake (genotype x sex x age: $F(2,87)=2.566$, $p=0.0826$, Fig. 3d,e,f), higher energy expenditure (EE, time x genotype x age: $F(46,2001)=1.783$, $p=0.0010$, Fig. 3g,h,i) and impaired lipid metabolism (time x genotype x sex x age: $F(46,1978)=2.467$, $p<0.0001$, Fig. 3j,k,l). Thus, while adult SW male

mice predominantly used carbohydrates (Respiratory Exchange Ratio, RER ~ 1), adult TH male subjects also used lipids as an energy source (RER ~ 0.9). Finally, absolute levels of locomotion were indistinguish- able between adult TH and SW mice (time x genotype x sex x age: F (46,2001)=1.278, respectively). In TH females, we only detected a small increment in EE (p<0.05 in post hoc tests, Fig. 3h) in young adulthood, but neither earlier nor later in development. Importantly, all experimental subjects maintained the predicted circadian rhythm of EE with highest values
attained during the dark active phase (i.e. lights off) of the diurnal cycle in adolescence (time: F(23,690)=6.216, p<0.0001), young adulthood (time: F(23,667)=7.427, p<0.0001), and adulthood (time: F(23,644)= 9.436, p<0.0001).

Behavioral testing

T-maze test

The limited number of trials performed by TH mice during adoles- cence and young adulthood did not allow us conducting statistical an- alyses during these life stages (see Supplementary Information). While adult SW mice exhibited an intact spontaneous alternation, with values significantly higher than 50 % (SW males: 95 % CI 55.81–74.19, SW females: 95 % CI 59.58–70.42), neither male nor female TH mice exhibited an alternation significantly different from chance (TH males: 95 % CI 48.42–70.09, TH females: 95 % CI 39.17–65.46).

Attentional set-shifting task

In the ASST, all experimental subjects completed the entire task within five days. Compared to SW, TH mice required a higher number of trials (genotype: F(1,102)=71.916, p<0.0001) and committed 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 sex (genotype x sex x age: F(2,102)=4.678, p=0.0114 and F(2,102)=4.244, p=0.0170 for was no longer present at the end of the acquisition phase. When tested for memory retention (Fig. 5g,h,I,j,k,l) experimental groups exhibited an indistinguishable phenotype. Noteworthy, the time spent in the target quadrant was al- ways p=0.1012, data not shown). Earlier developmental
stages were not associated with consistent metabolic differences. Specifically, young adult TH male subjects exhibited higher food intake, EE, and RER (genotype: F(1,14)=23.409, p=0.0003, Fig. 3e**;** F(1,14)= 13.149, p=0.0028, Fig. 3h; and F(1,14)=9.980, p=0.0070, Fig. 3k; trials and errors respectively). Specifically, between group differences were nearly absent during adolescence, were robust, albeit only in male mice, in young adulthood, and became fully consolidated in adulthood (see Fig. 4). During young adulthood, TH male mice required more trials than SW in CD and IDS (data not shown), and committed more errors in CD, CDre and IDS, to attain the learning criterion, while during adult- hood, TH mice – regardless of sex – exhibited significant impairments in CDre, IDS, and EDS.

Open field (OF) test

TH mice consistently exhibited reduced locomotion compared to SW (genotype: F(1,94)=118.253, p=<0.001, Fig. 5a,b,c). While general locomotion steadily increased throughout development (age: F(2,94)=30.965, p<0.001), TH mice of both sexes always travelled a shorter distance compared to SW (p<0.05 in post hoc tests).

Novel object recognition (NOR) test

In contrast with our predictions, we observed that all subjects, regardless of sex and age, failed to exhibit any preference for the novel object over the familiar one (Fig. S2a**-f**), although we have previously demonstrated that the test works in our laboratory in different strains (see discussion).

Barnes maze test

TH and SW mice exhibited a differential learning performance and an indistinguishable memory retention of the target location. The la- tency 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). Addi- tionally, 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 (p<0.05 in post hoc tests, Fig. 5d), young adulthood (p<0.05 in post hoc tests, Fig. 5e) and adulthood (p<0.05 in post hoc tests, Fig. 5f). This differential profile indistinguishable from chance (25 % chance level, minimum value: —0.33≤ CI ≤24.01, maximum value: 24.16≤ CI ≤64.74) regardless of genotype, age, and sex.

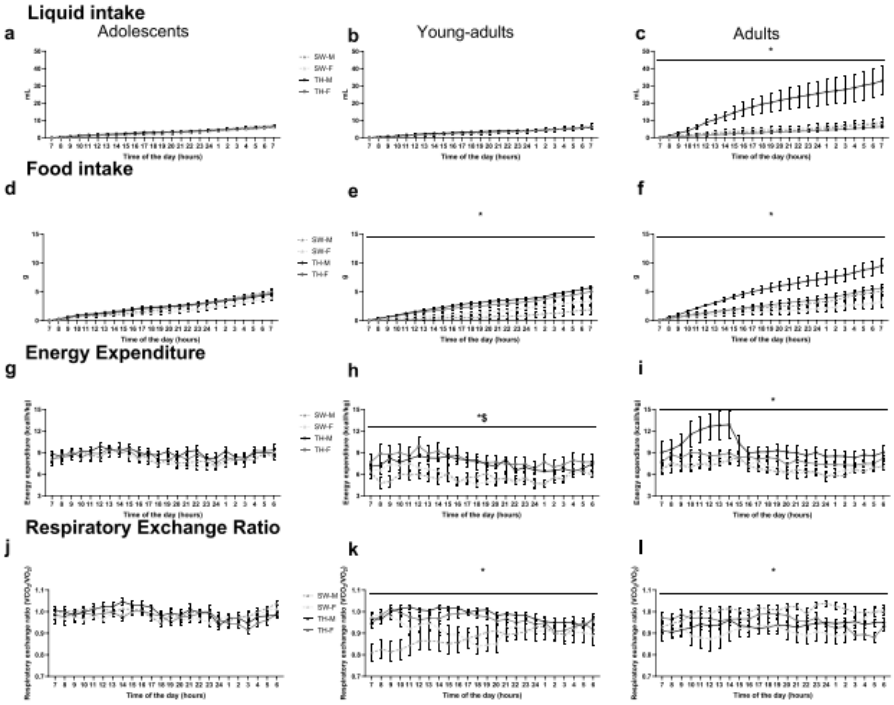

Fig. 3. Metabolic cages. Water intake (mL, mean ± SEM, a,b,c), food intake (g, mean ± SEM, d,e,f), energy expenditure (Kcal/h/Kg, mean ± SEM, g,h,i) and respiratory exchange ratio (VCO_2/ VO_2, mean ± SEM, j,k,l) in metabolic cages at (a,d,g,j) adolescence (SW males and females n=8, TH males and females 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.

Gut microbiota

Alpha- and beta-diversity

Alpha-diversity (Shannon) varied as a function of genotype and sex (genotype x sex: F(1,51)=2.29, $p_{permutation}$<.001, Table S2). While fe- male TH mice exhibited higher diversity compared to SW controls, male TH mice had lower diversity compared to SW mice (Fig. 6). These effects remained stable after adjusting for body weight ($p_{permutation}$<.001). Additionally, we observed significant associations between alpha-diversity, blood glucose and blood glucose x sex interaction (blood glucose x sex: β =.646, $p_{permutation}$ =.020, Table S2), both of which remained after adjusting for body weight ($p_{permutation}$<.029). Details on beta diversity are reported in the Supplementary Information, Table S3.

Taxonomy

An overview of the selection probability for each genus is provided in Table S4. Among the several genera for which we detected a variation as a function of genotype (see Supplementary Information and Table S5 for details), after Bonferroni correction (α = 0.05/5 = 0.01), we identified a significant association between *Parvibacter* abundance and genotype (Table S5). The effect was no longer statistically significant after adjustment for body weight ($p_{permutation}$=0.019). All other associations were non-significant (Table S5).

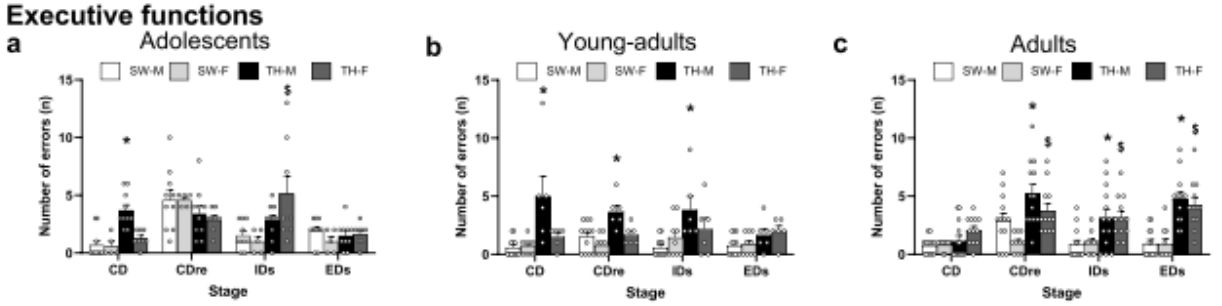

Fig. 4. Attentional set-shifting task. Number of errors (mean ± SEM) committed to complete each stage of the ASST task in adolescence (SW males and TH males n=10, SW females n=9 and TH females n=8), young adulthood (SW males and females n=12, TH males and females n=6) and adulthood (SW males, SW females and TH males n=13, and TH females n=12). CD: compound discrimination; CDre: compound discrimination reversal; IDS: intra-dimensional shift; EDS: extra-dimensional shift. * 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.

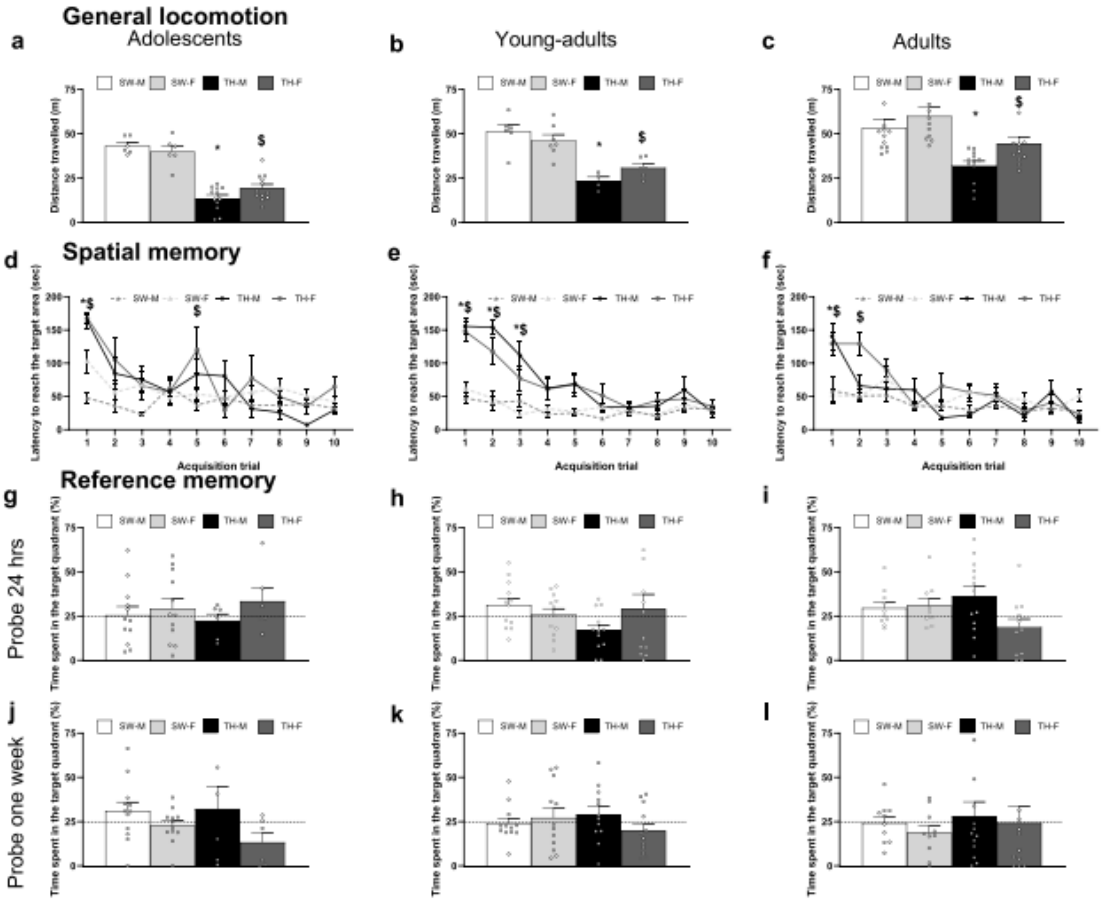

Fig. 5. Open field and Barnes maze tests. Distance travelled in meters (mean ± SEM) at adolescence (SW males and females n=6 and TH males and females n=12, a), young adulthood (SW males and TH females n=6, SW females n=7 and TH males n=4, b) and adulthood (SW females n=11, SW males and TH males and females n=12, c). Latency to enter the target area in seconds (mean ± SEM) during the acquisition phase at (d) adolescence (SW males and females n=12, TH males and females n=6), (e) young adulthood (n=12) and (f) 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 (g,h,i) and in probe 1 week trial (j,k,l) at adolescence (SW males and females n=12, TH males and females n=6, g,j), young adulthood (n=12, h,k) and adulthood (SW males and females n=10, TH males n=13 and TH females n=12, i,l). * 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.

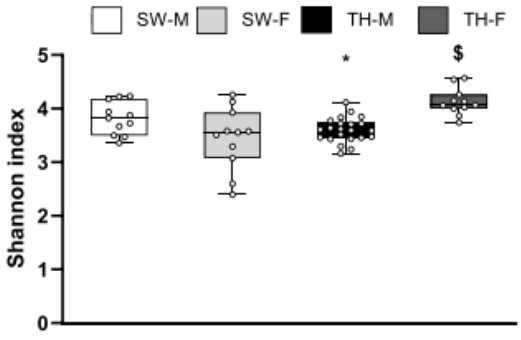

Fig. 6. Gut microbiota. Shannon alpha-diversity index as a function of genotype and sex in females (SW-F and TH-F n=11) and males (SW-M n=11 and TH-M n=22). $ $p<0.05$ TH-F significantly higher than SW-F in post hoc tests; * $p<0.05$ TH-M significantly lower than SW-M in post hoc tests.

Discussion

In the present study, we aimed to confirm that metabolic alterations isomorphic to T2DM are associated with cognitive impairments, to further detail the temporal association between these comorbid abnor- malities, and to identify potential common risk factors. Accordingly, adult male TH subjects exhibited a comprehensive T2DM-like phenotype (hyperglycemia, impaired glucose tolerance, insulin resistance, poly- dipsia, increased food intake and energy expenditure, and altered lipid metabolism) associated with deficits in executive functions (spanning from reversal learning, CDre of the ASST test, to attentional set shifting, IDS stage, learned irrelevance, EDS stage, and stuck-in-set perseveration, EDS stage and T-maze test). This comorbid phenotype developed gradually over time, with cognitive deficits emerging together with a pre-diabetic stage, and earlier than overt T2DM-like alterations. Similar, but less pronounced, comorbidities were also observed in adult female TH mice and also developed over time. Contrary to males, TH females failed to show any alteration in food and water intake, energy expenditure and lipid metabolism. The observation that gut microbiota diversity was lower than controls in males and higher in females, when their phenotypes were at their most extreme, may contribute to explain the reported sex differences.

Sex differences in diabetes have also been observed in humans (Kautzky-Willer et al., 2016; Tramunt et al., 2020). Sex steroids have been proposed to exert a protective role as: (i) the onset of menopause co-occurs with increased incidence of diabetes (Margolis et al., 2004; Mauvais-Jarvis et al., 2013); and (ii) rare mutations causing loss-of-function in genes involved in estrogen function result in impaired metabolism (Grumbach and Auchus, 1999). This role has been further substantiated in preclinical studies conducted in diet-induced mouse models of obesity, insulin resistance, and glucose tolerance (Handgraaf et al., 2013; Riant et al., 2009). Kim and collaborators (Kim et al., 2006, 2001) observed that, at 16 weeks of age, while TH male mice exhibited an abnormal structure of Langerhans islets and degranulation of beta-cells, females did not show this phenotype. Adult TH mice resemble human diabetes also with respect to the ontogenetic progression of the observed abnormalities (Kim and Saxton, 2012). Thus, just as diabetes progressively develops over time in humans (Fonseca, 2009), so also TH male mice exhibited a full-blown abnormal phenotype no earlier than adulthood (Kim and Saxton, 2012).

Our core aim was to determine whether deficits in insulin signaling were associated with alterations in CNS-dependent behavioral pheno- types isomorphic to the core symptoms of the brain disorders of interest based on genetic and demographic human findings (AD, OCD, and ADHD). With respect to AD, we conducted the Barnes maze test, a hippocampal-dependent paradigm (Barnes, 1979; Pitts, 2018), in which spatial memory is assessed via the measurement of the latency to reach an escape box (and time spent therein). Beside the neuroanatomical homology (Rao et al., 2022), the relevance of this task for AD is sup- ported by the fact that several experimental models of AD exhibit defi- cits when tested in this paradigm (Attar et al., 2013; Reiserer et al., 2007). Attentional deficits, typical of ADHD, have been investigated through the ASST, a prefrontal cortex-dependent task (Birrell and Brown, 2000; see also Tait et al., 2018 for a review), "devised after the intradimensional/extradimensional component of the Cambridge Neuropsychological Test Automated Battery (CANTAB)" (Heisler et al., 2015), which translated the Wisconsin card sorting task to laboratory mammals (Colacicco et al., 2002), and reported to be impaired in mouse models of ADHD (Chess et al., 2011; Tait et al., 2014). Last, together with the CDre stage of the ASST, the T-maze task evaluates potential impairments in behavioral flexibility (Tanimura et al., 2008), a hallmark of OCD (Gruner and Pittenger, 2017).

In accordance with previous observations (van de Vondervoort et al.,2016, 2019), the present data suggest that the main deficits observed in TH mice revolve around cognitive flexibility, i.e., the capability to oversee different perspectives and implement appropriate strategies upon changed circumstances (Diamond, 2013), and working memory, which is primarily mediated by the prefrontal cortex (Jos´e et al., 2020; Powell and Ragozzino, 2017). The deficits observed in adult TH male and female mice encompass three stages of the ASST, each of which maps to a specific cognitive domain: (i) the CDre assesses reversal learning capabilities (Birrell and Brown, 2000); (ii) the IDS evaluates rule abstraction capabilities (Garner et al., 2006); and (iii) the EDS directly addresses attentional set-shifting capabilities whereby it requires subjects to shift their attention from a previously relevant stimulus to a new stimulus that was previously irrelevant. The latest stage of the ASST can thus be used to evaluate learned irrelevance and stuck-in-set perseveration (Garner et al., 2006). Ultimately, TH mice seem to exhibit a profound deficit at the level of executive functions whereby it entails a large portion of its components, spanning from inhibitory control (the capability to inhibit actions that are no longer appropriate to the purpose (Aron, 2007)), to working memory (neces- sary to oversee and maintain the current goals (Baddeley and Hitch, 1974)), and then onto cognitive flexibility (the ability to update appropriate behaviors functional to the achievement of an individual goal (Dajani and Uddin, 2015)). Importantly, we observed that male mice exhibited deficits in the ASST earlier than adulthood. Specifically, they exhibited impaired CD in adolescence and impaired CD, CDre, and IDS in young adulthood. These data suggest that during the early stages of maturation, TH mice may have deficits that extend beyond reversal learning and rule abstraction, to encompass the formation of the attentional set required to acquire the compound discrimination (Garner et al., 2006).

The data on memory performance are puzzling. Specifically, both the Barnes maze and the NOR task entail internal controls (time spent in the target quadrant significantly higher than 25 % chance level and pref- erence index for the novel object significantly higher than 50 % chance level, respectively) to claim the presence of intact memory. Herein, neither SW nor TH mice exceeded the aforementioned chance level in neither the Barnes maze nor the NOR. These findings are thus open to two alternative interpretations: while, on the one hand, they may pur- port that TH and SW mice have remarkable memory impairments, on the other hand, they may simply reflect the possibility that we failed to appropriately execute these tasks in our conditions. We would favor the former, especially in the light of our own data, collected in the same facilities in other mouse strains (e.g. C57BL/6), wherein we observed intact memory in both the Barnes maze and the NOR adopting identical test procedures (Hauser et al., 2021; Pisa et al., 2021). Regardless of these considerations, the present data do not enable us to conclude that the memory deficits observed in TH mice are related to alterations in insulin signaling. A more parsimonious interpretation may contemplate the possibility that these deficits are related to genetic alterations pre- sent in both SW and TH mice. In line with this interpretation (Yang et al., 2011), Ritchie and Clapcote (Ritchie and Clapcote, 2013) observed that SW mice present a genetic mutation linked to memory deficits.

Methodological considerations may also devalue the magnitude of the learning deficits exhibited during the acquisition of the Barnes maze. Specifically, just as TH were significantly slower than SW in acquiring the target location, so also were they much slower than SW in the open field. Whether this reduced locomotion represents a natural tendency, a trade-off in energy utilization, in keeping with our findings from the metabolic cages, due to the higher effort required to fuel a heavier body, or an anxiety-related response is unclear. Albeit preliminary, we would favor the anxiety-related explanation whereby,

when tested in a non- challenging environment (metabolic cages), TH and SW mice did not differ in general locomotion.
Regardless of the ambiguous and inconclusive interpretation of memory tests, which await further experimental clarification, we observed that deficits in behavioral flexibility emerged either much earlier (males), or independent of (females), a comprehensive constel- lation of symptoms isomorphic to an overt T2DM-like phenotype. Importantly, both the ASST and the T-maze test are much less reliant on locomotion than the aforementioned tests. Thus, both tests require mice to perform a binary choice (baited vs. unbaited bowl and left vs. right arm, respectively) within a time-frame that is determined by the experimental subject itself. Although both tasks entailed a cut-off time, we observed that this was only attained in very few instances, which were indistinguishable across the different experimental groups. Since the dependent variable in both tests is constituted by relative fre- quencies, we posit that speed and locomotion do not play a major role. Last, dissociations between ASST performance and locomotion have also been reported elsewhere (Young et al., 2010; Zoratto et al., 2023). The nature of this association suggests that altered insulin signaling in TH mice, already present in adolescence (Sung et al., 2005), may skew developmental processes to influence the cognitive phenotype inde- pendent of metabolic disturbances. While many studies (Bennett and Knowler, 2005; Hawkins and Rossetti, 2005; Saltiel and Kahn, 2001) detailed the role of insulin signaling in T2DM, a much narrower fraction investigated its role in mental disturbances (Klimova et al., 2018; van de Vondervoort et al., 2016). Thielen and collaborators (Thielen et al., 2019) reported that altered insulin signaling 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 (Kleinridders et al., 2014). Moreover, Martin and collaborators (Martin et al., 2022) showed that intranasal insulin administration increased neuronal firing by acting upon postsynaptic serotonin 5-HT_{1A} receptors, which are abundant in the PFC, thus sug- gesting that insulin may potentially regulate emotional behavior (Martin et al., 2022). Future studies are needed to further detail the role of in- sulin in the PFC as a potential mediator of the observed deficits in ex- ecutive functions. Finally, the potential role of other brain regions involved in executive functions needs to be clarified. Van de Vonder- voort and collaborators (van de Vondervoort et al., 2019) hypothesized a potential role for the cerebellum, whereby they reported that deficits in behavioral flexibility and perseveration in TH mice are associated with reductions in IGF1 protein expression in this brain region (van de Vondervoort et al., 2019). 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 signaling, body weight balance (Elabd and Sabry, 2015) and dopaminergic transmission (Stouffer et al., 2015). Moreover, the mechanisms underlying such deficits appear to differ between sexes, given their age of onset and contrasting insulin-signaling profile.
Present data also suggest that the gut microbiota may partially modulate the observed phenotypic alterations (see Zhou et al. (Zhou et al., 2022) for a review entailing humans and animal models). Spe- cifically, we observed sex-dependent alterations in alpha diversity, with a reduced alpha diversity in males and an increased alpha diversity in females. Moreover, we observed increased *Parvibacter* abundance in TH mice. Increasing evidence shows that the gut microbiota can impact insulin signaling, for example through the production of short chain fatty acids and incretin (for a review, see (Baothman et al., 2016)). Thus, it is tenable that gut microbiota composition may act as a player in the development of T2DM or MS. The findings suggest that while reduced microbiota diversity may aggravate the observed abnormalities in males, increased diversity may have a protective effect in females. Capitalizing upon large population studies, Chen and collaborators (Chen et al., 2021) observed that diabetic patients were characterized by reduced gut microbial alpha-diversity, which was also associated to higher levels of insulin resistance. Furthermore, the findings provide preliminary evidence that *Parvibacter* abundance may constitute a risk factor for the development of T2DM. Accordingly, Qin and collaborators (Qin et al., 2022) reported that *Parvibacter* is reduced in an experimental model of diabetes, and Song and collaborators (Song et al., 2019) observed that a treatment capable of restoring *Parvibacter* colonization reduced metabolic disturbances in an experimental model of obesity. While the data do not allow a definitive conclusion on whether the role of gut microbiota in insulin-related comorbidities is correlational or causative in nature, they suggest that the modulation of microbiota composition may represent a promising therapeutic target. Specifically, treatments aimed at increasing microbiota diversity (e.g., diet, pre-biotics, probiotics, and fecal transplantation) and/or at promoting the growth of selected bacterial species (e.g., *Parvibacter*) may contrast or mitigate the altered phenotypes observed as a function of insulinopathies.
Our study provides additional experimental evidence regarding the potential association between insulin signaling and cognitive impairments. The consideration of sex differences, together with the compre- hensive 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 correlations may be related to impaired insulin signaling, which may cause the aforementioned symptoms via partly independent mechanisms. This proposition is substantiated by a systematic review (Ottomana et al., 2023), in which we recently analyzed the association between hyperglycemia and cognitive impairments in a wide range of experimental models in which hyperglycemia has been induced via diverse methodologies (e.g., dietary interventions, pharmacological manipulations, and genetic preparations). The vast majority of these studies highlighted the emergence of cognitive symptoms as a function of experimentally-induced hyperglycemia. At the same time, a comple- mentary approach in which the authors investigated glycemic control in experimental models of Alzheimer's disease reported that cognitive decline anticipated alterations in glucose metabolism (Vandal et al., 2015). Ultimately, although future research is needed to clarify this link, it is tenable to propose that glucose intolerance and cognitive decline may sustain each other in a potentially self-amplifying recurrent cycle. 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 dysfunctions. Analogous considerations pertain to the possibility to test whether treatments capable of normalizing gut dysbiosis may result beneficial in mental and somatic comorbidities.

CRediT authorship contribution statement

Danique Mulder: Writing – original draft, Investigation. **Francesca Zoratto:** Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Martina Presta:** Writing – original draft, Methodology, Investigation, Data curation. **Alejandro Arias-Va´squez:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation. **Edoardo Pisa:** Investigation. **Angela Maria Ottomana:** Investigation. **Simone Macri:** Writing – original draft, Supervision, Project administration, Method- ology, Funding acquisition, Data curation, Conceptualization. **Jeffrey C Glennon:** Writing – review & editing, Funding acquisition, Conceptu- alization. **David Anthony Slattery:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

None.

Declaration of Competing Interest

All authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2024.107102

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POST SCRIPTUM

As my final dissertation showed, my PhD project assessed the role of a gene in the brain, exploring its correlation with metabolic and cognitive disorders. I achieved that goal through specific scientific protocols of metabolic and cognitive experimental tests. However, my experience, over the last three years, goes further than laboratory data manipulation and has been not only a scientific challenge but also a personal and professional growth opportunity. My doctoral journey was more than a possibility to study a specific gene or a biological phenomenon but an experience that has shaped my vision of responsible scientific research. Firstly, I have delved into the theory underlying the crucial link between body and brain health, understanding how metabolic dysfunctions can impact cognitive activity and vice versa. The foundation of my work has been supported by a rigorous review of scientific literature, which allowed me to create a precise methodological plan. Analysis of preclinical previous researches has informed my experimental hypothesis, providing a key framework for the design of my study. Moreover, I have embraced the principles of the 3Rs (Reduction, Refinement, Replacement) in laboratory animal use, ensuring an ethical and responsible approach to experimentation. I faced the context of variability within preclinical studies, implementing strategies to minimize sources of error and maximize the reproducibility of results. I also learned how to emphasize the importance of heterogeneous study designs, as they more accurately represent human complexity and are more readily translatable to clinical settings. Lastly but most relevant, I have learned the immense benefit of collaborating with other talented and interdisciplinary researchers (and from now colleagues). Working alongside other individuals, with diverse expertise, has enriched my approaches to research, have allowed me to view challenges from different perspectives, and have led to deeper appreciate the multifaceted nature of scientific inquiry. I have learned valuable lessons from the mistakes I have made along the way and from whom with more experience in the field. I have worked to cultivate independence and autonomy, but also to appreciate the importance of help when faced with difficulties. For all, I am deeply grateful to those who allowed this journey, as well as to those who have offered guidance and support. Thanks to them, I have also realized how to properly share my personal research. My above final dissertation has attempted to do it.