

The impact of the 'Western Diet' on emotional, social and cognitive behaviours as revealed by a study on conventional and serotonin transporter-deficient mice

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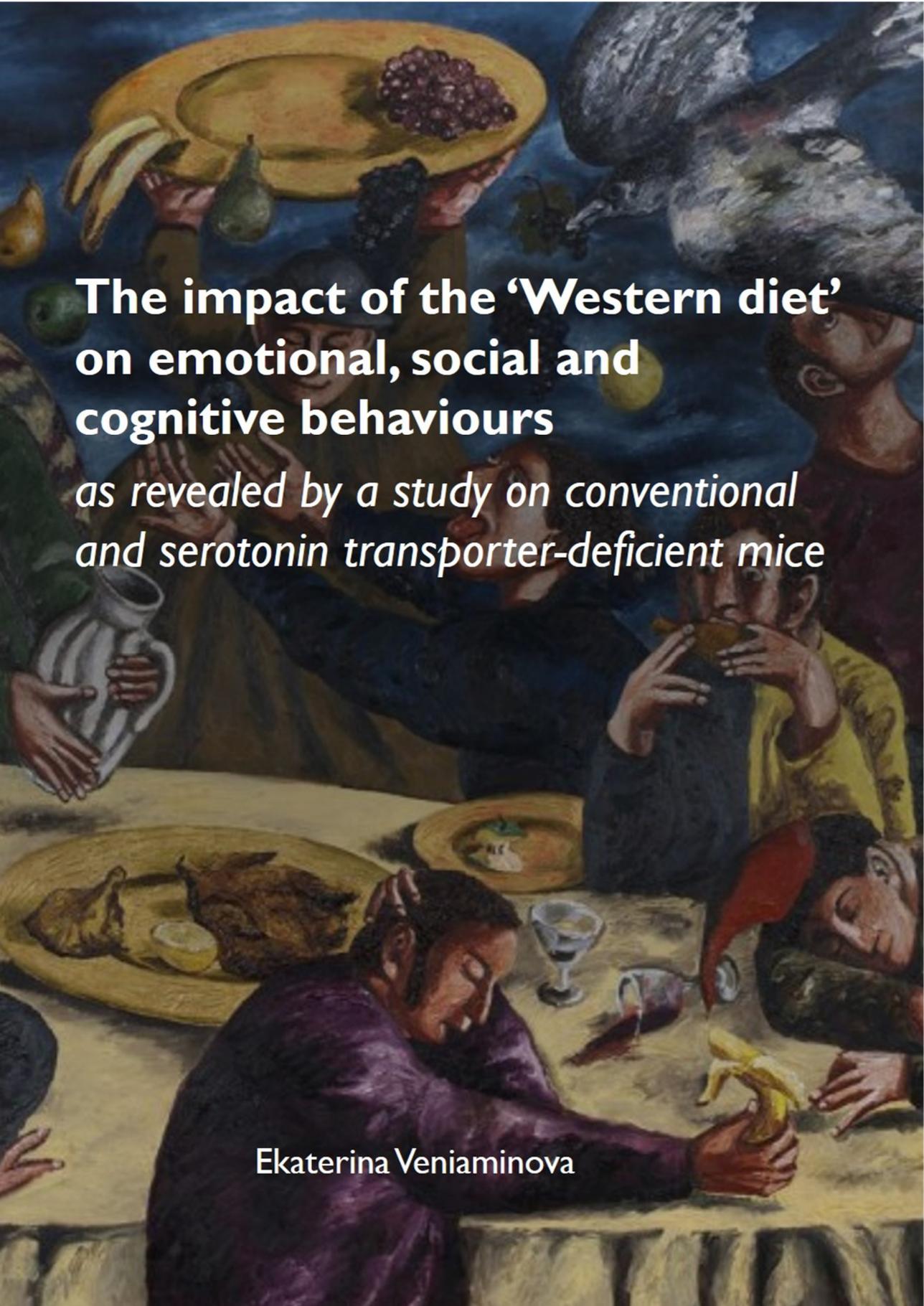
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A painting depicting a group of people gathered around a table, engaged in a meal. The scene is set in a dimly lit room with a dark blue background. In the foreground, a man in a purple tunic is seated at the table, looking down at a banana he is holding. To his right, a woman in a red headscarf is also seated, looking towards the camera. In the background, several other figures are visible, some holding plates of food and others holding glasses. The table is set with various dishes, including a large platter of roasted meat and a glass of wine. The overall atmosphere is one of a communal meal, possibly in a historical or biblical setting.

**The impact of the ‘Western diet’
on emotional, social and
cognitive behaviours**

*as revealed by a study on conventional
and serotonin transporter-deficient mice*

Ekaterina Veniaminova

MAASTRICHT UNIVERSITY

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Cover: Natalia Nesterova, “A feast in time of plague”, 1993, private collection

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**The impact of the ‘Western diet’ on
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serotonin transporter-deficient mice**

DISSERTATION

to obtain the degree of Doctor at the Maastricht University,

on the authority of the Rector Magnificus,

Prof.dr. Rianne M. Letschert

in accordance with the decision of the Board of Deans,

to be defended in public

on Thursday, March 5th 2020, at 12:00 hours

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Propositions

1. The Western diet suppresses social interactions, impairs cognition, induces impulsive and depressive-like behaviours, which are, together, all reminiscent of symptoms of autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) (*this thesis*);
2. The Western diet-induced abnormalities are associated with signs of oxidative stress, microglial activation, decreased gene expression of serotonin transporter in the prefrontal cortex as well as metabolic impairments (*this thesis*);
3. Complete genetic serotonin transporter inactivation exacerbates metabolic, molecular, and the behavioural consequences of the Western diet in aging mice (*this thesis*);
4. Mice with partial genetic serotonin transporter deficiency display a ‘rescued’ phenotype in dietary-induced abnormalities in glucose tolerance, neuroinflammation and hippocampus-dependent behaviour (*this thesis*);
5. Invention of a specific diet might provide relief for patients with co-occurring metabolic and neuropsychiatric disorders, which will decrease costs associated with disability and health management (*this thesis, valorization*);

6. Diet might impact on the pathogenesis of other neuropsychiatric disorders not considered in this thesis (for example, psychosis);
7. Other diets such as Mediterranean diet is likely to be protective in adulthood in cases of ADHD/ASD;
8. Gut microbiota has an important role in the interaction between diet and brain dysfunction;
9. “In food excellent medicine can be found, in food bad medicine can be found; good and bad are relative” (*Hippocrates, “De Alimento”, 5th-4th century BC*);
10. “Dilettante gardeners love the spring and summer; real gardeners also love the winter” (*Anne Scott-James, “Down to Earth”, 1971*);
11. “Today’s philosophy questions are tomorrow’s precision science experiments” (*Jorge Cham and Daniel Whiteson, “We Have No Idea: A Guide to the Unknown Universe”, 2017*).

Abbreviations

3-NT	3-nitrotyrosine
5-HIAA	5-hydroxyindoleacetic acid
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
8-oxo-dG	8-oxo-deoxyguanosine
8-oxoG	8-oxo-7,8-dihydroguanine
ADHD	Attention-deficit/hyperactivity disorder
Akt	Protein kinase B
AP-1	Activator protein-1
ASD	Autism spectrum disorder
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BH4	Tetrahydrobiopterin
CAT	Catalase
CNS	Central nervous system
COX	Cyclooxygenase
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CVO	Circumventricular organs
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EC	Enterochromaffin cell
EPA	Eicosapentaenoic acid

FFA	Free fatty acids
FK506	Protein binding protein 5
GLUT	Glucose transporter
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
HDL	High density lipoprotein
HDL	High density lipoprotein
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography
IDO	Indoleamine 2,3 dioxygenase
IFG	Impaired fasting glucose
IFN	Interferon
IGT	Impaired glucose tolerance
IKK	I κ B kinase
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
IR	Insulin receptors
IRS	Insulin receptor substrate
I κ B α	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
JNK	N-terminal kinase Jun
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase

MCP-1	Monocyte chemotactic protein-1
MDA	Malondialdehyde
MDD	Major depressive disorder
mtDNA	Mitochondrial DNA
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF- α B	Nuclear factor α B
NO	Nitric oxide
NOS	Nitric oxide synthase
OSI	Oxidative stress index
PDH	Pyruvate dehydrogenase
PET	Positron emission tomography
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PPARGC	Peroxisome proliferator-activated receptor gamma coactivator
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SERT	Serotonin transporter
SFA	Saturated fatty acids
SGLT	Sodium-dependent glucose transporters
SNP	Single nucleotide polymorphisms

SOD	Superoxide dismutase
SSRI	Selective serotonin reuptake inhibitor
STAT	Signal transducer and activator of transcription
T2D	Type 2 diabetes
TAS	Total antioxidant status
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOS	Total oxidant status
VMAT2	Vesicular monoamine transporter 2
WT	Wild type
XO	Xanthine oxidase

Chapter 1

General introduction

Chapter 1. General introduction

1.1 ‘Western diet’ as an environmental factor in psychiatric morbidities

The ‘Western diet’ is a dietary pattern that comprises a high intake of saturated fat, cholesterol, sugars, red meats, and salt, typically in the form of refined and processed foods. It is low in fresh fruits and vegetables, whole grains and seafood (Carrera-Bastos et al., 2011). Diet typical of Western societies was brought about by lifestyle changes following the 19th century Industrial Revolution, also known as the First Industrial Revolution, which introduced new methods of food processing, widespread use of refined vegetable oils and sugars and the advent of the ‘junk-food’ and ‘fast-food’ industry in the 1950s (Carrera-Bastos et al., 2011; Cordain et al., 2005). Western-style food consumption has been increasing consistently since then, especially in Western-culture countries (**Fig. 1.1**). The BBC Good Food Nation Survey 2016 found that people in the UK ate fast food on average two days per week, with young adults eating it 4.5 times a week. In the USA, on any given day, 34% of children consume fast-food and in general 44% eat fast-food once a week and 20% twice a week (Pew Research Center). Due to a growing economy, higher household income and increased globalization, the rates of fast-food consumption are also increasing in China (Wang et al., 2016), Japan (USDA Foreign Agricultural Service), India (Euromonitor International, 2017), and

Middle Eastern countries (Sarant et al., 2013, Naeem et al., 2012). Here, Western-style meals, snacks and junk food are at odds with the traditional food culture.

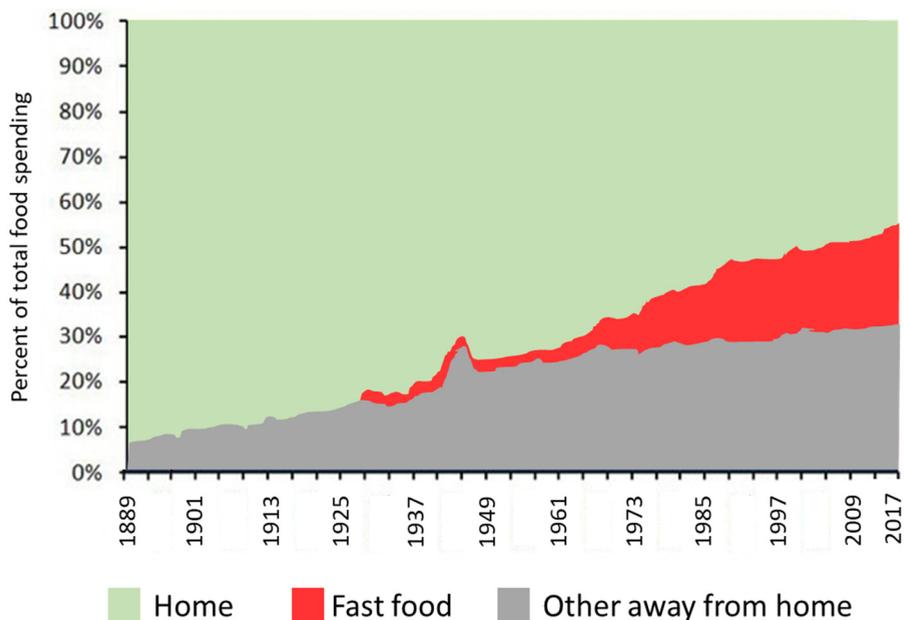


Figure 1.1. Eating habits dynamics in the USA from 1889 till 2017. Fast food expenditures were not tracked before 1929. In 1930-1960 fast food was not a significant expenditure, after which it rapidly gained in popularity. Today, fast food accounts for nearly 20% of total food expenditures. In 2017, less than half of food was consumed at home, the rest was consumed in either full-service or fast-food restaurants. Adapted from Guyenet, 2011 and USDA report, 2018.

The 21st century is also characterized by an increasing prevalence of psychiatric disorders, with challenges both for the individuals and wider society (Fuhrer and Keyes, 2019). Although the determinants of mental health are complex, there is emerging evidence for nutrition as an important factor in the prevalence of mental disorders (Sarris et al.,

2015). There is a growing amount of observational data regarding the association between dietary factors, such as Western diet consumption, and mental health. A higher risk of depression has been found in an adult populations with higher consumption of processed foods (Akbaraly et al., 2009) and fast food (Sánchez-Villegas et al., 2012). In women, Western diet adherence was found to be associated with major depression, dysthymia and anxiety disorders (Jacka et al., 2010). A systematic review has confirmed a relation between unhealthy dietary patterns and poorer mental health in children and adolescents (O'Neil et al., 2014).

On the other hand, adherence to Mediterranean and other regional traditional diets, which are typically high in vegetables, fruit, fish, nuts, beans and whole grains, were associated with reduced risk of depression (Le Port et al., 2012; Psaltopoulou et al., 2013) and cognitive impairment (Psaltopoulou et al., 2013). Dietary interventions, such as reducing intake of sugary, fried and processed food and fast-food were shown to decrease depressive symptomatology scores, assessed using the rating scale, in patients (Jacka et al., 2017; Reynolds et al., 2014). Although stress and depression can lead to unhealthy eating, recent studies have suggested that mental state-induced alterations in eating behaviour are not likely to explain long-term associations between depression and dietary pattern (Le Port et al., 2012).

Poor maternal dietary patterns are considered a risk factor for neurodevelopmental disorders such as autism spectrum disorder (ASD) (Li et al., 2018; Lyall et al., 2013) and attention-deficit/hyperactivity disorder (ADHD) (Millichap and Yee, 2012). Western-type diet consumption is associated with ADHD diagnosis in adolescents (Howard et al., 2011). Here, the causal link between dietary pattern and disorder development, including the underlying mechanisms involved, remain to be elucidated.

1.2 Pathophysiological mechanisms of negative effects of Western diet on the brain

Inflammation presents one potential mechanism through which nutrition may affect the brain (Minihane et al., 2015). This is supported by several studies showing that a higher dietary inflammatory index, the measure providing an estimate of the inflammatory potential of a diet from individual food parameters, is associated with increased risk of psychiatric disorders, including major depression and bipolar disorder (Firth et al., 2018; Oddy et al., 2018). One meta-analysis also found that a high dietary inflammatory index is more strongly associated with depression among females than males (Oddy et al., 2018). There is an emerging body of literature suggesting that elevated peripheral inflammatory markers are associated with deficits in cognitive function (Allison and Ditor, 2014; Carrard et al.,

2016) as well as worse disease prognosis (Firth et al., 2018) among patients with psychiatric disorders.

Though the specific mechanisms underlying this association remain unclear, diet-induced low-grade inflammation, characterized by chronic activation of the immune system, is thought to have a number of detrimental effects on brain structure and function (Minihane et al., 2015). Several studies propose that alterations in the gut microbiota triggered by dietary changes may be a key initial step in the development of low-grade systemic inflammation, affecting the whole body (Sanmiguel et al., 2015). Data from animal models support this hypothesis, demonstrating that germ-free mice exhibit neither obesity nor upregulation of intestinal tumor necrosis factor α (TNF) level compared to conventionalized mice when fed with high fat diet (Turnbaugh et al., 2008). Comparing microbiota of obese and lean subjects demonstrated that high bacterial richness (diversity) is associated with less significant adiposity and inflammation than found in subjects with low bacterial richness (Le Chatelier et al., 2013). Shifts in gut microbiota may lead to increased levels of lipopolysaccharides (LPS) and increased intestinal permeability through Toll-like receptor (TLR)-dependent mechanisms (Yiu et al., 2017) (**Fig. 1.2**), thus promoting increased translocation of LPS into the circulation (Bleau et al., 2015; Ding et al., 2010). High levels of glucose were also shown to stimulate serotonin release from enterochromaffin cells (EC) in the

gut (Kim et al., 2001; Racké et al., 1996), increasing extracellular serotonin levels. This resulted in the overstimulation of 5-HT₃ receptors and subsequent reduction of tight-junction proteins, ultimately leading to enhanced intestinal permeability (Yamada et al., 2003).

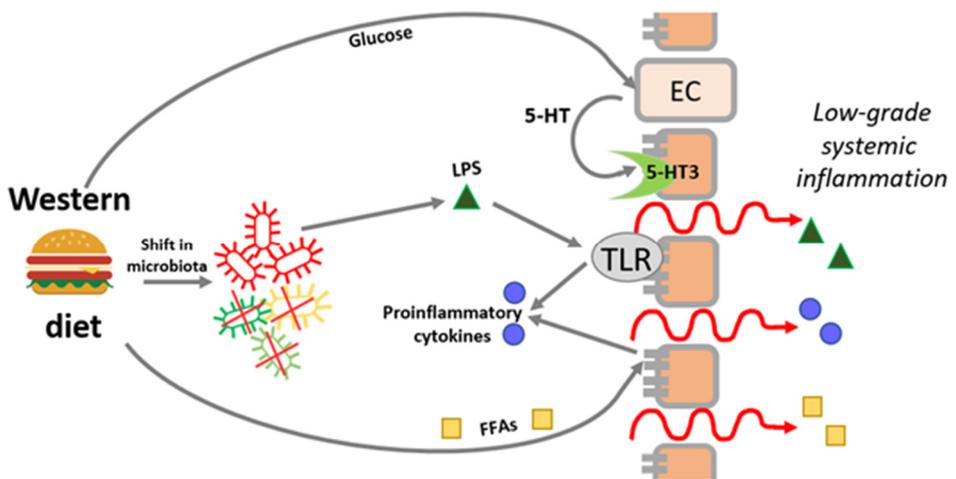


Figure 1.2. Development of systemic inflammation due to diet-induced shift in gut microbiota. Shifts in gut microbiota lead to increased levels of LPS and increased intestinal permeability due to TLR-dependent mechanisms. High levels of glucose stimulate serotonin release from the enterochromaffin cells (EC) increasing extracellular serotonin levels, which results in overstimulation of 5-HT₃ receptors, reduction of tight-junction proteins and enhanced intestinal permeability. Free fatty acids (FFAs), present in high amounts in Western diet, act on intestinal cells leading to increased production of proinflammatory cytokines in the gut. Intestinal endotoxin, proinflammatory cytokines and FFAs are then delivered into the systemic circulation leading to low-grade systemic inflammation.

In addition, free fatty acids (FFAs), prevalent in the Western diet, may act directly on intestinal and endothelial cells leading to

increased production of proinflammatory cytokines in the gut (Fujiyama et al., 2007; Konrad and Wueest, 2014; Yoshida et al., 2001). This way, intestinal LPS, proinflammatory cytokines and FFAs are delivered into the systemic circulation, leading to chronic low-grade systemic inflammation (Konrad and Wueest, 2014; Tsukumo et al., 2015).

Elevated plasma FFAs and LPS can then upregulate the expression of TLRs in circulating monocytes, leading to their activation and production of proinflammatory cytokines (Bleau et al., 2015; Kim et al., 2012). Activated inflammatory macrophages infiltrate adipose, muscle, and liver tissue, leading to a generalized peripheral inflammatory response (Bleau et al., 2015; Lumeng and Saltiel, 2011). The proinflammatory effect is mediated by at least two mechanisms, associated with the nuclear factor κ B (NF- κ B) and N-terminal kinase Jun (JNK) (Bastard et al., 2006) (**Fig. 1.3**). The NF- κ B pathway responds to diverse stimuli, including ligands of cytokine receptors, pattern-recognition receptors such as TLRs, as well as T-cell and B-cell receptors (Zhang and Sun, 2015). The primary mechanism for NF- κ B activation is the inducible degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha ($I\kappa$ B α) triggered through its site-specific phosphorylation by $I\kappa$ B kinase (IKK) complex (Liu et al., 2017b).

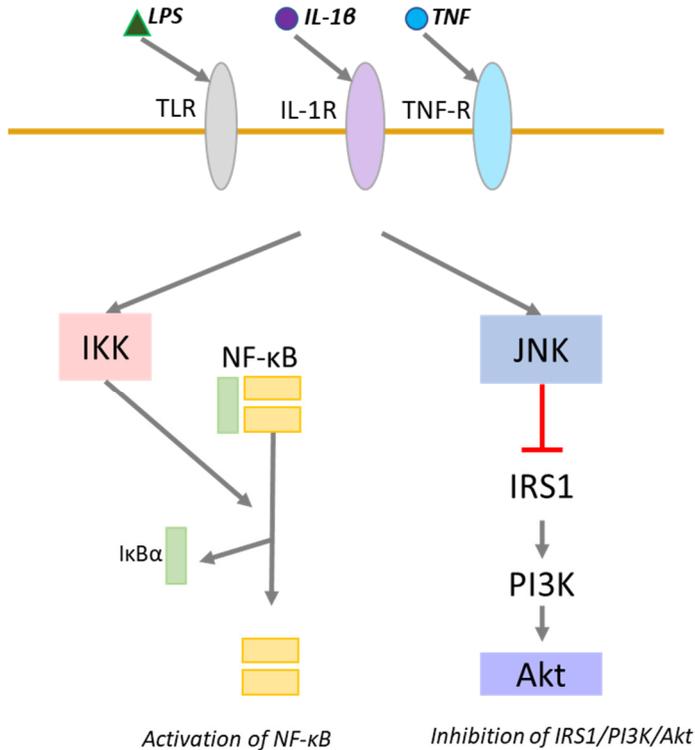


Figure 1.3. Mechanisms of NF- κ B pathway activation and of IRS1/PI3K/Akt pathway inhibition. I κ B kinase (IKK) is activated by different stimuli, including cytokines and microbial components. Upon activation, IKK phosphorylates I κ B α and triggers its ubiquitin-dependent degradation, resulting in activation and nuclear translocation of nuclear factor κ B (NF- κ B). Enhanced proinflammatory signaling also results in N-terminal kinase Jun (JNK) activation which inhibits activation of IRS1 protein and subsequently leads to the inhibition of PI3K and Akt.

In addition to mediating induction of various proinflammatory genes in innate immune cells, NF- κ B regulates the activation of inflammatory T cells and inflammasomes (Liu et al., 2017b). Enhanced proinflammatory signaling also results in a subsequent JNK activation,

which leads to the inhibition of signal transduction via pathways involving protein kinase B (Akt), and associated signal transducers like insulin receptor substrate 1 (IRS1) and phosphoinositide 3-kinases (PI3K) (Aguirre et al., 2000). This pathway is a key mechanism in the regulation of glucose metabolism in insulin-sensitive peripheral tissues (Aguirre et al., 2000) and is also implicated in the general response to extracellular stress (Karin and Gallagher, 2005) and inflammation (Bogoyevitch and Kobe, 2006; Karin and Gallagher, 2005).

Ectopic lipid accumulation aggravates systemic inflammation via the recruitment of M1 macrophages and increased expression of proinflammatory mediators (Caesar et al., 2015; Lee and Lee, 2014). Leptin produced by adipocytes triggers the activation of mitogen-activated protein kinase (MAPK) p38, and signal transducer and activator of transcription (STAT) 3, which lead to increased production of proinflammatory cytokines (Tilg and Moschen, 2006). Downstream effects include the activation, proliferation and migration of monocytes. In addition, resistin expressed by adipose tissue further enhances proinflammatory cytokine production (Tilg and Moschen, 2006).

There are several pathways by which peripheral inflammation could affect the brain (**Fig. 1.4**). Firstly, cytokines from the blood have been shown to be able to penetrate the blood brain barrier (BBB) in regions of high BBB permeability, for example the circumventricular

organs (CVO), and act directly on the neuronal and glial receptors, thus regulating the synthesis of cytokines in the CNS (Banks and Erickson, 2010). Cytokine influx into the CNS can also be increased in case of BBB disruption. A number of studies suggest that dietary and metabolic factors are related to disrupted BBB integrity. For example, adiposity in midlife female subjects is found to be strongly linked with reduced BBB integrity 24 years later (Gustafson et al., 2007). Increased BBB permeability has been shown in several models of the Western diet feeding in rodents (Banks et al., 2004; Kozler and Pokorný, 2003; Sparks, 2008; Wolburg and Lippoldt, 2002). Independent of BBB integrity, there are transport systems which are able to carry interleukin (IL)-1 α , IL-1 β , IL-1Ra, IL-6 and TNF through the BBB (Banks et al., 1995). Inflammation was shown up-regulate the influx carriers responsible for TNF (Osburg et al., 2002). Circulating cytokines activate the expression of cyclooxygenase (COX), leading to increased synthesis of prostaglandins, which in turn induce cytokine production in neurons. Systemic inflammation can promote leukocyte passage across the BBB through a paracellular or transcellular route (Bechmann et al., 2007), which was demonstrated for lymphocytes (Banks et al., 2012), neutrophils (Bohatschek et al., 2001) and monocytes (Wang et al., 2008). The activated systemic immune system can also signal to the brain via the nervous pathway involving both vagal and sympathetic nerves (Banks and Erickson, 2010).

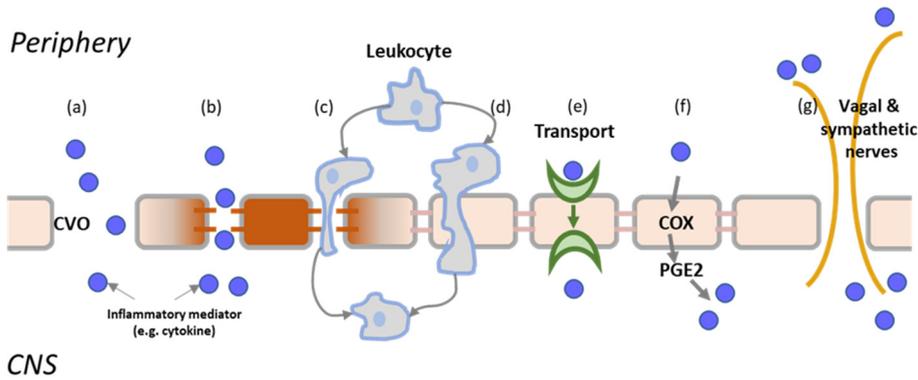


Figure 1.4. Pathways by which peripheral inflammation can affect the CNS. Inflammatory mediators (e.g. cytokines) can penetrate through the BBB in the regions of high permeability, such as (a) circumventricular organs (CVO), or (b) in case of impaired tight junction integrity. Passage of the leukocytes across the BBB may occur through (c) a paracellular or (d) transcellular route. (e) Some proinflammatory mediators can be directly transported through BBB. (f) Proinflammatory cytokines also promote cyclooxygenase (COX) expression that increases production of prostaglandin E2 (PGE2) which induces inflammatory CNS changes. (g) Vagus and sympathetic nerves are involved in signal transduction from activated immune system into the brain via the nervous pathway.

Once proinflammatory signals reach the CNS, their signaling pathways may affect neurotransmitter systems including serotonin, norepinephrine, dopamine and glutamate systems, all of which when perturbed are known to be associated with the development of mental disorders (Miller et al., 2013). First, CNS inflammation can influence the synthesis and reuptake of monoamine neurotransmitters. Inflammatory cytokines and their signaling pathways can activate the indoleamine 2,3 dioxygenase (IDO) which converts tryptophan, the primary amino acid of serotonin, into kynurenine, thus potentially

depleting the availability of serotonin in the brain (Dantzer et al., 2008). Another mechanism by which inflammatory cytokines can influence the synthesis of monoamine neurotransmitters is through the disruption of tetrahydrobiopterin (BH4) which is an enzyme co-factor for the rate limiting enzymes for the synthesis of serotonin, dopamine and norepinephrine (Haroon et al., 2012). High levels of pro-inflammatory cytokines can affect dopamine packaging by decreasing expression of vesicular monoamine transporter 2 (VMAT2) (Kazumori et al., 2004). Activation of MAPK pathways was also shown to increase the expression of dopamine transporter (Morón et al., 2003). Another mechanism by which inflammatory cytokines can influence neurotransmitter function is through effects on release. Administration of interferon (IFN) α has been shown to increase the reuptake and decrease the release of dopa, the primary precursor of dopamine (Capuron et al., 2012). Inflammatory cytokines have also been shown to stimulate the release of glutamate from astrocytes and reduce astrocytic expression of glutamate transporters, potentially leading to increased glutamate excitotoxicity (Ida et al., 2008; Matute et al., 2006).

It is well-established that high intakes of fat, sugars and cholesterol can promote oxidative stress and subsequently contribute to inflammation (Aksenova et al., 2005). Despite this, the mechanisms underlying the elevation of oxidative stress in metabolic disorders are

not fully understood. It is proposed that mitochondrial dysfunction (Yuzefovych et al., 2013) and increased fatty acid oxidation (Lee et al., 2015) contribute to these phenomena. Obese subjects are characterized by enhanced superoxic lipid oxidation in adipocytes (Furukawa et al., 2004), increased nitric oxide (NO) production and concentration of oxidative stress markers in the blood (Codoñer-Franch et al., 2011), and decreased expression of antioxidative enzymes (Le Lay et al., 2014). Reactive oxygen species (ROS) can stimulate signal transduction pathways promoting production of IL-6, TNF and monocyte chemoattractant protein-1 (MCP-1) (Elmarakby and Sullivan, 2012; Park et al., 2006; Rahman et al., 2002; Sung et al., 2002). Further, oxidative stress can also induce cellular senescence, particularly adipocyte senescence, via cellular oxidation damage (MacKellar et al., 2010; Minamino et al., 2009). Adipocyte senescence leads to macrophage recruitment and increases production of proinflammatory cytokines (Lafontan, 2014; Minamino et al., 2009).

Impaired by prolonged exposure to the Western diet, peripheral insulin sensitivity and regulation may affect CNS function by influencing the efficacy of insulin signaling in the brain directly. Insulin receptors (IR) are abundantly expressed in the hypothalamus, hippocampus, cerebral cortex, cerebellum and olfactory bulb. Astrocytes express both IRA and IRB isoforms, while neurons express exclusively the IRA (Pomytkin et al., 2018). Numerous studies have

revealed the involvement of IR in a broad spectrum of functions in the CNS, such as synapse formation and remodeling, neuronal survival and synaptic plasticity. Deficiency in IR-mediated signaling in the brain is associated with neurodevelopmental, affective and neurodegenerative disorders (Cline et al., 2012; Pomytkin et al., 2018).

Evidence suggests that levels of CNS insulin depend upon transport of peripheral insulin into the brain, as little or no insulin is produced in the CNS (Woods et al., 2003). Increased dietary fat intake was shown to reduce insulin transport into the CNS in a dog model of high-fat diet feeding (Kaiyala et al., 2000). Reduced insulin signaling in the hippocampus as well as peripheral insulin resistance were demonstrated in mice fed with the Western diet (Mielke et al., 2006). The adverse effect of excessive dietary fat and sugar intake on both peripheral and brain insulin signaling may partially explain the comorbidity of metabolic and psychiatric disorders. Together, it can be suggested that the interplay between oxidative stress and inflammation induced by the Western diet may also affect the brain, leading to the destabilization of various signaling systems including insulin signaling and neurotransmitter systems.

1.3 Peripheral effects of Western diet relevant to neuropsychiatric conditions

Nutrition is one of the most important factors affecting the development of metabolic disorders and their complications (Maugeri

and Barchitta, 2019). A strong relationship can be drawn between Western-style food consumption and obesity (Drewnowski, 2007; Kant, 2004; Yang et al., 2012). Current rates of obesity have reached epidemic levels, for example, in the EU about 20-30% of citizens are obese (Webber et al., 2014). Aside from the myriad of consequences to metabolic health, obesity is also associated with the diagnosis of mood and anxiety disorders (Bodenlos et al., 2011). Thus, major depressive disorder and obesity demonstrate a bidirectional relationship: the presence of either condition at baseline leads to a greater risk of developing the other condition (Bodenlos et al., 2011). Increased prevalence of being overweight has been noted in drug-naïve patients with bipolar disorder (Maina et al., 2008).

Children with ASD suffer from obesity and other metabolic disorders approximately two times more frequently than controls without ASD (Shedlock et al., 2016) (**Fig. 1.5**). ADHD has also been demonstrated to be associated with greater likelihood of being overweight (Pagoto et al., 2009) or obese (Cortese and Tessari, 2017; Fleming et al., 2005; Pagoto et al., 2009). Several studies demonstrated that obesity and associated factors, such as sleep disorders, could lead to ADHD development (Cortese et al., 2008; Gami et al., 2003; Weinberg and Brumback, 1990). Comorbid obesity and ADHD resulted in less successful weight-control treatment compared to patients with no ADHD symptoms (Altfas, 2002). In addition, paternal

and maternal obesity were described as risk factors for both ADHD (Andersen et al., 2018; Li et al., 2016) and ASD development in children (Andersen et al., 2018; Li et al., 2016; Suren et al., 2014).

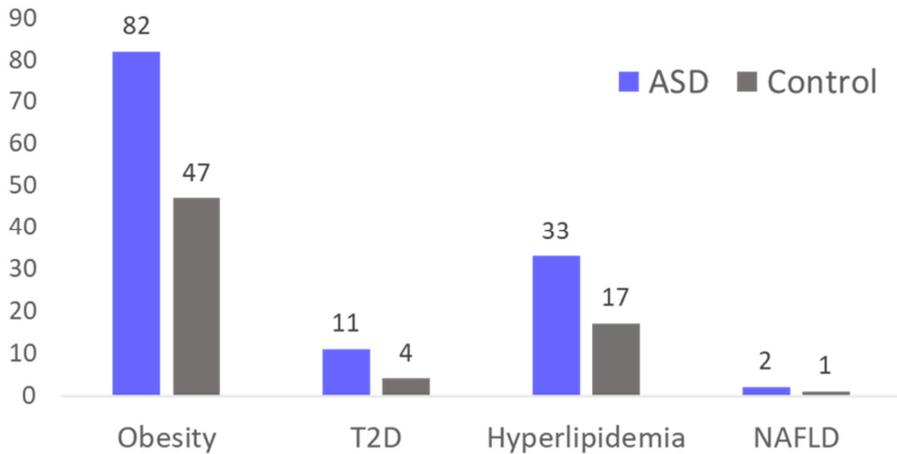


Figure 1.5. Metabolic disorders among children with ASD. The prevalence of obesity, type 2 diabetes (T2D), hyperlipidemia and non-alcoholic fatty liver disease (NAFLD) are approximately two-fold increased in children with ASD compared to controls. Adapted from Shedlock et al., 2016.

Dietary habits and sedentary lifestyle are the major factors for the rapidly rising incidence of diabetes mellitus (Sami et al., 2017). Diet higher in glycemic index and glycemic load drives glucose metabolism abnormalities such as impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and can ultimately lead to a diagnosis of type 2 diabetes (T2D) (Brand-Miller et al., 2003; Salmerón, 1997; Salmeron et al., 1997). Certain foods, especially sugar-sweetened beverages (Malik et al., 2010; Schulze, 2004), as well as dietary patterns, including a Western dietary pattern (Fung et al., 2004; van

Dam et al., 2002) are related to an increased risk of developing T2D. Dietary interventions were shown to decrease the incidence of diabetes later in life among people with IGT (Pan et al., 1997).

Disturbances in brain glucose metabolism are a feature of many psychiatric disorders, including depression (Li et al., 2015; Schweiger et al., 2008), ASD (Anil Kumar et al., 2017; Mitelman et al., 2018) and ADHD (Zametkin et al., 1990). There is an increased rate of depressive symptoms in women with impaired glucose metabolism (Adriaanse et al., 2008) and patients with T2D (Bouwman et al., 2010). Higher fasting blood glucose concentrations and IGT have been found in depressed individuals who have demonstrated suicidal behaviour (Koponen et al., 2015). Positive dietary interventions, such as adherence to a Mediterranean diet, can have a beneficial effect on the risk of depression in patients with T2D (Sánchez-Villegas et al., 2013).

Maternal gestational diabetes and T2D are associated with increased odds of having a child with an ASD (Connolly et al., 2016; Li et al., 2016) or other developmental disorder (Krakowiak et al., 2012). Children and adolescents with type 1 diabetes suffer from psychiatric disorders, including ADHD, more frequently than those without diabetes (Butwicka et al., 2015). This further highlights the comorbidity between diabetes and psychiatric disorders, and the likelihood of a common pathophysiological mechanism linked to deficits

in central insulin signaling (Kleinridders et al., 2015; Lyra E Silva et al., 2019; Pomytkin et al., 2015).

In addition to disturbances in glucose metabolism, a Western style diet can also impair metabolism of lipids (van Dam et al., 2002). This dietary pattern is positively associated with increased blood levels of total cholesterol and low density lipoprotein (LDL) cholesterol (Na et al., 2019; Sadakane et al., 2008; van Dam et al., 2003). Hypertriglyceridemia is observed in people with high intake of refined sugars (Bray and Popkin, 2014; Rippe and Angelopoulos, 2015). Total and LDL cholesterol and triglyceride levels are also higher among depressed individuals with suicidal behaviour (Koponen et al., 2015) and those with a history of anxiety disorders (Bajwa et al., 1992).

Maternal low blood level of high density lipoprotein (HDL) is associated with ADHD in offspring (Ji et al., 2017). While in some cases of ASD, deficit in cholesterol synthesis is thought to underlie the pathology (Gillberg et al., 2017), a matched case-control study found no difference in total cholesterol blood level between children with autism compared to children without autism (Kim et al., 2010). In addition, increased triglyceride level and LDL/HDL ratio were demonstrated in autistic children (Kim et al., 2010).

Dysregulated lipid metabolism is characteristic of liver diseases, such as non-alcoholic fatty liver disease (NAFLD) (Ipsen et al., 2018). NAFLD affects an estimated 25% of adults living in Western countries,

with up to 80% of obese individuals being affected (Ruhl and Everhart, 2004). Importantly, liver steatosis, a key feature of NAFLD, can be diagnosed in the absence of obesity (Margariti et al., 2012). Diets that are high in fat and sugar, which characterize Western diets, have been shown to induce NAFLD (Hosseini et al., 2016). Several studies in patients with biopsy-proven NAFLD have linked depression with non-alcoholic steatohepatitis (NASH) (Elwing et al., 2006), hepatocyte ballooning (Youssef et al., 2013), hepatic steatosis (Weinstein et al., 2011) and NAFLD activity score (Tomeno et al., 2015). NAFLD patients also experience anxiety (Mahmood et al., 2008) and cognitive impairment (Filipović et al., 2018) more often than the general population. Children with autism show an increased risk of NAFLD development (Shedlock et al., 2016). Cumulatively, this points to a strong association between metabolic and psychiatric disorders, and in many cases there are indications of a bidirectional relationship. It can be hypothesized that the presence of one condition may exacerbate the symptoms of another due to common pathophysiological mechanisms. Moreover, both metabolic and psychiatric disorders may share predisposing lifestyle factors or genetic susceptibility.

1.4 Role of decreased function of the serotonin transporter (SERT) in metabolic and mental disorders

The serotonin transporter (SERT) plays a central role in the regulation of serotonergic neurotransmission (Collier et al., 1996; Murphy et al., 2004) and is implicated in the regulation of metabolic processes as well as mechanisms of psychiatric disorders (Homberg and Lesch, 2011; Margoob and Mushtaq, 2011). In humans, two key variants in the regulatory region (5-HTTLPR) of the *SERT* (*SLC6A4*) gene exist, the so-called short (s) allele, which in comparison with the long (l) allele, reduces the expression of *SERT*. This is associated with higher body mass index (BMI) (Fuemmeler et al., 2008; Sookoian et al., 2007) and incidence of T2D (Iordanidou et al., 2010). Down-regulation of SERT in platelet membranes (Giannaccini et al., 2013) and cerebellum (Erritzoe et al., 2010) is also shown in obese people. Excessive intake of a hypercaloric diet enriched with saturated fat and sugars is shown to suppress the binding of hypothalamic SERT in obese subjects and diencephalon SERT in insulin-resistant subjects (Koopman et al., 2013; Versteeg et al., 2017).

Carriers of the s allele were also reported to be more susceptible to development of anxiety-related traits (Lesch et al., 1996) and depression (Caspi et al., 2003) in the context of adverse environmental challenge, although these findings have controversially been discussed in recent years. Multiple studies have shown that *SLC6A4* represents

a susceptibility locus for autism-spectrum disorders (Sutcliffe et al., 2005). Studies of 5-HTTLPR association with autism has yielded inconsistent results, with associations shown mostly to the s allele (Cook et al., 1997; Devlin et al., 2005; Tordjman et al., 2001) but also some to l allele (Yirmiya et al., 2001). Autism is an extremely heterogeneous disease with numerous single nucleotide polymorphisms (SNPs) found in ASD patients, which makes it possible that multiple different alleles at SLC6A4 contribute to the genetic risk of the disorder (Devlin et al., 2005; McCauley et al., 2004). Genome-wide linkage scans have demonstrated a positive linkage of ADHD and *SERT* (Fisher et al., 2002). Reduced availability of SERT in the brain is shown in patients with ADHD and conduct disorder (Chang et al., 2017; Vanicek et al., 2017).

Decreased SERT functioning was proposed to contribute to the development of both mental and metabolic disorders through elevated LPS levels and increased production of proinflammatory cytokines in the periphery (Pomytkin et al., 2015). Uptake by SERT is a key mechanism controlling the availability of serotonin in the gastrointestinal tract (Racké et al., 1996), meaning that decreased SERT would lead to excessive extracellular serotonin concentrations and increased gut permeability mediated by the 5-HT₃ receptor (Yamada et al., 2003) (*see section 1.2*). This hypothesis was supported by a study in *Sert* knockout mice which demonstrated enhanced

intestinal permeability and LPS leakage (El Aidy et al., 2017). If translated to humans, the 5-HTTLPR might result in similar effects related to endotoxemia and subsequent peripheral inflammation, which could contribute to the mechanisms of susceptibility to mental and metabolic disorders.

Thus, such overlapping psychiatric disorders as ADHD, ASD and depression (Taurines et al., 2012), as well as metabolic disorders have shared genetic susceptibility that could possibly interact with environmental factors, for example, diet. However, the mechanisms of the interplay between genetic SERT deficiency and metabolic dysregulation need further investigation.

1.5 Studying the effects of Western diet on brain functions using animal models

Animal feed enriched with fat, sugars and cholesterol, which mimics some aspects of a human Western dietary pattern, is typically used to study the mechanisms of diet on systemic physiology *in vivo*. Western diet-fed rodents are widely and successfully used to model such conditions as obesity, metabolic disorder, and insulin resistance (Hariri and Thibault, 2010; Hintze et al., 2018). More recently, Western diet-feeding in animals has been employed to investigate the nutritional consequences on behaviour and brain functioning.

Considerable variation exists in the use of, and experimental conditions associated with Western diet analogs in research (**Fig. 1.6**).

Fat content usually varies between 40% and 75% of total calories (Hintze et al., 2018), and diet composition may include different animal-derived fats or plant oils, which is known to elicit distinct effects on physiology (Buettner et al., 2006; Hryhorczuk et al., 2013). Duration of exposure to Western diet may vary from 1 week (Kaczmarczyk et al., 2013) to several months (André et al., 2014; Yamada et al., 2011). The age at which Western diet is administered also varies, with experiments utilizing young adult (Papazoglou et al., 2015; Strekalova et al., 2015, 2016; Yamada et al., 2011), aging (Kesby et al., 2015), or juvenile mice and rats (André et al., 2014; Kaczmarczyk et al., 2013). Exposure can also start in the early postnatal period (Carlin et al., 2013) or prenatally (Buffington et al., 2016). Different models involving adult and juvenile rodents provide an opportunity to explore diet-induced effects both on mature and developing organisms.

Peripheral changes during exposure to the Western diet differ between sexes, possibly contributing to the difference in dietary effects on the CNS. Male mice display faster body weight increases and greater dyslipidemia while female mice are more susceptible to liver steatosis and inflammation (Comhair et al., 2011; Dong et al., 2018; Gasparin et al., 2018; Homberg et al., 2010; Li et al., 2017; Link et al., 2015). Sex differences in the metabolic effects of diet-induced obesity were also shown to vary by age of onset of dietary intervention. In juvenile

mice the Western diet caused a greater increase in body weight and greater impairment in glucose tolerance in males than in female mice (Freire-Regatillo et al., 2019; Salinero et al., 2018). In later in life, when mice were 32 weeks old, the sex difference was reversed (Salinero et al., 2018). Despite the fact that the number of women affected by mental disorders is very high, the current literature on dietary effects on males is more abundant than the effects of diet on females.

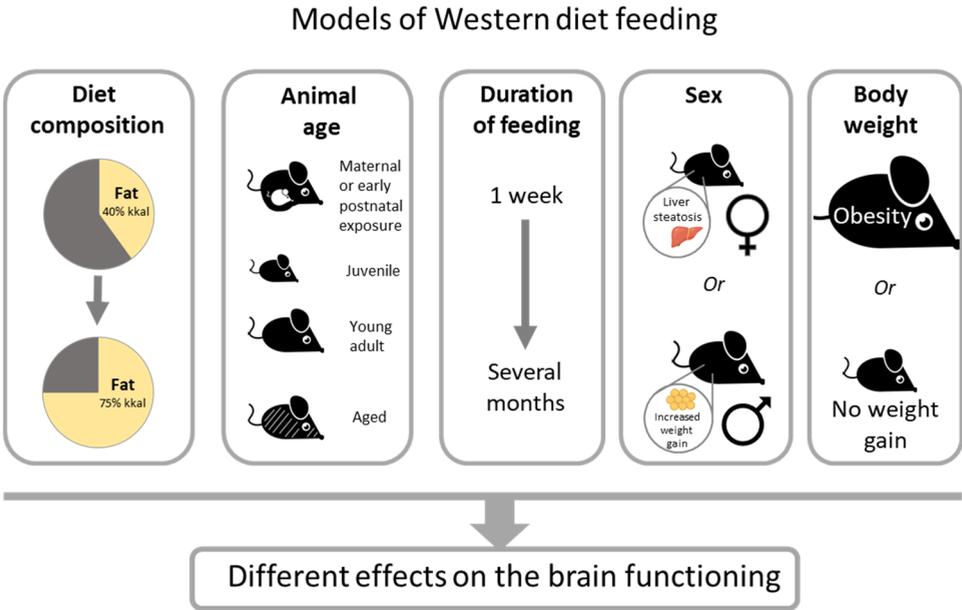


Figure 1.6. Variable parameters in animal models of dietary exposure. Models may vary in diet composition, animal age of dietary intervention onset, duration of feeding, animal sex, and effects of the diet on body weight.

Many studies have shown cognitive deficit, depressive-like behaviours and anxiety in diet-induced obesity in rodents (Abbott et al., 2019; André et al., 2014; Carlin et al., 2013; Nguyen et al., 2014a; Papazoglou et al., 2015; Vagena et al., 2019). This is associated with neuroinflammation, oxidative stress, changes in metabolome and BBB permeability as well as alterations in brain signaling systems. However, it remains unclear as to whether the dietary effects on CNS functioning in obesity and in non-obesogenic Western diet feeding have similar mechanisms. In addition, increased body weight in animal models can make it difficult to interpret test results.

Few studies have used non-obese animals fed with Western diet for a short period of time to examine behavioural and molecular brain changes. For example, memory deficit were reported in rats fed with Western diet without developing obesity using an 8-arm radial maze test of hippocampus-dependent working memory (Granholm et al., 2008; Kanoski and Davidson, 2010; Murray et al., 2009). It was shown that Western diet feeding for 1 week caused anxiety-like behaviors and impaired memory while body weight gain was significant only after 3 weeks of the diet feeding (Kaczmarczyk et al., 2013). Young female mice fed with the Western diet for 3 weeks demonstrated anxiety and depressive-like behaviour accompanied by TLR4 activation and constant body weight (Strekalova et al., 2015).

While impairments in social behaviours and motor coordination are important components of some disorders including ASD and ADHD, few studies have examined the effects of Western diet on these behaviours in animal models. One study using long-term high fat diet feeding in mice showed time-dependent reduction in motor coordinative skills measured by rotarod, pole test, stepping test and hind-limb clasping test. Muscle strength, locomotor activity and body weight were unaffected (Stojakovic et al., 2018). Social behaviours have mainly been studied in juvenile mice and rats, showing that Western diet can induce social recognition deficits (Buffington et al., 2016; Reichelt et al., 2019). Rodent models allow us to study not only the effects of the Western diet on behaviours reminiscent of mental disorder symptomology, but also to explore the mechanisms underlying behavioural changes.

Hypothesis and aim

In this study we used a model in which female C57BL/6J mice are fed with the Western diet for three weeks. Based on the previous experiments in this model, which revealed increased anxiety and depressive-like behaviour in young mice housed on the Western diet, and human data regarding cooccurrence of ASD and ADHD with depression and impaired metabolism, we hypothesized that Western diet may affect behaviours associated with these disorders. We aimed

to investigate the effects of the Western diet on social interactions, cognitive functions, emotionality and motor coordination (**Chapters 2 and 3**). In addition, we investigated diet-induced metabolic changes which are relevant to the development of behavioural abnormalities including glucose tolerance and insulin resistance, blood levels of lipids, liver injury markers and liver steatosis (**Chapters 2 and 3**). To explore the mechanisms underlying behavioural changes, we studied lipid peroxidation, microglial activation and gene expression of the mitochondrial biogenesis marker, and expression of the serotonin transporter in the brain of mice fed with the Western diet (**Chapters 2 and 3**).

Based on the well-established relationship between genetic deficit in SERT activity and susceptibility to mental and metabolic aberrations in humans, we next studied how Western diet, which in our model decreases *Sert* expression (**Chapter 3**), interferes with genetic *Sert* deficiency in mice (**Chapter 4**). It was hypothesized that complete and partial absence of *Sert* may differentially affect Western diet-induced changes. Using mice with a heterozygous and complete knockout for the *Sert* gene, we investigated diet-induced behavioural, metabolic changes and analyzed them in connection to molecular changes in the brain.

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Chapter 2

**Autism-like behaviours and
memory deficits result from
a Western diet in mice**

Chapter 2. Autism-like behaviours and memory deficits result from a Western diet in mice

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Abstract

Nonalcoholic fatty liver disease, induced by a Western diet (WD), evokes central and peripheral inflammation that is accompanied by altered emotionality. These changes can be associated with abnormalities in social behaviour, hippocampus-dependent cognitive functions, and metabolism. Female C57BL/6J mice were fed with a regular chow or with a WD containing 0.2% of cholesterol and 21% of saturated fat for three weeks. WD-treated mice exhibited increased social avoidance, crawl-over and digging behaviours, decreased body-body contacts, and hyperlocomotion. The WD-fed group also displayed deficits in hippocampal-dependent performance such as contextual memory in a fear conditioning and pellet displacement paradigms. A reduction in glucose tolerance and elevated levels of serum cholesterol and leptin were also associated with the WD. The peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1a) mRNA, a marker of mitochondrial activity, was decreased in the prefrontal cortex, hippocampus, hypothalamus, and dorsal raphe, suggesting suppressed brain mitochondrial functions, but not in the liver. This is the first report to show that a WD can profoundly suppress social interactions and induce dominant-like behaviours in naïve adult mice. The spectrum of behaviours that were found to be induced are reminiscent of symptoms associated with autism, and, if paralleled in humans, suggest that a WD might exacerbate autism spectrum disorder.

1. Introduction

In the context of increasing societal preference for “cafeteria-type diets” and “comfortable food” enriched with unsaturated fat and sugars, as well as the so-called “Western-type diet”, which is predominantly based on a heightened intake of saturated fat and cholesterol, there is an urgent need to study the physiological consequences of these diets. Excessive consumption of the Western diet has been shown to generate obesity, insulin resistance, hypercholesterolemia, and neuroinflammation in many organs, including the brain [1–6]. In addition, a Western-type diet may impact on reciprocal cognition [5, 7] and social interactions [3]. Human and animal studies suggest important roles of increased fat/cholesterol-containing dietary regimen in behavioural abnormalities associated with social behaviour, aggression, and brain plasticity [7–12].

Recent experiments showed aberrant social interactions and increased measures of aggression associated with diets containing high amounts of fat and cholesterol. Dietary exposure to increased amounts of cholesterol and fat was shown to increase male aggression in monkeys [13], elevate the risk of autism spectrum disorder in offspring of humans approximately 1.5 times [14], and suppress social exploratory interactions in offspring of mice [15, 16]. Also, a combination of elevated contents of cholesterol and saturated fat was found to aggravate cognitive rigidity and social deficiency in the mouse

model of autism [3]. Yet, these and other recently reported results provide limited information regarding the effects of a “Western”/westernized diet on social behaviours of adult individuals not predisposed to any abnormalities, including the changes typical for autism-like spectrum disorder.

The mechanisms that underlie deficient social interactions and brain plasticity associated with westernized diets can overlap. Increased rates of aggression-like traits are suggested to be related to reduced cognitive control and deficiencies in cognitive functions in general [17, 18]. Most of available literature reports decreased learning abilities in animals exposed to high-fat and high-cholesterol diets. Housing of rats on a high-cholesterol and fat-containing diet containing 2% cholesterol for 4 months led to impaired learning in the Morris water maze [19]; a similar diet induced altered stress response [20]. Spatial learning in an 8-arm maze was shown to be impaired in mice and rats housed on high-cholesterol and fat-containing diets [9, 12, 21, 22]. A high-fat diet containing 2% cholesterol induced deficits in contextual conditioning that were accompanied by altered hippocampal structure, such as a reduction in microtubule-associated proteins and elevation of markers of microglial activation in a rat [21]. Yet, very few studies addressing the effects of a westernized-type diet in the fear conditioning paradigms have been reported so far, while this paradigm provides higher sensitivity than other models to explore the

integrity of hippocampal functions [23]. In addition, a high-fat/high-cholesterol diet was also associated with increased immobility in the forced swim test indicating that the diet also generates a negative affect [21].

Several metabolic endpoints in the high-fat/high-cholesterol diet-fed mice have been noted that may underpin the behavioural changes, and these include increased insulin resistance, which is associated with a reduction in the expression of peroxisome proliferator-activated receptor gamma coactivator 1a (PPARGC1a), a marker of mitochondrial disbalance and impaired mitochondrial activity in dietary-induced type 2 diabetes [16, 24–26]. Diminished expression of this gene has been reported in the human hippocampus in patients with Alzheimer's disorder, and its expression level correlates with the clinical progression of dementia [27]. Mice genetically lacking the PPARGC1a gene exhibit an imbalance between inhibitory and excitatory synaptic transmission in the hippocampus, a mechanism that is suggested to be an important pathogenetic factor of autism [28, 29]. Thus, cognitive and other behavioural deficits reported in this work on mice fed with high-fat/high-cholesterol diet can result not only from lowered mitochondrial activity that is associated with decreased PPARGC1a brain levels but also from the specific role of this molecule in synaptic plasticity.

Our previous studies in mice employing a high-fat/high-cholesterol diet to induce nonalcoholic fatty liver disease (NAFLD) revealed that the diet increased levels of impulsivity, behavioural despair, anxiety, and reduced exploration of novel objects [30–32]. However, to date, the effects of excessive dietary cholesterol on social behaviours and aggression were not studied in this model. Here, we exposed female C57BL/6J mice to the Western diet (WD) [30, 31] to investigate the impact of the diet on social interactions, hippocampal function, and metabolism in normal mice exposed to the westernized diet during their adulthood. We are able to report, for the first time, that experimental exposure of mice to the WD profoundly affects their social interactions, substantially suppressing social exploratory contacts, inducing dominant-like behaviours and aberrant patterns of social behaviour. This diet also caused a deficient hippocampus-dependent performance, increased locomotion, downregulated mitochondrial activity marker PPARGC1a in the brain but not in the liver, and induced glucose intolerance and hyperleptin / hypercholesterolemia in mice. Thus, under conditions of excessive intake of fat and cholesterol which do not alter gross physiological measures such as body weight, substantial changes in behaviour, some of which are reminiscent of symptoms associated with autism, can occur.

2. Materials and methods

2.1. Animals

Studies were performed using 3-month-old female C57BL/6J from Janvier, Charles River, France. Mice were housed five per cage during the study, under a 12 h light-dark cycle (lights on: 19:00 h) with food and water ad libitum and under controllable laboratory conditions ($22 \pm 1^\circ\text{C}$, 55% humidity). All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals (2010/63/EU) upon approval by the Ethical Committee of the C. Bernard University on animal care and welfare.

2.2. Study design and dietary challenge

Mice were fed with a regular laboratory diet with an energy content of 3.0 kcal/g, 6.55% unsaturated fat, and 65% carbohydrates (Mucedola s.r.l., Settimo, Italy) or with a diet containing 0.2% (w/w) cholesterol, 20% of saturated fat, 39% carbohydrates, and an energy content of 4.6 kcal/g, “Western diet” (WD), (Research Diet Inc., New Brunswick, NJ, USA) for three weeks as described elsewhere [30–32]. The content of the nutrients in calories and weight is indicated in **Figure 1(a)** and Supplementary Table 1 available online at <https://doi.org/10.1155/2017/9498247>. Mice from the two groups were compared for the parameters of social interaction, hippocampus-dependent performance, and several metabolic read-outs, in three

separate experiments. Body weight and food intake were monitored weekly in each study, as described elsewhere (**Figures 1(b)** and **1(c)**; [30–32]) and were calculated as normalized to the mean of a control group.

After a three-week period of dietary challenge, a cohort of mice was studied for social interactions in a home cage and in a food competition test, as well as for an acquisition and an extinction of contextual fear in the fear conditioning paradigm (study 1; $n = 14$ for the control group and $n = 20$ for the group fed with a high-cholesterol and fat-containing diet; **Figure 1(d)**). Another cohort of animals was tested in the pellet displacement tube test, a rodent paradigm for a hippocampus-dependent performance [33, 34], and a glucose tolerance test, followed by blood collection for the analysis of leptin, triglyceride, and cholesterol content (study 2; $n = 8$ for the control group and $n = 7$ for the group fed with a high-cholesterol and fat-containing diet; **Figure 1(e)**). Finally, a portion of mice was sacrificed and dissected for the analysis of gene expression of a mitochondrial activity marker PPARGC1a, in the prefrontal cortex, hippocampus, hypothalamus, dorsal raphe, and liver (study 3; $n = 5$ for each group; **Figure 1(f)**). In the first two experiments, behavioural and biochemical assays were carried out during four consequent days following a period of a dietary intervention; gene expression was analysed at the midpoint of this testing period (**Figures 1(d)**, **1(e)**, and **1(f)**).

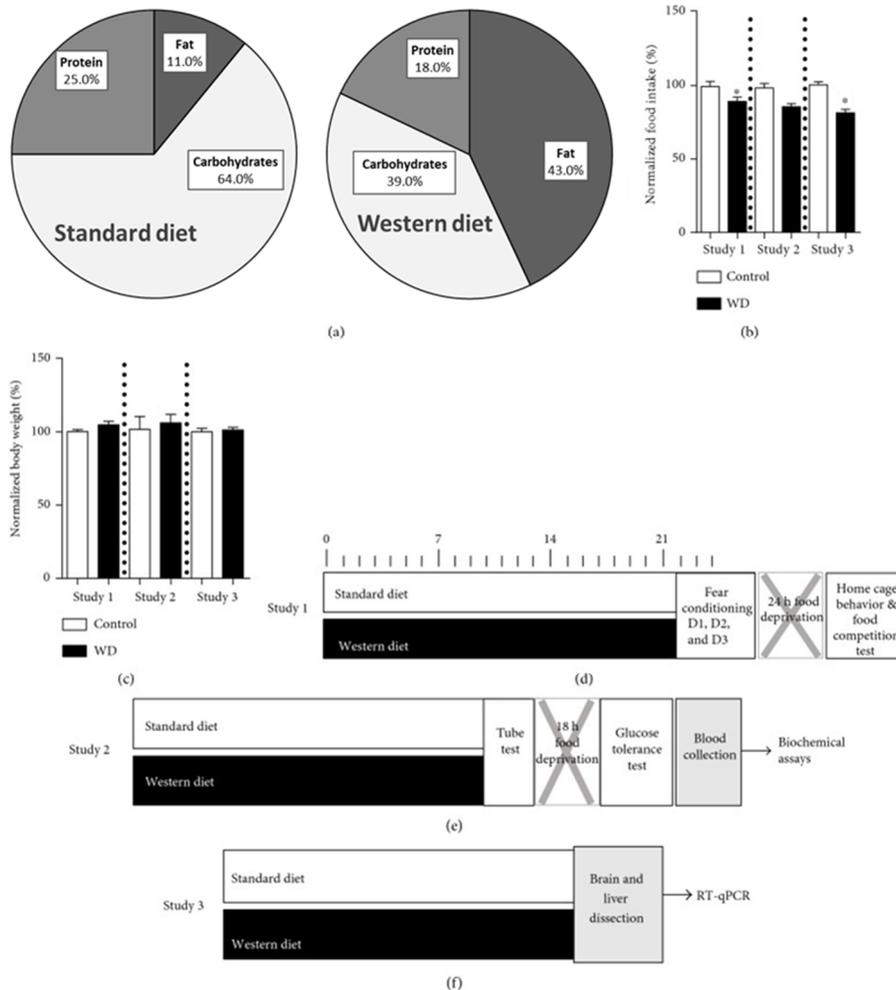


Figure 1. Experimental design. (a) Composition of the diets and percentage of total calories. Food intake (b) and body weight (c) normalized to control on day 21 of a dietary challenge (control - control diet, WD - Western diet; * $p < 0.05$ versus that in the control diet, t-test). (d) Study of the effects of high-fat/high-cholesterol diet exposure on mouse behaviour in fear conditioning test, home cage, and food competition test. (e) Study of the effects of high-fat/high-cholesterol diet exposure on mouse performance in pellet displacement tube test, glucose tolerance, and blood biochemical parameters. (f) Study of the effects of cholesterol-enriched diet exposure on gene expression in the brain and liver.

2.3. Behavioural Testing

All behavioural tests were carried out during an active period of animals' light cycle (09:00–21:00); behaviour was recorded and manually scored offline using behavioural criteria that were previously validated with automated scoring [35, 36]. The experimenter was blind for the diet used in the mice subjected to the testing.

2.3.1. Social interactions and solitary behavioural activities in a home cage

In both experimental groups, social behaviours and other activities in a home cage were assessed 24 h after a food deprivation, immediately prior to the food competition test. Mice were food-deprived in order to potentiate their social interactions [32]. The top of a home cage was replaced by a transparent cover, and mouse behaviours were recorded during 10 min under the subtle lighting (light intensity 5 lux). The following behavioural parameters were evaluated: group huddle, “sitting alone”, time spent in motion, and digging behaviour. The group huddle was defined as sitting of a mouse in a physical contact to a body of at least one cage mate [36, 37]. “Sitting alone” defined as a position of a mouse in a cage without any physical contacts to another cage mate(s). Time spent in motion was defined as an appearance of horizontal movement of an animal where a position of a centre of a body changed in a cage with a speed > 2 cm/sec or an animal would display a rearing by taking a vertical position for >1 sec,

as defined previously [38]. To evaluate digging (burrowing) behaviour, a species-specific behaviour of a displacement of bedding material using the snout and forepaws, percentage of mice that display this form of behaviour was calculated in each group; its total duration for an observation period was evaluated as well.

2.3.2. Social interactions during a food competition test

In the food competition test, two 24 h food-deprived mice from different cages and the same experimental group were placed in a plastic observation cage (21 cm × 27 cm × 14 cm) which contained a piece of beef meat (2 g) for 10 min under subtle lighting (light intensity: 5 lux) and allowed to compete for food as described previously [32]. Our previous studies showed that these conditions, which were adapted from earlier studies [39], female mice which are genetically prone to display aggression, are triggered for agonistic behaviours, competition for food, and aggressive behaviours (Strekalova et al., unpublished data). While previous studies revealed a lack of direct evidences for proaggressive effects of a high-fat/cholesterol diet on female mice in a food competition test [32], the same paradigm was used to study detailed analysis of social interactions in the current study.

During this test, animals were analysed for crawl-over behaviour, as well as body-body, nose-anal, and nose-nose contacts as described elsewhere [40]. The crawl-over behaviour was defined as climbing over the back and head of another animal. “Nose-nose” contacts were defined

as maintaining vibrissae for longer than 1 sec. “Nose-anal” contacts were defined as an examination of the anogenital area of another mouse. “Body-body” contacts were defined as other physical contacts that did not fall under the criteria of above-described forms of interactions. For each type of social contacts, the latency, duration, and a number of behavioural events were scored.

Additionally, during a food competition test, each mouse was scored for horizontal activity by counting the number of lines crossed where squares were app 10×10 cm in size, for the entire observation period. Their vertical activity was scored by counting the total number of rearings; a latency to rear was registered as well.

2.3.3. Acquisition and extinction of contextual fear in the fear conditioning paradigm

The apparatus (Open Science, Russia and Technosmart, Rome, Italy) consisted of a transparent plastic cubicle (25×25×50 cm) with a stainless-steel grid floor (33 rods, 2 mm in diameter). A single alternating electric current (AC, 50 Hz; 0.5 mA, 1 sec, Evolocus LLC, Tarrytown, NY, USA) was delivered after a 2 min acclimatization period. After delivery of the current, the mouse was immediately placed back in the home cage. Freezing behaviour was scored by visual observation during a test of memory recall that was carried out 24 h later as described elsewhere [41, 42]. The occurrence of freezing behaviour was assessed every 10 s for 180 s; each 10 s score was assigned

to a freezing or nonfreezing period, and the percentage of time spent in freezing was calculated. Mice that spent $\geq 50\%$ of time in freezing were defined as “good learners”. After scoring of memory recall, mice were left for another seven minutes in the apparatus for memory extinction; no foot shock was applied during this period. 24 h later, freezing behaviour was scored again in a 180 s recall of extinction session as in the previous trial and percentage of time spent in freezing was calculated.

2.3.4. Pellet displacement tube test

In order to further assess hippocampal function, all experimental groups were tested for pellet displacement in a tube test [33, 34]. A tendency to displace small objects, for example, small stones or food pellets from a tube inside the cage, is species-specific in mice and has been demonstrated to depend on an intact hippocampal formation [33]. Using a paper tube (internal diameter 4 cm, length 10 cm), filled with 20 food pellets and placed in the middle of a cage (21 cm \times 27 cm \times 14 cm), the latency to displace the first food pellet and time required for 50% and 100% tube emptying were assessed in mice.

2.4. Glucose tolerance test

The animals from both groups underwent an oral glucose tolerance test. Mice were fasted overnight during 18 h, starting at 17:00; thereafter, glucose solution (2 g/kg, 1.8 g/l) was delivered into

the stomach by oral gavage and blood was sampled from the tail vein. Samples were obtained before glucose administration at time point 0 and 5, 15, 30, 60, 90, and 120 minutes after. The level of blood glucose was analysed using the OneTouch UltraEasy glucometer and strips (LifeScan OneTouch, Dubai, UAE). Because there were no group differences between basal blood glucose levels, absolute values of glucose concentrations were analysed. The area under a curve for the dynamics of this parameter was calculated as well.

2.5. Brain dissection, RNA extraction, and RT-qPCR

Mice were sacrificed by cervical dislocation as described elsewhere [30, 40]. The brain of each mouse was dissected, and the prefrontal cortex was isolated and stored at -80°C until use. mRNA was extracted by using TRI Reagent (MRC, USA). First-strand cDNA synthesis was performed using random primers and Superscript III transcriptase (Invitrogen, Darmstadt, Germany); $1\ \mu\text{g}$ total RNA was converted into cDNA. Quantitative PCR for the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1a) gene and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using the SYBR Green master mix (Bio-Rad Laboratories, Philadelphia, USA) and the CFX96 Real-time System (Bio-Rad Laboratories, Philadelphia, USA). Sequences of primers used are indicated in Supplementary Table 2. Data were normalized to GAPDH mRNA expression and calculated as relative fold changes

compared to that in control mice as described elsewhere [30, 40]. Results of qRT-PCR measurement were expressed as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product was 0.05% of normalized maximal signal. We used the comparative Ct method and computed the difference between the expression of the gene of interest and GAPDH in each cDNA sample ($2^{-\Delta\Delta Ct}$ method). Data are given as expression folds compared to the mean expression values in control mice.

2.6. Blood biochemical analysis

Trunk blood was collected by decapitation during animals' sacrifice, stored at 4°C overnight, and centrifuged at 10000g for 10 min at 4°C. Serum was collected and stored at -20°C until use. A commercially available Mouse Leptin (OB) ELISA Kit (Sigma-Aldrich, MA, USA) was used to measure leptin level; the optical densities of experimental plates were measured at 450 nm using a plate reader (Wallac 1420 VICTOR, Waltham, MA, USA). All samples were run in duplicate. Quantitative determination of cholesterol and triglycerides in mouse serum was performed on a biochemistry analyzer Konelab 30i (Thermo Fisher Scientific, MA, USA) using a cholesterol kit and triglyceride kit (Thermo Fisher Scientific). All procedures were done according to the instruction manual.

2.7. Statistics

Data were analysed using GraphPad Prism version 5.0 (San Diego, CA, USA). All quantitative data sets were first analysed for normal distribution using Shapiro-Wilk normality test; then, t-test and two-way ANOVA with Bonferroni post hoc testing were used for normally distributed data and Mann-Whitney test for not normally distributed data. Two-way ANOVA was used to analyse the results from the glucose tolerance test. Fisher's exact test was used for categorical data. The level of significance was set at $p < 0.05$.

3. Results

3.1. Changes in food intake and body weight

In accordance with the previous findings, intake of the high-cholesterol and fat-containing diet was lower than that of the control diet, likely due to the adjustment of mice to the higher caloric value of the WD. This difference was significant in studies 2 and 3 ($t = 3.35$, $p = 0.004$ and $t = 5.97$, $p = 0.0001$, respectively, **Figure 1(b)**) and close to a level of significance in study 2 ($t = 2.18$, $p = 0.061$). Body weight did not differ significantly between groups in all three studies, suggesting that the dietary intervention did not grossly alter body composition ($p > 0.05$, t-test, **Figure 1(c)**); however, a tendency to an increased body mass was found in study 1 ($t = 1.79$, $p = 0.093$). These findings are in line with our previous results that showed a lack

of body weight changes and a decrease in intake of a high-cholesterol and fat-containing diet [24–26].

3.2. Aberrant social interactions, hyperlocomotion, and excessive digging behaviour in home cage conditions and during the food competition test in dietary-induced mice

During study 1, we found that in a home cage, in comparison with controls, mice fed with the high-cholesterol and fat-containing diet showed a significant decrease in the duration of group huddle behaviour ($t = 16.49$, $p < 0.001$, t-test; **Figure 2(a)**) and a significant increase in the duration of “sitting alone” behaviour ($t = 5.81$, $p < 0.001$, t-test; **Figure 2(b)**), as well as the time spent in motion ($t = 13.39$, $p < 0.001$, t-test; **Figure 2(c)**). The number of animals expressing digging behaviour during this test and duration of digging (burrowing) behaviour, which is a sign of active copying and a correlate of social dominance, were significantly increased in the group fed with the fat/cholesterol-enriched diet compared to the control ($p = 0.003$, Fisher's exact test and $U = 24.00$, $p = 0.003$, Mann-Whitney test, **Figures 2(d)** and **2(e)**). Thus, consumption of the high-cholesterol and fat-containing diet induces social avoidance, hyperlocomotion, and excessive digging behaviour in home cage conditions.

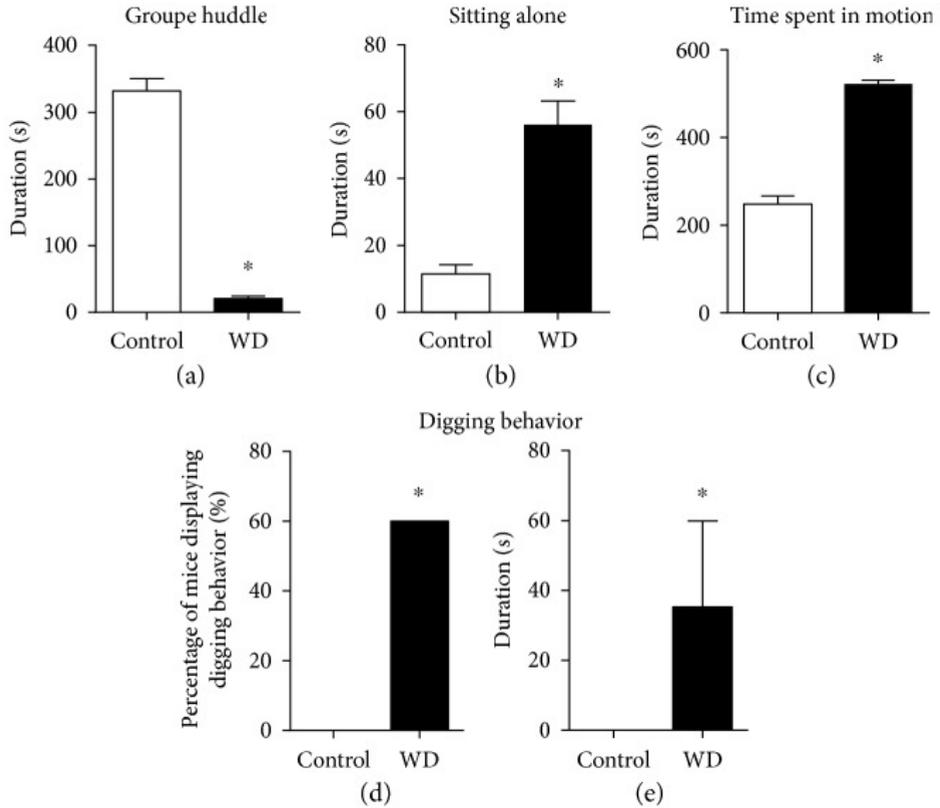


Figure 2. Dietary challenge with the Western diet results in aberrant home cage social behaviour. In comparison to control mice, the dietary-challenged group displayed (a) a significant decrease in the duration of group huddle, (b) a significant prolongation of the duration of “sitting alone” behaviour, and (c) a significant increase in the time spent in motion in the cage (* $p < 0.05$ versus that in the control group, t-test). As compared to control animals, a group fed with the high-fat/high-cholesterol diet showed a significant elevation of (d) the percentage of mice displaying digging behaviour (* $p < 0.05$ versus that in the control group, Fisher's exact test) and (e) the duration of digging behaviour (* $p < 0.05$ versus that in the control group, Mann-Whitney test). Control—standard diet, WD—Western diet. Data are shown as mean \pm SEMs.

During the food competition test, in comparison with controls, the dietary-challenged group showed a significant decrease in the latency of crawl-over behaviour ($U = 25.50$, $p = 0.020$; Mann-Whitney test, **Figure 3(a)**) and a significant increase in the number of crawl-over behavioural events ($U = 25.50$, $p = 0.020$, Mann-Whitney test, **Figure 3(b)**) and the duration of this behaviour ($U = 19.00$, $p = 0.005$, Mann-Whitney test, **Figure 3(c)**). In comparison to mice housed on a regular diet, mice fed with the high-cholesterol and fat-containing diet showed no difference in the latency of body-body contacts between the groups ($U = 10.00$, $p = 0.398$; Mann-Whitney test, **Figure 3(d)**); however, the number of the body-body contacts was significantly increased ($U = 3.00$, $p = 0.026$; Mann-Whitney test, **Figure 3(e)**), while the total duration of this behaviour was decreased ($U = 1.00$, $p = 0.009$, Mann-Whitney test, **Figure 3(f)**). The latency, number, and duration of nose-anal and nose-nose contacts were not different between the groups ($U = 49.50$, $p = 0.502$; $U = 53.50$, $p = 0.615$; $U = 53.50$, $p = 0.615$ and $U = 10.00$, $p = 0.416$; $U = 7.00$, $p = 0.171$; $U = 8.00$, $p = 0.197$, respectively; Mann-Whitney test, **Figures 3(g), 3(h), 3(i), 3(j), 3(k), and 3(l)**).

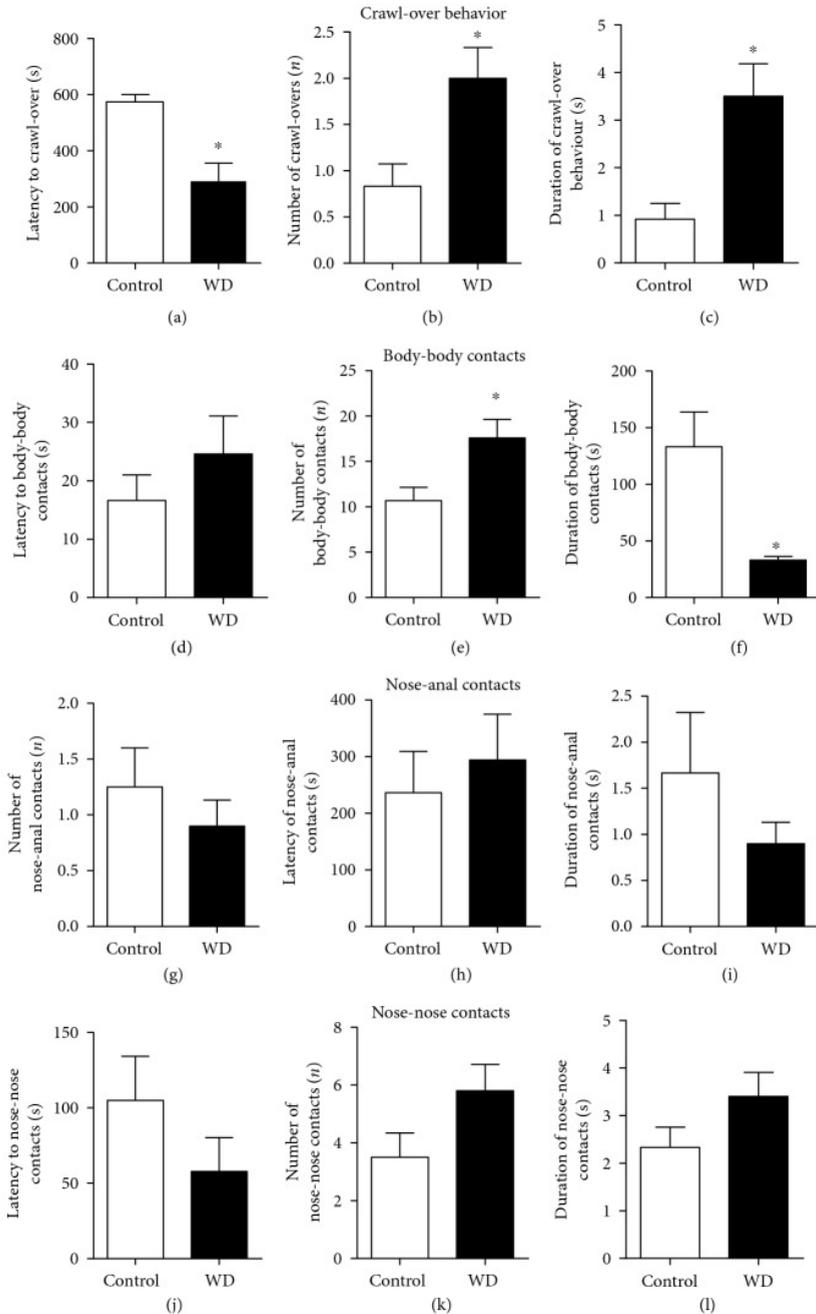


Figure 3. The high-fat/high-cholesterol diet potentiates dominant-like behaviour and suppresses sociability in a food competition test. In comparison to the control group, mice housed on the high-fat/high-cholesterol diet

showed (a) a significant decrease in the latency to crawl-over behaviour and (b) a significant increase in a number of crawl-overs, as well as (c) prolonged total duration of crawl-over behaviour (* $p < 0.05$ versus that in the control group; Mann-Whitney test). In comparison to the control group, mice exposed to the high-fat/high-cholesterol diet had (d) no significant changes in the latency to body-body contacts and had (e) a significant increase in a number of body-body contacts (* $p < 0.05$ versus that in the control group; Mann-Whitney test) and (f) a significant decrease in the duration of body-body contacts (* $p < 0.05$ versus that in the control group; Mann-Whitney test). No significant group differences were observed between the groups in the parameters of nose-anal contacts: (g) the number of nose-anal contacts, (h) latency to nose-anal contacts, and (i) total duration of nose-anal contacts (* $p > 0.05$ versus that in the control group; Mann-Whitney test). There were no significant group differences in the parameters of nose-nose contacts: (j) latency of nose-nose contacts, (k) number of nose-nose contacts, and (l) total duration of nose-nose contacts (* $p > 0.05$ versus that in the control group; Mann-Whitney test). Control—standard diet, WD—Western diet. Data are shown as mean \pm SEMs.

During the food competition test, in comparison with control mice, the group fed with the high-cholesterol and fat-containing diet showed a significant increase in the total number of line crossings ($t = 4.18$, $p < 0.001$, t-test, **Figure 4(a)**) and in the total number of rearings ($U = 17.50$, $p = 0.004$, Mann-Whitney test, **Figure 4(b)**) and a significant decrease in the latency of the first rearing event ($t = 4.42$, $p = 0.001$, t-test, **Figure 4(c)**). Together, these data suggest increased dominant-like behaviours, reduced sociability associated with abnormal social behaviour, and hyperlocomotion in mice housed on the WD.

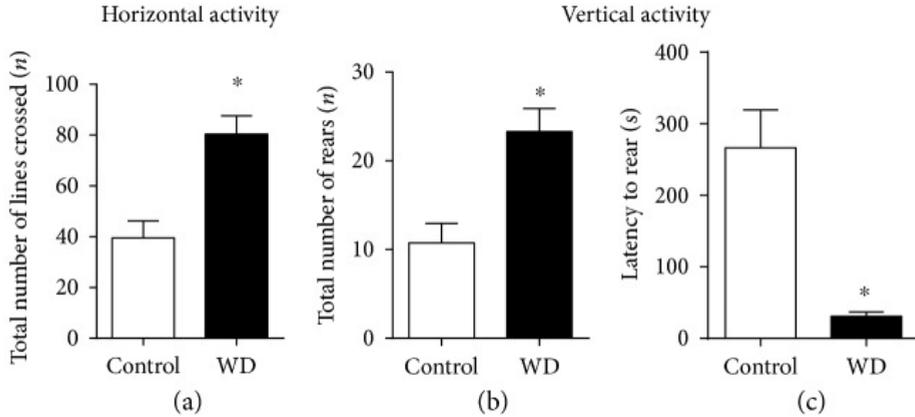


Figure 4. The high-fat/high-cholesterol diet enhances horizontal and vertical activity during food competition test. In comparison to control mice, animals exposed to the high-fat/high-cholesterol diet showed (a) a significant increase in the total number of line crossing (* $p < 0.05$ versus that in the control group, t-test), (b) significant elevation of the total number of rearings (* $p < 0.05$ versus that in the control group, Mann-Whitney test), (c) and a significant decrease in the latency of rearings (* $p < 0.05$ versus that in the control group, t-test). Control—standard diet, WD—Western diet. Data are shown as mean \pm SEM (a, c) and median with interquartile range (b).

3.3. Exposure to a diet enriched with fat and cholesterol results in the deficient hippocampus-dependent performance in the fear conditioning and pellet displacement tests

In the fear conditioning test, in comparison to that in control mice, the number of “good learners” defined by the percentage of time spent with freezing $\geq 50\%$ at the memory recall session was significantly diminished in the dietary-challenged group ($p = 0.017$, Fisher's exact test, **Figure 5(a)**); also, there was a strong trend to a decreased duration of freezing in this group ($t = 1.96$, $p = 0.059$, t-test,

Figure 5(b)), suggesting reduced contextual memory in mice housed on the high-cholesterol and fat-containing diet.

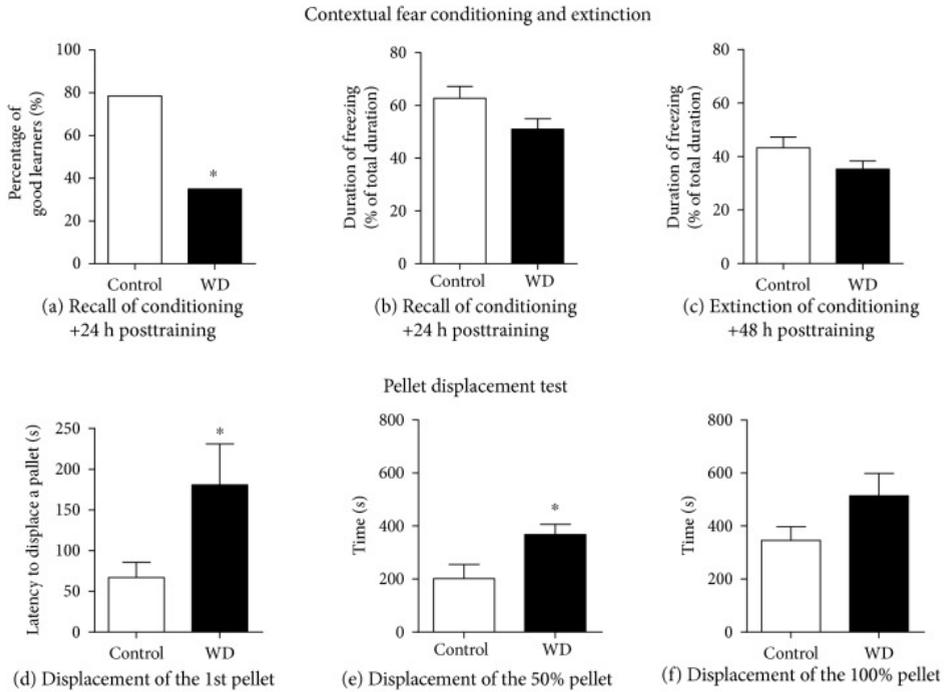


Figure 5. The high-fat/high-cholesterol diet compromises the hippocampus-dependent performance. In the fear conditioning test, in comparison to control mice, the dietary-challenged group showed (a) significant decreased number of “good learners” (* $p < 0.05$ versus that in the control group, Fisher's exact test), (b) a strong tendency to a reduced time spent with freezing during recall of conditioning ($p = 0.055$ versus that in the control group, t-test), and (c) a significant decrease in time spent with freezing in a memory extinction protocol (* $p < 0.05$ versus that in the control group, t-test). In the pellet displacement tube test, in comparison to control animals, mice exposed to the high-fat/high-cholesterol diet showed significantly prolonged (d) latency of a displacement of the 1st pellet and (e) the duration of displacement of 50% pellets (* $p < 0.05$ versus that in the control group, t-test) and (f) did not differ in the duration of a displacement of 100% pellets ($p > 0.05$ versus that in the control group, t-test). Control—standard diet, WD—Western diet. Data are shown as mean \pm SEM.

During the recall of memory extinction, mice housed on the WD showed significantly shorter duration of freezing than control mice, suggesting faster extinguishing of contextual memory due to its weaker acquisition ($t = 3.024$, $p = 0.005$, t-test, **Figure 5(c)**). During study 2, in the pellet displacement tube test, the latency to displace a pellet and time required for a 50% emptying the tube with pellets were significantly increased in the high-fat/high-cholesterol diet group, in comparison to control animals ($t = 2.26$, $p = 0.044$ and $t = 2.62$, $p = 0.020$, respectively, t-test, **Figures 5(d)** and **5(e)**). The time required for a 100% emptying of the tube did not differ significantly between the groups ($t = 1.74$, $p = 0.105$, t-test, **Figure 5(f)**). Together, these data suggest a moderate deficiency in the hippocampus-dependent performance in mice fed with the high-cholesterol and fat-containing diet.

3.4. Altered central and peripheral metabolic markers in mice housed on the high-cholesterol and fat-containing diet

At study 2, in the glucose tolerance test, two-way ANOVA revealed a significant effect of both the diet and time after glucose load on blood glucose level ($F = 10.16$, $p = 0.013$ and $F = 19.15$, $p < 0.001$; **Figure 6(a)**).

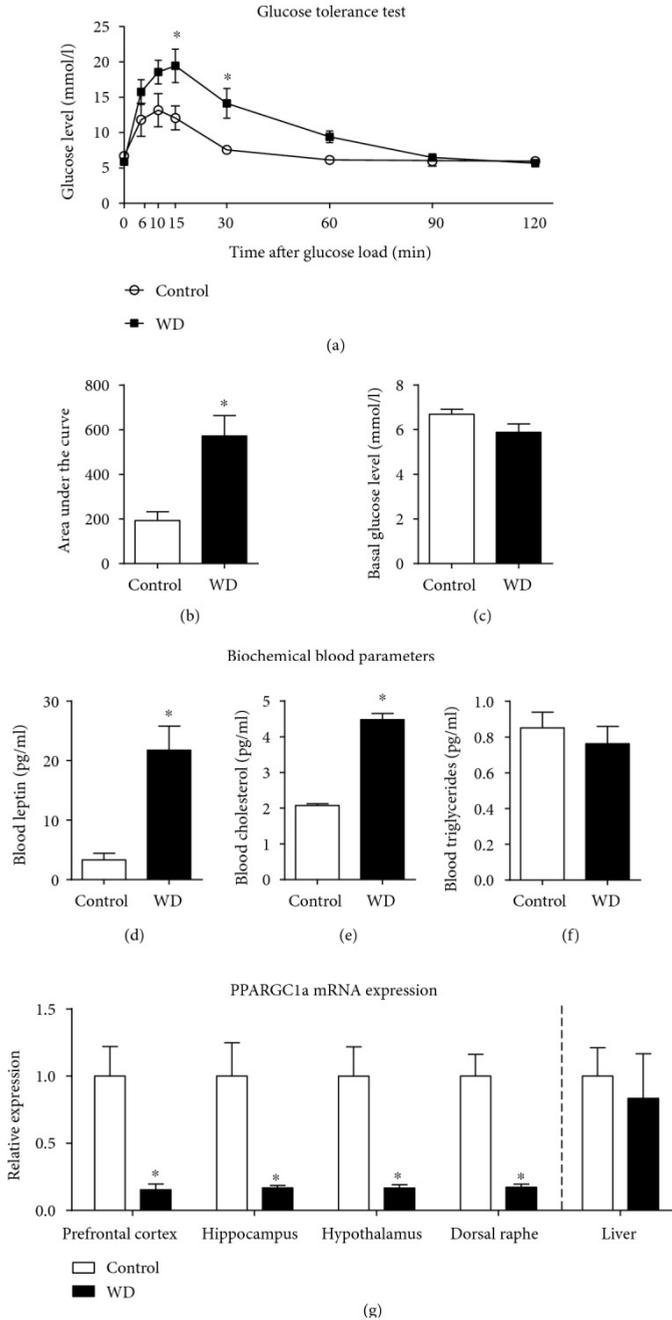


Figure 6. Effects of high-fat/high-cholesterol diet exposure on glucose tolerance, biochemical blood parameters, and PPARGC1a gene expression. (a) In comparison to that in the control, there was an increase in

blood glucose level at 15 and 30 min after glucose load in the glucose tolerance test (* $p < 0.05$ versus that in the control group, 2-way ANOVA and Bonferroni post hoc test). (b) There was a significant increase in the area under curve in glucose concentration in dietary-challenged mice in comparison to controls (* $p < 0.05$ versus control group, t-test). (c) There was no significant difference in basal glucose levels between mice housed on the standard and high-fat/high-cholesterol diets. Mice housed on the high-fat/high-cholesterol diet, as compared to the control group, showed significantly increased (d) blood leptin levels, (e) blood cholesterol levels (* $p < 0.05$ versus that in the control group, t-test), and (f) unaltered blood level of triglycerides ($p > 0.05$ versus that in the control group, t-test). (f) In comparison to control mice, animals fed with the high-fat/high-cholesterol diet had significantly decreased PPARGC1a mRNA in all brain areas (* $p < 0.05$ versus that in the control group, 2-way ANOVA and Bonferroni post hoc test) but not in the liver ($p > 0.05$ versus that in the control group, 2-way ANOVA and Bonferroni post hoc test). Control—standard diet, WD—Western diet. Data are shown as mean \pm SEM.

In comparison with control mice, the dietary-challenged group showed a significant increase in blood glucose levels at the time points 15 and 30 min of the test and a tendency to increase at time point 10 min (10 min: $t = 2.54$, $p = 0.108$; 15 min: $t = 3.48$, $p = 0.007$; 30 min: $t = 3.09$, $p = 0.024$, Bonferroni test), suggesting a decrease in glucose tolerance in the latter group. No significant group differences were found at other time points. The area under a curve calculated for a 2 h period of the afterload measurements of blood glucose levels normalized to the baseline was significantly increased in the high-cholesterol and high-fat diet group as compared to the control ($t = 3.21$, $p = 0.012$, t-test, **Figure 6(b)**). There was no difference in basal glucose level between the groups ($t = 1.67$, $p = 0.137$, t-test; **Figure 6(c)**). Serum levels of leptin and cholesterol were significantly increased in the group

fed with the high-fat/cholesterol diet as compared to control animals ($t = 4.40$, $p = 0.037$ and $t = 13.47$, $p < 0.001$, respectively; t-test; **Figures 6(d)** and **6(e)**); no difference was found in triglyceride level ($t = 0.68$, $p = 0.529$; t-test; **Figure 6(f)**).

Two-way ANOVA showed significant differences between the groups investigated in study 3 in the PPARGC1a mRNA concentrations in the brain ($F = 56.12$, $p < 0.001$), which were independent of the brain area ($F = 0.28$, $p = 0.841$; two-way ANOVA; **Figure 6(g)**). The PPARGC1a mRNA levels were significantly lower in all investigated brain regions in mice fed with the high-cholesterol and fat-containing diet, in comparison to control mice (prefrontal cortex: $t = 4.00$, $p = 0.001$; hippocampus: $t = 3.92$, $p = 0.002$; hypothalamus: $t = 3.93$; $p = 0.002$; and dorsal raphe: $t = 3.13$, $p = 0.015$, Bonferroni test). Hepatic PPARGC1a mRNA levels did not significantly differ between the groups ($t = 0.42$, $p = 0.687$, t-test).

4. Discussion

In this study, we showed, for the first time, that chronic exposure of naïve adult mice in the NAFLD model employed here to a high-cholesterol and fat-containing diet induces substantial changes in their social behaviour, comprising of a reduction in sociability and an increase in a dominant-like behaviour. These changes were accompanied by altered patterns of social behaviours and hyperactivity

in a context of social interactions. Furthermore, dietary challenge with the high-cholesterol and fat-containing diet resulted in a decreased acquisition and enhanced extinction of contextual fear conditioning and slower hippocampus-dependent performance in pellet displacement tube test. Glucose intolerance, elevated plasma leptin and cholesterol levels, and decreased expression of the PPARGC1a gene in the brain but not in the liver paralleled above-described behavioural abnormalities.

The analysis of social interactions both in a home cage and during a food competition test demonstrated that in comparison to control animals, dietary-challenged mice were less sociable, spending less time huddling and longer time “sitting alone” in a home cage, as well as shorter duration of body-body contacts during the food competition model. These data can be interpreted as a sign of social avoidance of both familiar and unfamiliar mice displayed by animals fed with the WD. They also rule out a factor of neophobia that could explain lower social interactions in cases where only the interactions with unfamiliar mouse would be reduced in mice exposed to the WD.

Our results are generally in line with several studies that reported diminished sociability in rodents after exposures to diets containing high amounts of fat/cholesterol; however, previous works largely dealt with their effects on the offspring [3, 15]. In one of the most recent studies, Buffington and colleagues found that the offspring of dams fed

with a high-fat diet containing high amounts of cholesterol displayed markedly dropped number, frequency, and duration of social contacts both with familiar and unfamiliar mouse, as well as impaired long-term potentiation in the ventral tegmental area, a sign of a deficient synaptic plasticity [15]. These deficits were rescued by supplementary oxytocin and a restoration of normal microbiota parameters, which were affected in the offspring of dietary-challenged mice and can potentially mediate reported effects here. Moreover, another study with a high-fat diet containing high amounts of cholesterol showed that it can exacerbate social deficiency and cognitive rigidity in BTBR T+tf/J inbred mouse line, a model of autism. BTBR mice, after housing on a high-fat diet containing high amounts of cholesterol starting at weaning, demonstrated greater deficits in social memory, lowered preference for social novelty, and impaired learning of the T-maze than these mice fed with a regular diet [3].

In the current study, the group fed with the WD revealed heightened scores of crawl-over behaviour in a food competition test and burrowing activity in a home cage, which are generally accounted for the manifestations of a dominant-like behaviour [40, 43]. Burrowing behaviour was shown to correlate with elevated levels of social dominancy and not be displayed by mice with subordinate social traits [43]. Of note, signs of burrowing behaviour were completely absent in the control group in the current study and were expressed only by

dietary-challenged animals. Other studies suggest that burrowing behaviour is associated with repetitive behaviour and impulsivity that was recently reported in a study with C57 female mice fed with a fat and high-cholesterol diet [44].

In many studies with dietary interventions and assessment of social behaviours, a factor of a gain/loss of body weight was suggested to determine the outcome [45]. In the current work, we choose to apply a dietary challenge that is selectively enhanced in a content of cholesterol and also does not induce a change in body weight, as such, excluding a factor of obesity in the induction of negative consequences of dietary cholesterol. Thus, potential confounds related to altered body mass in the evaluation of social and other behaviours could be ruled out.

Our study has also revealed the alternations in the basic patterns of social interactions. An increase in the number of body-body contacts associated with a decrease in their duration can be interpreted as a sign of impulsive behaviour that was previously shown in an employed model under different experimental settings from those used in the present work, which did not include the elements of social interaction unlike the current work [30]. In line with this result and the previous findings, we found increased vertical and horizontal locomotion during both home cage testing and food competition test in Western diet-challenged mice, suggesting their impulsivity which was displayed in a

context of social interactions. Thus, mice challenged with high amounts of dietary cholesterol and fat display hyperlocomotion/impulsivity regardless of whether or not social element presents in their environment.

Similar to our results, a combination of exposure to a high-fat/high-cholesterol diet with food deprivation was recently found to enhance behavioural signs of impulsivity and upregulation of several molecular factors involved in the pathophysiology of impulsivity and addiction [45, 46]. Exposure of rodents to this type of diet has been shown to evoke substantial alternations in dopamine signalling in several brain structures including the prefrontal cortex [46, 47], the nucleus accumbens [47], and the hypothalamus [48]. As these changes have been associated with the mechanisms in social interactions, motivation, and various other behaviours [49–52], they can underlie the above-described behavioural signs of hyperactivity and impulsivity observed in our and previous studies.

Increased rates of dominant behaviours and decreased signs of sociability can be related to deficient cognitive functions in general and hippocampal plasticity in particular [17, 18]. The current study showed that applied exposure to a high-fat/high-cholesterol diet reduces contextual fear conditioning in mice that is in line with the previous findings in this paradigm [21, 53]. Mice housed on a diet enriched with cholesterol were previously found to show increased anxiety [30, 31]

generally known to potentiate fear learning. Yet, animals from this group displayed lowered percentage of “good learners” and a strong tendency to a reduction in freezing behaviour, suggesting deficient contextual learning. Significantly increased extinction of contextual conditioning as shown by diminished duration of freezing behaviour further suggests weaker learning abilities of mice housed on the high-fat/high-cholesterol diet. Other animal studies found altered learning deficits in mice and rats that were housed on a high-cholesterol/high-fat diet and tested in a range of learning and memory tasks including water mazes, variable interval delayed alternation task, object recognition test, and operant bar-pressing task [7–12, 54, 55].

Findings described here in the fear conditioning test were further supported by the data obtained in the pellet displacement tube test. Dietary-challenged mice showed a slower performance of emptying a tube filled with pellets in this test, which is regarded as a sign of reduced hippocampus-dependent functions. Previous studies validated the food pellet displacement test as a paradigm, in which rodent behaviour of object displacement from a tube was found sensitive to the lesions of dorsal hippocampus [33] and accompanies the deficits in the hippocampal plasticity during depressive-like syndrome [34] and in a model of Alzheimer's disease [56]. Pellet displacement in rodents was shown to be suppressed by systemic inflammation [56, 57], a feature of animal models of a high-fat/high-cholesterol diet [6, 9, 26, 30].

As deficient cognition, reduced exploration, aggressive behaviour, attention deficit, depressive-like and anxiety-like changes, neophobia, and impulsivity constitute the elements of autistic behavioural profile [18, 58], behavioural changes in mice fed with the WD resembled proautistic behavioural repertoire.

Behavioural changes found in mice exposed to dietary cholesterol and fat were accompanied by pronounced metabolic changes. We found increased blood concentrations of cholesterol and leptin, while the triglyceride level was unaltered. Dietary-induced hyperleptinemia and hypercholesterolemia were reported to be consistent in high-fat/high-cholesterol models [22, 59, 60], whereas similar to our study, the changes in blood triglyceride levels can vary [22, 59].

The present work has demonstrated delayed restoration of normal glucose levels after glucose load in the WD-challenged mice, suggesting their impaired glucose tolerance and insulin resistance that were not reported before in the NAFLD model employed here. Together with marketable inflammation that was previously demonstrated in an applied model here [30] and that can be a causal factor of insulin resistance [61], this finding led to suggesting altered insulin-mediated signalling in the brain, where insulin resistance in the hippocampus may account for reported behavioural abnormalities here. The use of high-fat/high-cholesterol diets showed that behavioural changes of rodents housed on these diets are accompanied by type 2 diabetes

mellitus [8, 10, 21, 53]. Importantly, heightened occurrence of diabetes and reduced glucose tolerance during autistic spectrum disorder is well established in the literature [62, 63].

Epidemiological and clinical studies provide evidence for the role of metabolic syndrome on the occurrence of dementia and cognitive decline, in particular in the presence of inflammation [64, 65]. Mitochondrial dysfunction was found to be associated with social deficits in young individuals [62, 66, 67] and cognitive impairment in aging people and animals [68, 69]. Clinical and animal studies suggest a common neurobiological basis and interrelationship for aggression, diminished cognitive abilities, behavioural disinhibition, and metabolic abnormalities. Remarkably, pharmacological enhancement of insulin receptor signalling was demonstrated to rescue normal emotional behaviours in an employed paradigm here [31]. Since central and peripheral glucose intolerance is well documented to underlie numerous affective and cognitive abnormalities [70–74], this mechanism is likely to explain described behavioural aberrations here.

Our study revealed a decrease in PPARGC1a, a marker of diminished mitochondrial activity during dietary-induced type 2 diabetes [16, 31, 75, 76], in the brain of mice housed on the high-fat/high-cholesterol diet. This finding is in line with reported reduced glucose tolerance here in the WD group and further supports the view that suppressed mitochondrial functions in the brain may underlie

described behavioural abnormalities here of mice housed on the WD. Interestingly, diminished expression of this gene was also found in the human hippocampus, correlating with clinical progression of dementia in patients with Alzheimer's disorder [27]. Mice genetically lacking the PPARGC1a gene exhibited an imbalance between inhibitory and excitatory synaptic transmission in the hippocampus, a mechanism that is suggested to be an important pathogenetic factor of autism [28, 77]. Thus, cognitive and other behavioural deficits reported in this work on mice fed with the high-fat/high-cholesterol diet can result not only from lowered mitochondrial activity that is associated with decreased PPARGC1a brain levels but also specific roles of this molecule in synaptic plasticity.

Previously, we showed a decreased gene expression in an employed model here of another member of peroxisome proliferator-activated receptor family member, PPARGC1b, both in the brain and in the liver [31], whereas no changes in the hepatic PPARGC1a were found in the present study. These results highlight functional differences between two molecules as suggested earlier [31, 77].

Together, our data are generally consistent with so far obtained results with rodent models suggesting that diets containing high amounts of fat/cholesterol can evoke social deficiencies which resemble the features of autism spectrum disorders. Our results correspond to the current epidemiological findings, demonstrating a link between

obesity and autism [14, 62], and to reports on ameliorated cognitive and behavioural symptoms of autism due to a diet [63]. Thus, the results reported here, along with currently accumulated data considering behavioural abnormalities in the light of proautistic changes, allow speculation that preference for the “Western diet” can be a potential risk factor for this spectrum of diseases in humans. These findings provide evidence for a possible environmental risk factor that can contribute to the production of autistic-like symptoms.

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Conflicts of Interest

All contributing authors have no conflict of interests.

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Supplementary Data

Table 1. The composition of the diets with respect to the content of carbohydrates, saturated / unsaturated fat and protein, w/w

	Standard chow, %	Western diet, %
Total fat	5	21
Saturated fat	1	20
Polyunsaturated and monounsaturated fat	4	1
Carbohydrates: starch and sugars	61	50
Protein	24	19
Fiber	5	5
Cholesterol	0	0.2

Table 2. Sequences of primers used

mRNA target	Primer sequence (5' → 3')	
PPARGC1a	Forward	CTCCAGTTCCGGCTCCTC
	Reverse	CCCTGTGCTCTCACGTCTG
Gapdh	Forward	ACCCCTTCATTGACCTCAACTACATG
	Reverse	CCTTCTCCATGGTGGTGAAGAC

Chapter 3

**Prefrontal cortex
inflammation and liver
pathologies accompany
cognitive and motor deficits
following Western diet
consumption in non-obese
female mice**

Chapter 3. Prefrontal cortex inflammation and liver pathologies accompany cognitive and motor deficits following Western diet consumption in non-obese female mice

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Veniaminova E, Oplatchikova M, Bettendorff L, Kotenkova E, Lysko A, Vasilevskaya E, Kalueff AV, Fedulova L, Umriukhin A, Lesch KP, Anthony DC, Strekalova T. Prefrontal cortex inflammation and liver pathologies accompany cognitive and motor deficits following Western diet consumption in non-obese female mice. Life Sciences. 2020;241:117163.

Abstract

Aims

The high sugar and lipid content of the Western diet (WD) is associated with metabolic dysfunction, non-alcoholic steatohepatitis, and it is an established risk factor for neuropsychiatric disorders. Our previous studies reported negative effects of the WD on rodent emotionality, impulsivity, and sociability in adulthood. Here, we investigated the effect of the WD on motor coordination, novelty recognition, and affective behavior in mice as well as molecular and cellular endpoints in brain and peripheral tissues.

Main methods

Female C57BL/6 J mice were fed the WD for three weeks and were investigated for glucose tolerance, insulin resistance, liver steatosis, and changes in motor coordination, object recognition, and despair behavior in the swim test. Lipids and liver injury markers, including aspartate-transaminase, alanine-transaminase and urea were measured in blood. Serotonin transporter (SERT) expression, the density of Iba1-positive cells and concentration of malondialdehyde were measured in brain.

Key findings

WD-fed mice exhibited impaired glucose tolerance and insulin resistance, a loss of motor coordination, deficits in novel object

exploration and recognition, increased helplessness, dyslipidemia, as well as signs of a non-alcoholic steatohepatitis (NASH)-like syndrome: liver steatosis and increased liver injury markers. Importantly, these changes were accompanied by decreased SERT expression, elevated numbers of microglia cells and malondialdehyde levels in, and restricted to, the prefrontal cortex.

Significance

The WD induces a spectrum of behaviors that are more reminiscent of ADHD and ASD than previously recognized and suggests that, in addition to the impairment of impulsivity and sociability, the consumption of a WD might be expected to exacerbate motor dysfunction that is also known to be associated with adult ADHD and ASD.

Keywords

Western diet, Non-alcoholic steatohepatitis (NASH), Motor coordination, Cognitive deficits, Microglia, Oxidative stress, Prefrontal cortex, Attention Deficit Hyperactivity Disorder (ADHD), Autism Spectrum Disorder (ASD), Mice

1. Introduction

The “Western diet” (WD) is characterized by high intake of saturated fats, cholesterol and refined sugars, and is recognized as a serious risk factor for a number of medical conditions [1,2], including neuropsychiatric disorders [3-5]. The effect of a WD on mood, cognition and associated psychiatric syndromes, including autism spectrum disorder (ASD) [6,7] and attention-deficit/hyperactivity disorder (ADHD) [8], has been most extensively studied during development or in the early postnatally period. However, recent studies, including our own, have revealed that the WD can also induce changes in mood and cognition in adult mice that are reminiscent of the symptoms that are characteristic of ASD and ADHD [9-13].

Mice fed the WD for three weeks exhibit open field hyperactivity, increased impulsivity, anxiety and depression-like behaviors, and altered social interactions [11-14]. These characteristics overlap with the ASD and ADHD syndromes [15-17]. It is of interest to note that ASD and ADHD in adolescents and young adults are frequently comorbid with metabolic syndrome and diabetes [18-20]. The WD has also been shown to modify motor coordination both in humans and in mice given the WD for extended periods [9,21,22]. Attentional control to engage motor/cognitive tasks involves the prefrontal cortex [23], where decreased activity within this part of the brain is argued to underpin ADHD [24] and ASD [25]. We hypothesized that the WD

regime might induce an ADHD/ASD-like syndrome, which would also include deficits in motor coordination, and would be associated with cellular and molecular changes the prefrontal cortex. As a prolonged dietary challenge with a WD induces widespread changes in the brain [26], we employed a short dietary regime in the hope that we might reveal early regional vulnerabilities that could provide insight into the sites that are responsible for the diet-induced behavioral changes.

Female mice display a greater vulnerability to negative effects of the WD [27]. For example, female C57BL6J mice fed the WD for three weeks exhibited a more marked liver steatosis, as shown by increased level of liver triglycerides, as well as increased levels of inflammatory markers in the liver, such as increased expression of CD68, TNF and CCL2, than male mice.

Contrastingly, male mice exhibit greater dyslipidaemia, displaying increased level of triglycerides and decreased level of non-esterified fatty acids in blood and weight gain, which is not observed in female mice. While there is a higher prevalence of males with ASD than females, it would be incorrect to suggest that ASD/ADHD is restricted to males, thus, the use of female mice remains a valid approach. In adulthood, the overall prevalence of adult ADHD is 4.4%, and the figure is, higher for males (5.4%) than for females (3.2%), but a conservative estimate still reveals that 124 million women worldwide live with ADHD. For ASD, the male/female ratio may be as high as

4:1 and discrete mechanisms may exist that confer “resilience” in women, but ASD prevalence in the population is estimated to be between 2.3% and 9.9% [28] and thus the number of women affected is still very high compared to most other pathologies. Hence, there is certainly a need to understand the impact of the WD in females as the number of women by these conditions worldwide is enormous.

In the present experiments, we also investigated the impact of the WD on glucose and insulin tolerance, liver steatosis, serum concentrations of lipids, urea and liver injury markers. Microglial activation in the hippocampus, prefrontal and motor cortex were evaluated. Malondialdehyde concentration, an oxidative stress marker, was measured in prefrontal cortex, hippocampus, hypothalamus and dorsal raphe. Serotonin transporter (SERT) expression, which is decreased by the WD, obesity [29-31], and ADHD [32,33] was also evaluated in the prefrontal cortex and hippocampus.

2. Methods

2.1. Animals

Experiments were performed using 3-month-old female C57BL/6J mice obtained from certified Charles River provider IPAC RAS, Moscow region. Mice were housed 2–3 per cage during the study, under a 12 h light-dark cycle (lights on: 21:00 h) with food and water ad libitum and under controllable laboratory conditions (22 ± 1 °C,

55% humidity). Laboratory housing conditions and experimental procedures were set up and maintained in accordance with the European Communities Council Directive for the care and use of laboratory animals (2010/63/EU) and approved by the local ethics committee (Ethics Committee of Sechenov First MSMU, #11–18).

2.2. Study design and diets

3-month-old female C57BL/6 J mice were fed with a standard laboratory diet (control diet, CD) with an energy content of 3.2 kcal/g and 3.3% of fat (0.6% of saturated fat) (V1534–000, Ssniff Spezialdiäten GmbH, Soest, Germany) or with a diet containing 0.2% cholesterol, 21% of fat (9% of saturated fat), and an energy content of 4.6 kcal/g, ‘Western diet’ (WD), (D12079B, Research Diet Inc., New Brunswick, NJ, USA, Supplementary Table 1) for three weeks as described elsewhere [11–13]. Body weight, and food and water intake were monitored daily during the first 4 days of dietary challenge and each 3rd day thereafter. The experiment was carried out in three runs, 6–7 mice per group were used in each run (**Fig. 1**).

After the three-week dietary challenge, mice from cohort 1 were studied for depressive-like behavior in the two-day swim test (days 21 and 22) and a glucose tolerance test (day 23) and were culled on day 24. Their blood and liver were collected for biochemical analysis and histological assessment. Spleen, kidney, muscle gastrocnemius, heart,

abdominal fat were harvested and weighed; the brain was dissected and the hippocampus and prefrontal cortex were collected for analysis of SERT gene expression. Dietary and control mice from cohort 2 were studied for muscle strength using the inverted screen test and weights test on day 19, in motor tests: rotarod, wire test and pole tests on Days 20–21, followed by a glucose tolerance test on day 22.

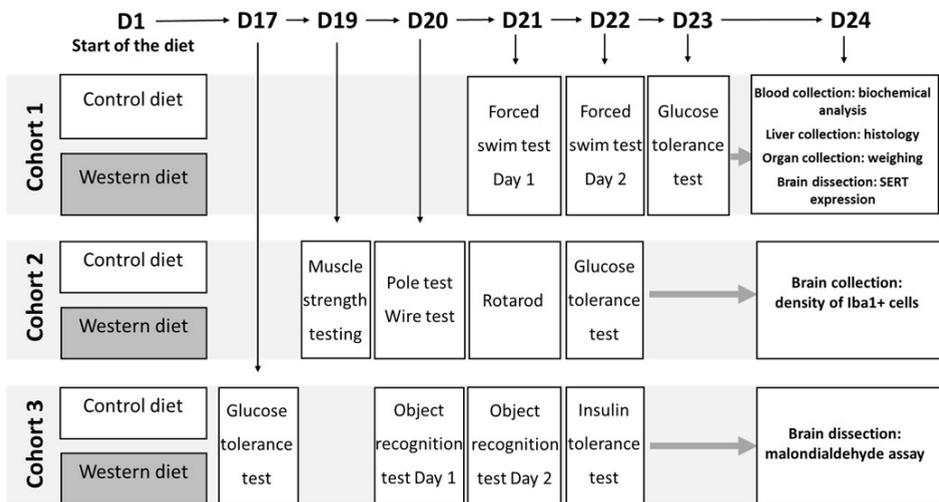


Fig. 1. Experiment design. Three cohorts of mice were fed either control or WD for 3 weeks. Thereafter, mice from cohort 1 were studied in the two-day forced swim test, glucose tolerance test and were culled. Their blood and liver were collected for the biochemical blood analysis and histological assessment of lipid accumulation; spleen, kidney, heart, and abdominal fat were harvested, brain was dissected for SERT expression measurement. Mice from cohort 2 were studied for muscle strength in the Weights test and inverted screen, and in motor tests: rotarod, wire test and pole test, and glucose tolerance test. Thereafter animals were culled and their brains were dissected for Iba1-staining. Mice from cohort 3 were tested for glucose tolerance on day 17, and thereafter for object exploration/recognition, and insulin resistance. Mice were culled and their brains were dissected for the measurement of the malondialdehyde concentration in the prefrontal cortex, hippocampus, dorsal raphe and hypothalamus.

Thereafter, the animals were culled and their brains were collected for Iba1 immunohistochemistry. Mice from cohort 3 were tested for glucose tolerance on day 17, object exploration / recognition, on days 20–21 and insulin tolerance test on day 22.

In order to prevent potential confounds owing to serial behavioral tests in the mice, and to avoid the effects of long interruptions in diet intake during the glucose-tolerance and insulin-tolerance tests, we extended the duration of our battery of behavioral tests to either side of the 21-day-long period of dietary intervention. As such, the onset and the end of testing was extended by 1–2 days. Thus, a small increase in the total duration of the experimental period was necessary to compensate for the periods of food deprivation during the glucose and insulin tolerance tests, in order to comply with the original 21-day long feeding protocol [27]. These adjustments were also employed in our previous studies and were shown to have no effect on the outcomes, as cognitive and emotional aberrations in WD-challenged mice were observed on days 20 and 21 of the dietary challenge [10-13]. Mice were culled and their brains were dissected for the measurement of the concentration of malondialdehyde (MDA), oxidative stress marker, in the prefrontal cortex, hippocampus, dorsal raphe and hypothalamus.

2.3. Behavioral testing

Behavioral tests were carried out during an active period of the animals' light cycle (09:00–21:00) and analyzed offline by the

experimenter who was unaware of diet each animal had received. Behavioral equipment was thoroughly cleaned with water between each test.

2.3.1. Rotarod

Motor coordination was studied in seven consequent trials of non-accelerating rotarod as described elsewhere [34]. As previous experiments revealed the occurrence of motor abnormalities in the mice housed on a WD in the rotarod test at day 19, (Veniaminova, unpublished data; Supplementary Fig. S1), here began testing the mice for abnormal motor functions from day 19. Rotarod set-up consisted of a cylinder with a diameter of 3.5 cm, which rotated at a constant speed (10 rpm). The animals were first trained to stay on the rotating rod for as long as they could over 6 trials. The maximum latency of falling was set at 10 min. If a mouse successfully completed the task it was not subjected to further trials and the latency for all the further trials was set as 10 min. Then all the mice participated in final 7th trial. The learning curve was drawn and the total time that an animal could stay on the rod before falling in the 7th trial was used as a measure of the task acquisition.

2.3.2. Wire test

The wire hanging test is based on the instinct of a mouse to avoid falling. Mice were allowed to grip a horizontally hanging wire (diameter 0.3 cm, height above surface 60 cm) with four limbs [34]. Their ability

to keep a balance on a wire and grip was measured by scoring a latency to fall. For each mouse the best result of two trials was analyzed.

2.3.3. Pole test

Mice were placed on top of a vertically standing bar (diameter 1.1 cm, height 60 cm) and allowed to climb down to a horizontal surface [34]. The latency to reach the ground with all four paws, events of jumping, sliding and falling were scored. For each mouse the best result of the two trials was analyzed.

2.3.4. Weights test

The 'Weights' test was used to assess muscular strength and was performed as described elsewhere [35]. Each weight consisted of a ball of tangled fine gauge stainless steel wire (7 g) attached to a series of steel chain links, each weighing 13 g. The number of links ranged from one to seven. The mouse was held by the base of the tail and allowed to grasp the first weight (20 g) which was lying on the laboratory bench. As it grasped the wire scale collector with its forepaws it was raised until the link was clear of the bench. A hold of three seconds was used as a criterion. If the mouse dropped the weight in <3 s, the time it held the weight was noted. The mouse was given three chances to hold the weight for 3 s. If it failed three times the mouse was assigned the maximum time/weight achieved. If it held the weight for 3 s then the next heaviest weight was tried. A final total score was calculated as the product of the number of links in the heaviest chain

held for the full 3 s, multiplied by the time (sec) it is held. If the heaviest weight was dropped before 3 s an appropriate intermediate value was calculated.

2.3.5. Inverted screen test

Inverted screen test was used to test muscle strength according to published protocols [35,36]. A 43 cm square of wire mesh consisting of 12 mm squares of 1 mm diameter wire was surrounded by a 4 cm deep wooden beading (which prevented the mouse from climbing on to the other side). The mouse was placed in the center of the wire mesh screen, the screen was rotated to an inverted position over 2 s, with the mouse's head declining first and hold steadily 50 cm above a padded surface. Latency to fall off was used as a measure of muscle strength. 180 s was set as the maximum latency.

2.3.6. Object exploration/recognition test

Mice were studied for new object exploration / recognition in a 2-day test, as described elsewhere [37]. The apparatus for the object exploration / recognition test consisted of a plastic cage (21×27×14 cm) with opaque walls and two objects (“brush” and “flower”, 7×4×3 cm, attached to 15 ml tube, Supplementary Fig.S2) placed symmetrically 2 cm away from the cage wall in the opposite corners of the cage. The lightning of 5 lx intensity was used. Objects used in the new object recognition test were either disposable and were new for each mouse (flowers were made from paper) or changeable and washable with water

and mild detergent (plastic brush), so that any contamination (the smell of a preceding mouse) was virtually excluded. This methodology has been extensively validated in previous studies and has been successfully employed in different paradigms [37,38].

On the day 1 of the test, two identical objects were used, and a mouse was placed in the cage at an equal distance from the objects and allowed to explore the area freely for 15 min. On the day 2, one object was replaced by the new object and mouse was placed in the cage for 15 min. The duration of object exploration, which was defined as the mouse's nose being directed towards the object while it is situated at a distance <2 cm from the object, was scored off-line for each object and both objects combined. A percentage of time of exploration for the “new object”, that has replaced the “familiar object” from Day 1 to Day 2, over the total duration of exploration was compared against 50%-chance level of approaching either object and was taken as a parameter of object recognition memory.

2.3.7. Swim test

The test was performed as described elsewhere [11,12,39]. Mice were placed into a plastic transparent cylinder (\emptyset 17 cm) filled with water (+23 °C, water height 13 cm, cylinder height 20 cm) under the red lightning for 6 min. The test was repeated with a 24 h interval. Duration of floating behavior, a sign of “despair” and helplessness, defined by the absence of any directed movements of animals' head

and body, was scored offline for 2-min intervals and for the whole test period. Latency to float was evaluated as well. Additionally, the total duration of climbing behavior, defined as quick movements of the forelimbs causing the front paws break the surface of the water near the cylinder wall, and active swimming, defined as movement of forelimbs or hind limbs in a paddling fashion, were scored. Behavioral protocol for the swim test was previously validated using imipramine [39-41].

2.4. Glucose tolerance test

Oral glucose tolerance test (OGTT) was performed according to a published protocol [13]. We sought to match, as closely as possible, the 21-day dietary exposure regime in accordance with the original description of the model [27]. The glucose tolerance and insulin tolerance tests require prolonged food deprivation, and, in order to avoid any confounding effects of the diet deprivation on the metabolic and molecular parameters, the duration of the study was increased so that actual dietary exposure would be 21–22 days for each cohort (see Supplementary file). The test mice were fasted overnight for 18 h, beginning at 1600. Thereafter, a glucose solution (2 g/kg, 1.8 g/l) was delivered by oral gavage and blood was sampled from the tail vein. Samples were obtained prior to glucose administration at time point 0 and 5, 15, 30, 60, 90 min afterwards. The level of blood glucose was

analyzed using the OneTouch UltraEasy glucometer and strips (LifeScan OneTouch, Dubai, UAE). Blood glucose concentrations and the area under a curve (AUC) for the values normalized to baseline glucose levels, were analyzed.

2.5. Insulin tolerance test

Insulin tolerance test (ITT) was performed as previously described [42]. Before the test, mice underwent a 5 h morning fast that began at 1030. Thereafter, a human insulin solution (0.25 IU/kg, diluted in saline) was injected intraperitoneally and blood was sampled from the tail vein. Samples were obtained before insulin injection at time point 0 and 15, 30, 45 and 60 min afterwards. The level of blood glucose was analyzed using the OneTouch UltraEasy glucometer and strips (LifeScan OneTouch, Dubai, UAE). If, during this test, blood glucose levels dropped to below 20 mg/dl, 1 g/kg glucose was administered to counteract the effects of insulin, in order to reduce animal suffering). Blood glucose concentrations and the area under a curve (AUC) for the values normalized to baseline glucose levels, were analyzed.

2.6. Culling, blood and tissue collection

Mice were terminally anaesthetized with isoflurane inhalation for a subsequent material collection. Blood was collected by cardiac puncture from mice from cohort 1 into ethylenediaminetetraacetic acid

(EDTA)-coated tubes and centrifuged at 2260 g for 8 min at 4 °C. Plasma was collected and stored at -80 °C until use for biochemical analysis. Mice were then transcardially perfused with 10 ml ice-cold saline, internal organs (liver, spleen, heart, left kidney, right kidney) and visceral fat were isolated and weighted. Relative weight was calculated as organ weight divided by body weight. The liver left medial lobe was immediately used for Oil Red O staining. Brain was dissected and prefrontal cortex and hippocampus were isolated and stored at -80 °C until use for gene expression analysis. Mice from cohort 2 were perfused with 10 ml ice-cold saline followed by 4% paraformaldehyde via left ventricle, brains were removed, post-fixed in PFA for 12 h and cryoprotected in 30% sucrose for 12 h [43,44] and then embedded in a mold filled with OCT compound and snap-frozen in dry ice-cooled isopentane. Samples were stored at -30 °C until use for immunohistochemical analysis. The right gastrocnemius muscles were isolated and weighted. Mice from cohort 3 were subjected after anesthesia to a perfusion of the left ventricle with 10 ml ice-cold saline, their brains were isolated, prefrontal cortex, hippocampus, hypothalamus and dorsal raphe were dissected and stored at -80 °C until use.

2.7. Blood biochemical analysis

Plasma concentrations of total cholesterol, triglyceride, low density lipoprotein (LDL) and high-density lipoprotein (HDL), glucose,

aspartate transaminase (AST), alanine transaminase (ALT) and urea were measured using the semiautomatic analyzer BioChem SA and commercial kits (High Technology Inc., Walpole, MA, USA) according to the manufacturers' instructions. Remnant cholesterol level was calculated as the difference between total cholesterol, LDL and HDL cholesterol.

2.8. Liver Oil Red O staining

Oil Red O staining of the liver was performed according to a published protocol [45]. 10- μ m-thick sections of fresh frozen liver tissue were cut on a cryostat microtome Microm HM-525 (Carl Zeiss, Oberkochen, Germany). Sections were rinsed with 60% isopropanol, incubated in Oil Red O solution (Sigma-Aldrich) for 10 min and again rinsed with 60% isopropanol. Slides were coverslipped using an aqueous mounting medium (Leika, Wetzlar, Germany). Staining was examined with a light microscope AxioImaiger A1 (Carl Zeiss) using image analysis system AxioVision 4.7.1.0 (Carl Zeiss) at 20 \times magnification. Two to four images per section and two liver sections per animal were analyzed for staining density using the ImageJ software. The density of Oil Red O staining was evaluated in WD-group in percent from mean values of controls and was used as a measure of liver steatosis.

2.9. RNA extraction and quantitative reverse transcription PCR

Total mRNA was extracted by using TRI Reagent (MRC, Cincinnati, OH, USA) according to the manufacturer instructions. Quantitative reverse transcription PCR (RT-qPCR) was carried out as described elsewhere [13,46]. Briefly, 1 µg total RNA was converted into cDNA using random primers and Superscript III transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed using the SYBR Green master mix (Bio-Rad Laboratories, Philadelphia, PA, USA) and the CFX96 Deep Well Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The sequences of the primers can be found in Supplementary Table 2. SERT expression was normalized to reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and calculated as relative-fold changes compared to control mice as described elsewhere [13,46].

2.10. Immunohistochemical analysis of Iba1-positive cells in the brain

Immunostaining with Iba1-antibody and the evaluation of the density of Iba1-positive cells in the prefrontal cortex, hippocampus and dorsal raphe were performed as described elsewhere [46]. Sagittal 10 µm-thick sections were cut on a cryostat microtome (Leica Biosystems, Wetzlar, Germany) and mounted on gelatin-coated slides. Slides were washed in PBS and blocked for non-specific protein binding

with 10% goat serum in PBS for 1 h. Then, sections were incubated in primary antibody (anti-Iba1, 1:2000, ab178846, AbCam, Cambridge, UK) in 1% normal goat serum at 4 °C for 12 h. Visualization was performed using biotinylated secondary antibodies in 1% serum in PBS (Vector Laboratories, Peterborough UK) for 1 h at room temperature and a standard avidin–biotin amplification step (Vector Laboratories) followed by development in diaminobenzidine hydrochloride (DAB, 0.5 µg/ml) in 0.1 M phosphate buffer. Immunostaining was examined using a light microscope Leitz Dialux 20 (Leica, Wetzlar, Germany) and digital camera Basler ACE (Basler Group, Ahrensburg, Germany). Microvisioneer slide scanning software (GT Vision, UK) was used to visualize brain areas at 20× magnification. The area of prefrontal cortex was specifically delineated according to the Paxinos and Franklin's the Mouse brain in stereotaxic coordinates (lateral 0.12–0.36, Supplementary Fig.S3A), and Iba1-positive cells were counted within this. The hippocampus (lateral 0.60–1.20) and motor cortex (lateral 0.60–1.20) areas, which appeared on the same slides were also examined (Supplementary Figs. S3B,C). Cell counting was carried out using ImageJ software. Three sections per each structure per animal were analyzed. The density of Iba1-positive cells was calculated as number of Iba1-positive cells divided by examined area.

2.11. Malondialdehyde assay

Concentrations of MDA were measured following Abcam ab118970 kit instructions (Abcam, Eugene, OR, USA). Briefly, the tissue was washed in cold PBS and homogenized in lysis solution, centrifuged at 13000 g for 10 min. TBA reagent was added to a supernatant and incubated at 95 °C for 60 min; the supernatant was analyzed at 532 nm in a 96-well microplate as described elsewhere [44].

2.12. Statistics

Data were analyzed using GraphPad Prism version 8.01 (San Diego, CA, USA). Repeated measurements were analyzed using a two-way ANOVA for repeated measures with Sidak's multiple comparisons test. For data with a normal distribution, unpaired t-tests were used, and where normality was not observed a Mann-Whitney test was employed. The level of significance was set at $P < .05$. Data with a normal distribution were presented as Mean \pm SEM, and data that were not normally distributed were presented as Median or Median with interquartile range.

3. Results

3.1. Physiological and metabolic effects of the Western diet

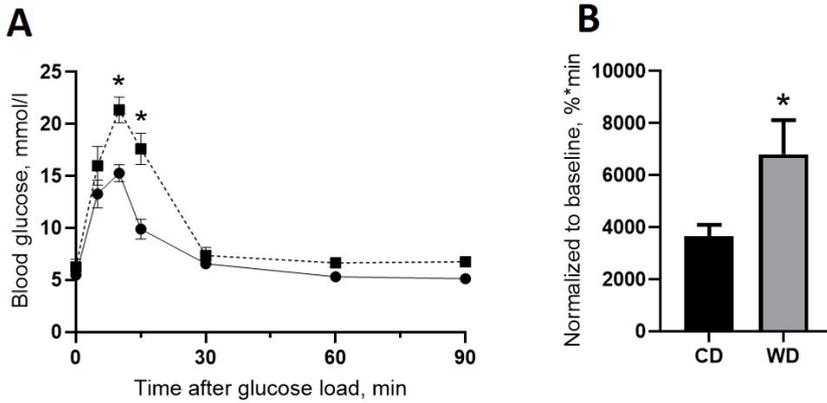
The body weight of mice housed on WD did not differ from control group at any time point of the study across all cohorts

(Supplementary Figs. S4AC), which is consistent with our previous findings [11-13]. No differences were found in the weight of the liver, kidneys, spleen, heart or visceral fat relative to total body mass (Supplementary Figs. S4DI). Daily intake of calories and water intake, normalized to body, weight did not differ between the groups (Supplementary Figs. S4JK). These data rule out uneven weight gain as a potential confound in the subsequent behavioral and molecular studies, which might have been expected to be a consequence of such a highly calorific diet.

In animals fed the WD for 21 days, a two-way ANOVA for repeated measures revealed a significant interaction between diet and the clearance of glucose from the blood over time in OGTT ($F = 5.245$, $P = .0002$; **Fig. 2A**). Compared to control mice, the WD-fed group showed a significant increase in blood glucose levels at 10 and 15 min after the glucose bolus ($t = 4.131$, $P = .0179$ and $t = 4.313$, $P = .0144$, respectively, Sidak's multiple comparisons test). The AUC was significantly increased in the WD-fed group compared to the control group ($t = 3.605$, $P = .0082$, Unpaired t-test, **Fig. 2B**). In another cohort, glucose tolerance was also decreased on day 23 (Supplementary Fig. S5A). At baseline, blood glucose concentration did not differ between the groups that were subjected to 18 h of fasting prior to the OGTT (Supplementary Fig. S5B). These findings are also consistent with our previously reported results [12]. After 16 days of diet feeding,

no difference in glucose tolerance was found between the groups (Supplementary Fig. S5C), supporting the importance of the 21-day feeding regime.

Glucose tolerance test D22



Insulin tolerance test D22

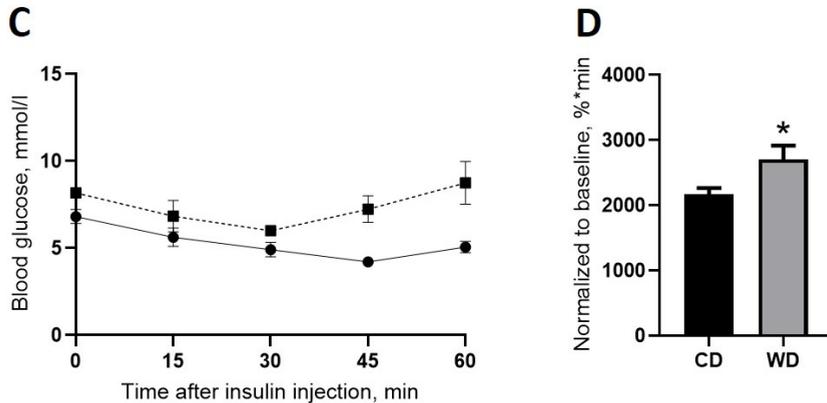


Fig. 2. Effects of housing on the Western diet on glucose tolerance and insulin resistance. Mice fed the Western diet for 3 weeks displayed (A-B) impaired glucose tolerance and (C-D) insulin tolerance. CD – Control diet, WD – Western diet, AUC – area under the curve. * - $P < .05$ compared to CD (Unpaired t-test or Sidak's multiple comparisons test). Data are presented as Mean \pm SEM.

A two-way ANOVA for repeated measures revealed a significant interaction between diet and time after insulin injection in the glucose level measured in the insulin tolerance test on day 22 ($F = 5.802$, $P = .0010$, **Fig. 2C**). The AUC for insulin tolerance was significantly increased in mice fed with the WD compared to controls ($t = 2.677$, $P = .0363$, Unpaired t-test, **Fig. 2D**). At baseline, blood glucose concentration was increased in the animals fed WD compared to controls when animals were subjected to 5 h of fasting prior to the insulin tolerance test (Supplementary Fig. S5D).

3.2. Dyslipidemia, hypercholesterolemia and increased glucose blood levels in mice fed with the Western diet

Compared to controls, in unfasted animals, basal blood glucose levels were significantly increased in the WD group ($t = 4.484$, $P = .0019$, Unpaired t-test, **Fig. 3A**). Basal (dark cycle) blood levels of total cholesterol, LDL, HDL and remnant cholesterol were increased in the mice fed the WD compared to control animals ($t = 10.65$, $P < .0001$, $t = 7.656$, $P < .0001$; $t = 11.01$, $P < .0001$ and $t = 3.314$, $P = .0109$, respectively, Unpaired t-test, **Fig. 3B–E**). There was no significant difference between the groups for the blood concentration of triglyceride ($t = 1.196$, $P = .2630$, Unpaired t-test, **Fig. 3F**).

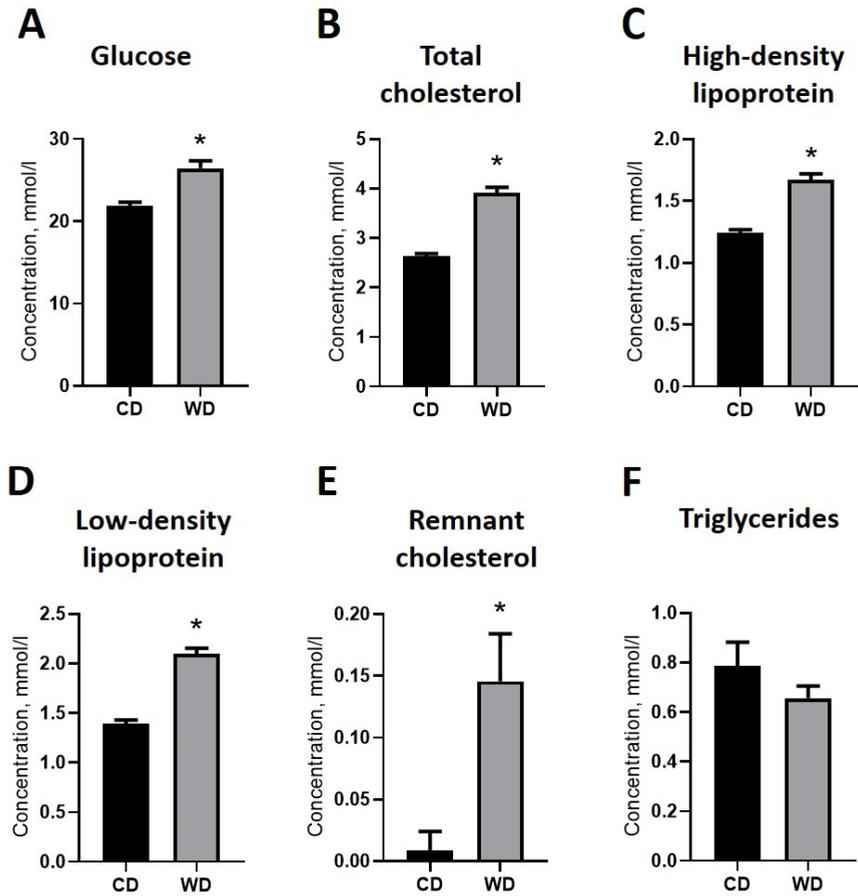


Fig. 3. The Western diet induces hypercholesterinemia and hyperglycemia. Blood concentrations of (A) non-fasting glucose, (B) total cholesterol, (C) low-density lipoproteins, (D) high-density lipoproteins and (E) remnant cholesterol were increased in mice fed a WD for 3 weeks compared to controls. (F) No difference was found in blood triglyceride concentration. CD – Control diet, WD – Western diet * - $P < .05$ compared to CD (Unpaired t-test). Data are presented as Mean \pm SEM.

3.3. A NASH-like syndrome induced by the Western diet

The group receiving the WD displayed marked liver steatosis (**Fig. 4A**); the density of Oil Red O staining, which reveals liver triglycerides and lipids, was significantly increased in this group compared to controls ($t = 26.57$, $P < .0001$, Unpaired t-test, **Fig. 4B**). Significant increases in the blood concentration of ALT and AST, markers of hepatocellular injury, in WD-fed animals compared to control mice ($t = 2.517$, $P = .0362$ and $t = 2.793$, $P = .0213$, Unpaired t-test, **Fig. 4C–D**), which suggest the development of a NASH-like syndrome. No significant difference was found in blood urea concentration between the groups ($t = 1.254$, $P = .2430$, Unpaired t-test, **Fig. 4E**).

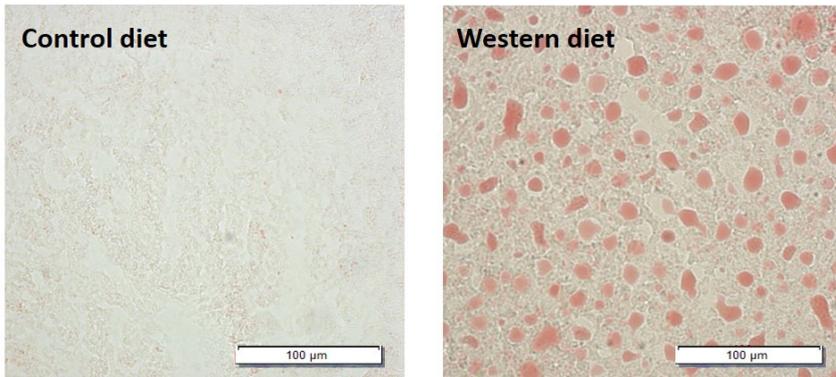
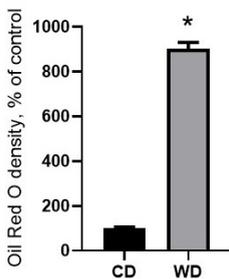
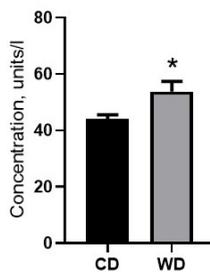
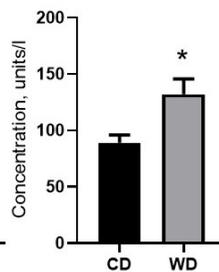
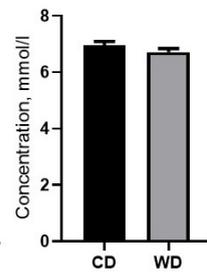
A**B****Liver
Oil Red O****C****Alanine
transaminase****D****Aspartate
transaminase****E****Urea**

Fig. 4. NASH-like syndrome in mice fed with the Western diet. (A-B) In comparison to controls, mice fed a WD displayed marked liver steatosis: density of Oil Red O staining was increased in WD-fed mice compared controls. Compared to controls, there was a significant increase of blood concentrations of markers of hepatocellular injury (C) aspartate transaminase and (D) alanine transaminase in mice fed a WD. (E) No difference between the groups was found in urea blood concentration. CD – Control diet, WD – Western diet. * - $P < .05$ compared to CD (Unpaired t-test). Data are presented as Mean \pm SEM.

3.4. Decreased serotonin transporter expression, microglia activation and elevated marker of oxidative stress malondialdehyde in the prefrontal cortex of mice fed the Western diet

Levels of SERT expression were significantly decreased in the WD-fed group in the prefrontal cortex ($t = 1.471$, $P < .0001$, Unpaired t-test, **Fig. 5A**), but not in the hippocampus ($t = 0.1133$, $P = .9119$, Unpaired t-test, **Fig. 5B**) compared to controls. In mice fed the WD, the number of Iba1-positive cells was significantly increased in prefrontal cortex ($t = 2.670$, $P = .0234$, Unpaired t-test), but not in the hippocampus ($t = 0.1089$, $P = .9173$, Unpaired t-test) or in the motor cortex ($t = 0.3374$, $P = .7414$, Unpaired t-test, **Fig. 5C–F**). The malondialdehyde concentration was significantly increased in animals fed the WD compared to mice fed control diet in the prefrontal cortex ($t = 2.515$, $P = .0456$, Unpaired t-test, **Fig. 5G**), but level was unchanged in all the other sites in which it was measured (**Fig. 5H–J**).

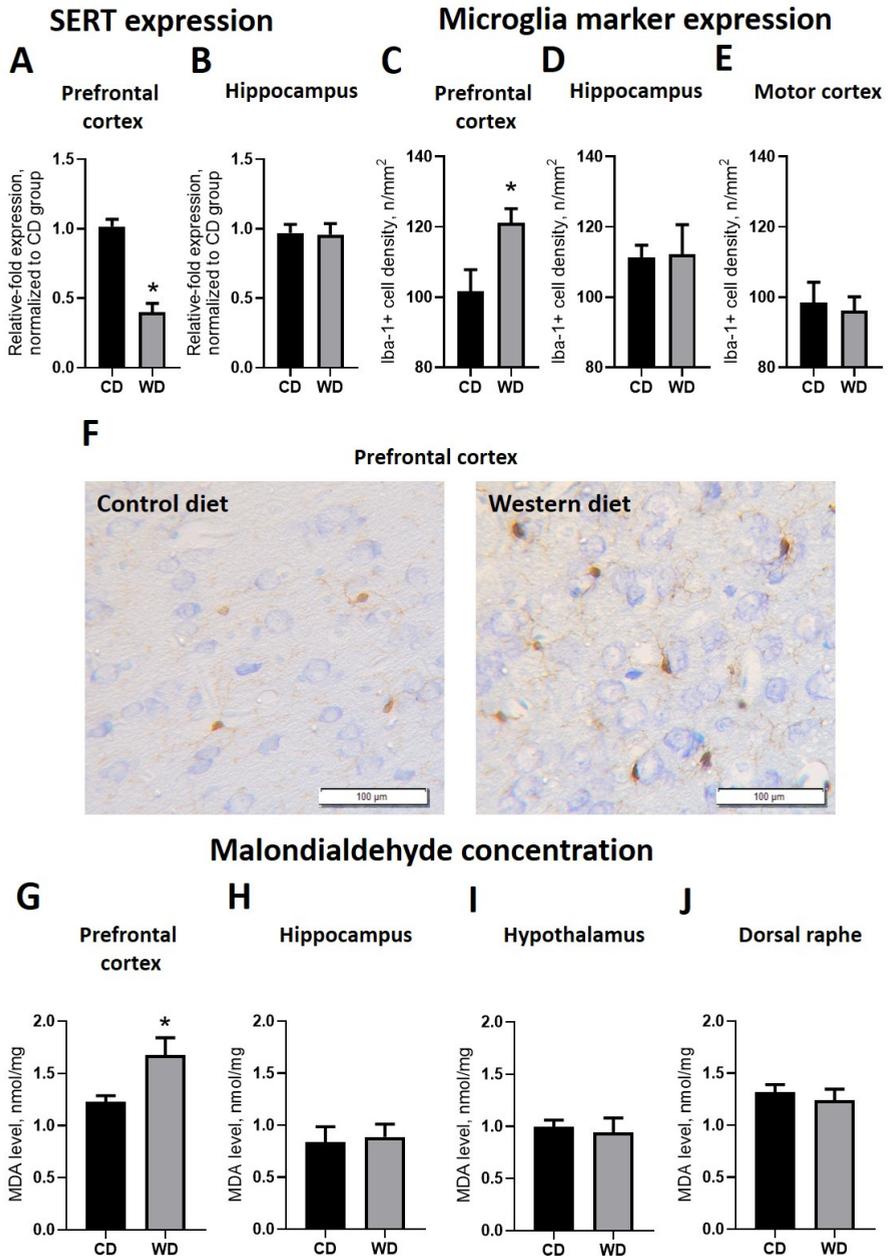


Fig. 5. The Western diet induces decreased serotonin transporter expression, signs of microglia activation and oxidative stress in prefrontal cortex. (A-B) Serotonin transporter expression in prefrontal cortex

and in hippocampus. (C) The density of Iba1-positive cells in prefrontal cortex, (D) the hippocampus, and (E) motor cortex. (F) Representative staining of microglia in the prefrontal cortex of mice fed control or WD (magnification x 40). (G) Malondialdehyde concentration in the prefrontal cortex, (H) the hippocampus, (I) hypothalamus and (J) dorsal raphe. CD – Control diet, WD – Western diet. * - $P < .05$ compared to CD (Unpaired t-test). Data are presented as Mean \pm SEM.

3.5. The Western diet causes impaired motor performance and exploration, and behavioral despair

In the last trial of the rotarod test, the latency to fall was significantly decreased in the WD-fed group compared to control mice ($U = 5$, $P = .0346$, Mann-Whitney test, **Fig. 6A&B**). In the wire test, mice fed the WD demonstrated decreased latency to fall compared to controls ($U = 3$, $P = .0152$, Mann-Whitney test, **Fig. 6C**). Data from the two separate motor tests suggest that the WD-fed mice have impaired motor function.

In the pole test, which is a short-test requiring muscle strength and less coordination, there was no difference between the groups ($U = 17$, $P = .9416$, Mann-Whitney test, **Fig. 6D**). There was also no significant difference between the groups in gastrocnemius muscle weight ($t = 0.3773$, $P = .7174$, Unpaired t-test, **Fig. 6E**) or the performance in ‘Weights test’ of muscle strength ($t = 0.2758$, $P = .7890$, Unpaired t-test, **Fig. 6F**).

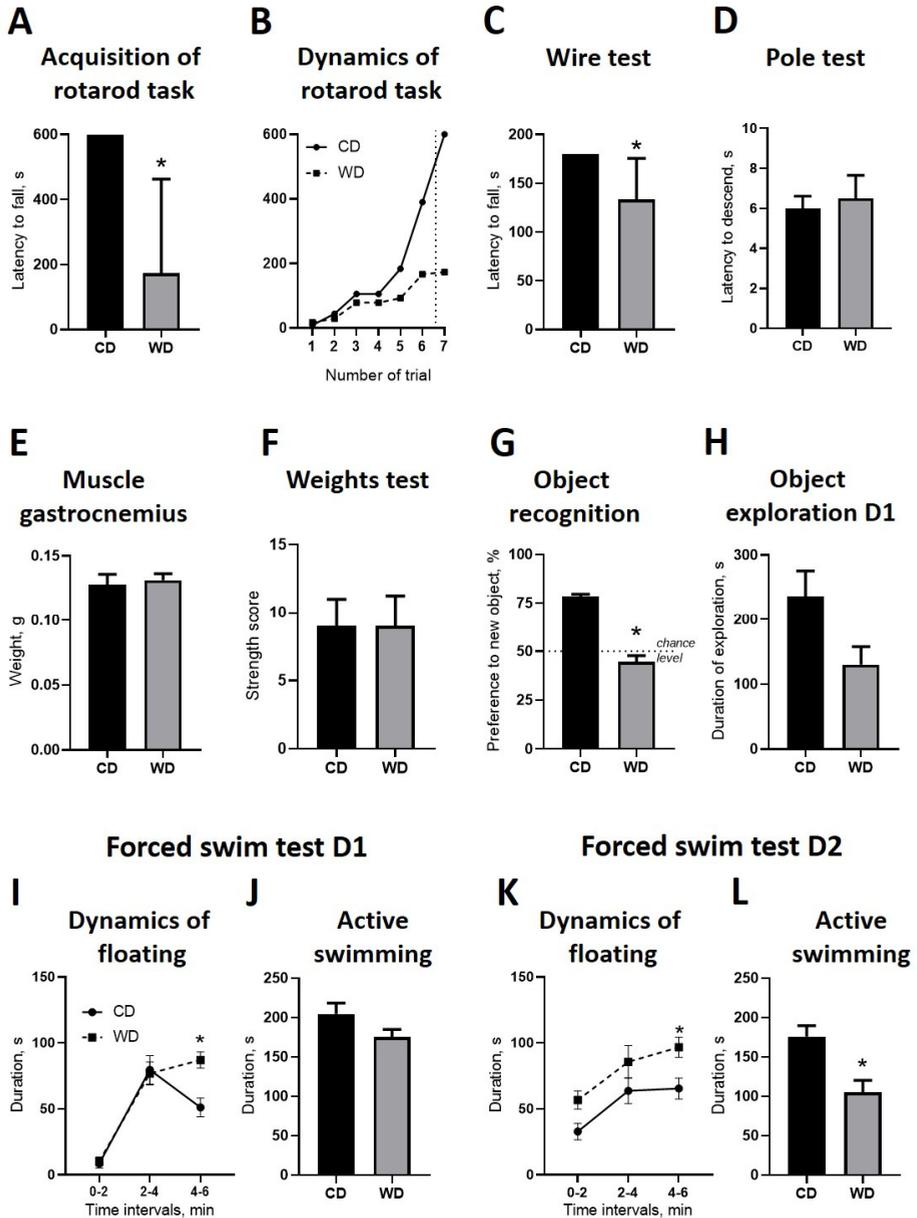


Fig. 6. Impaired motor performance, object recognition and behavioral despair in the forced swim test of mice fed with the Western diet. (A) The latency to fall in the last trial of the rotarod. Compared to controls, mice fed with the WD resulted in a significantly decreased. (B) The latency to fall during the repeated rotarod testing period. Control mice demonstrated an

increased compared to the WD-fed animals. (C) Latency to fall in the wire test. Note the latency was significantly decreased in mice housed on a WD compared to control animals. (D) Latency to fall in the pole test. (E) Gastrocnemius muscle weight. (F) Muscle strength measured in the Weights test. (G) Object exploration/recognition test. The animals fed with the WD demonstrated significantly decreased preference to explore the novel object compared to control group. (H) Time spent with the objects on Day 1: there were no significant differences between groups. (I) Forced swim test. On Day 1 of the, the WD diet-fed group showed significantly increased duration of floating during 4–6 min of the test and (J) unaltered active swimming compared to mice fed control diet. (K) Day 2 of the forced swim test results. The mice fed the WD displayed significantly increased duration of floating during 4–6 min of testing and (L) decreased duration of active swimming compared to controls. CD – Control diet, WD – Western diet. * - $P < .05$ compared to CD (Unpaired t-test or Sidak's multiple comparisons test). Data are presented as Median with interquartile range (A,C,D), Median (B) or Mean \pm SEM (E-L).

There were no differences between the groups in the latency to fall from the inverted screen (Supplementary Fig. S6A). These tests argue against the possibility of muscle dystrophy in the WD-fed group as a contributor to the outcomes.

The preference to explore the novel object was decreased in mice housed on WD compared to mice housed on control diet ($t = 2.219$, $P = .05$, Unpaired t-test, **Fig. 6G**, Supplementary Fig. S6B). These data suggest reduced novelty exploration in the WD-fed animals, which serves to demonstrate deficits in cognition in these mice. There was some evidence, from day 1 of the trial, that the animals fed the WD diet were less exploratory in the object training session, but, though close, the differences in time spent with the objects were not significant between groups ($t = 2.219$, $P = .0609$, Unpaired t-test, **Fig. 6H**).

In the swim test, on day 1, two-way ANOVA for repeated measures revealed a significant interaction between test time interval and dietary group on floating duration ($F = 5.123$, $P = .0140$). WD-fed mice displayed a significantly increased duration of floating during 4–6 min of the test compared to controls ($t = 3.831$, $P = .0075$, Sidak's multiple comparisons test), but not at other time intervals (**Fig. 6I**). The latency to float, total duration of floating, climbing behaviors and active swimming did not differ between the groups (Supplementary Fig. S7A–C, **Fig. 6J**).

In the swim test, on day 2, two-way ANOVA for repeated measures revealed significant effect of the diet ($F = 8.550$, $P = .0127$) on the duration of floating. WD-fed mice displayed a significant increase in the duration of floating during 4–6 min of the test compared to controls ($t = 2.844$, $P = .0438$, Sidak's multiple comparisons test, **Fig. 6K**), no significant differences were found for other intervals. Total duration of floating was increased in mice fed the WD (Supplementary Fig. S6E) compared to controls. The latency to float and duration of climbing behavior on day 2 did not differ between the groups (Supplementary Fig. S6D,F). The duration of active swimming on day 2 was increased in WD-fed mice compared to mice fed control ($t = 3.376$, $P = .0056$, Unpaired t-test, **Fig. 6L**).

Separate studies have revealed unaltered open field horizontal locomotion of WD-exposed mice, under stress-free low light conditions

(Supplementary Fig. S7). This observation argues against the possibility that the changes in the swim test, novelty exploration and memorizing, as well as altered floating behavior could be ascribed to general locomotor disturbances. Moreover, previous experiments revealed a lack of differences in the open field test activity in control and dietary challenged mice under conditions of stress-free subtle lighting [11]. In these studies, the dietary-challenged mice, which displayed unaltered speed and distance moved in the open field, had increased scores for floating in the swim test and immobilization in the tail suspension test, and spent a shorter time in the open arms of elevated O-maze and lit box of the dark/light box [11].

4. Discussion

The results presented here indicate that consumption of the WD changes gene expression and the cellularity of the prefrontal cortex, which is known to be involved in inhibitory behavioral control [47,48]. The three-week long period of WD feeding impaired motor coordination in the rotarod and wire test, in the absence of changes to muscle weight, strength, or body weight. Thus, the motor deficits were unlikely to be of neuro-muscular origin, and were most likely to be related to altered impulse control, and cognitive and emotional disturbances. The WD-fed mice exhibited down-regulated SERT expression, signs of microglial activation and oxidative stress, all in the

prefrontal cortex, but not in the other structures of the brain that were investigated here. Impaired glucose and insulin tolerance, increased liver injury markers (AST and ALT), liver steatosis, and signs of dyslipidemia provided evidence for the development of metabolic and NASH syndromes in the WD-fed mice. However, unaltered blood urea levels suggest compensated liver function, ruling out a possibility of that the negative behavioral effects of the WD are simply due to encephalopathy-related mechanisms.

In our previous studies, mice fed the WD for 21-days were found to display behavioral abnormalities that were reminiscent of certain key ADHD-like symptoms: impulsivity [11], hyperactivity [13], autistic-like traits in social interaction tests [13], and emotional disturbances in classic tests for anxiety and behavioral despair [12]. These data, together with the present findings, show that the mice fed the WD for 3 weeks can generate motor deficits and signs of compromised function of the prefrontal cortex further supports the argument that the mouse behaviors resemble those of the ADHD and ASD. It has been extensively reported that ADHD and ASD are characterized in humans by decreased activation in the medial prefrontal cortex [25,49], where suppressed integrative functionality is thought to underpin many of the behaviors. In our experiments, a number of the changes, such as a reduction in the SERT expression, the appearance of oxidative stress markers and microglial activation,

occurred in in the prefrontal cortex alone, but the explanation for why this part of the brain should be sensitive to the WD remains unclear. An increase in the susceptibility of prefrontal cortex to the WD was also highlighted in a previous study where we found increased protein expression of Toll-like receptor 4 (TLR4), a major regulator of pro-inflammatory response and microglial activation, in the prefrontal cortex but not elsewhere [11]. It is a part of the brain region that is most sensitive to the effects of stress exposure. Even mild acute uncontrollable stress can cause a rapid and dramatic loss of prefrontal cognitive abilities, and more prolonged stress exposure causes architectural changes in prefrontal dendrites. Others have shown that there is an interaction between stress and diet on the structure of the prefrontal cortex [50], but the relationship is complex and it remains unclear why regional differences exist.

Decreased SERT expression, observed in the brain of the WD-fed animals, has previously been reported to be associated with depressive symptoms [51], decreased sociability, impaired glucose tolerance [52,53], neuroinflammation [54] and the mechanisms underpinning ASD [55,56] and ADHD [32,57]. Thus, a downregulation of SERT in the prefrontal cortex might also contribute to the deficits reported in the present study. Other animal studies have also reported changes in the expression of SERT mRNA/protein in peripheral organs in rodents fed with the WD [29]. Human studies describe reduced SERT expression

in a brain and periphery of subjects with metabolic syndrome and obesity [30,31] and suggest a role of SERT polymorphism, regulating its activity, in controlling body weight [58,59], development of diabetes type II [60] and eating behavior [61].

The present literature also reports a negative relationship between SERT expression and neuroinflammation [54,62], which appears to be a key feature in mice fed a WD. Remarkably, the present study revealed increases of Iba1-positive cells and the concentration of MDA, a marker of oxidative stress, which is suggestive of pro-inflammatory processes [63]. The increase in Iba1-positive cells in the prefrontal cortex of WD-fed animals is in keeping with previous observations in rat and mouse models of obesity, after prolonged feeding, that have reported increases in Iba1-positive cell in the hippocampus [64,65], prefrontal cortex [66] and hypothalamus [67,68]. The increase in the oxidative stress marker (MDA) is also in keeping with the evidence for similar changes in a periphery in obese humans [69-71]. Juvenile mice fed a WD for 4 weeks displayed increased protein oxidation and lipid peroxidation in the frontal cortex [72]. Most animal studies that have described elevated brain oxidative stress markers in models of prolonged intake of the WD are also associated with obesity [73-76]. As a consequence, it has been hard to determine whether the downstream obesity or the WD per se is responsible for the molecular and cellular changes. Here, our results demonstrate that the

inflammatory changes develop in non-obese rodents after a relatively short period of WD feeding.

Previous studies employing longer feeding periods than employed here have demonstrated that motor deficits can be induced by the WD. These motor deficits include impaired performance in the balance beam test [9], rotarod, pole, stepping and hind-limb clasping tests [22]. The present study is one of few observations that reports motor deficits to be associated with a short period of housing on the WD, and excludes potential artifacts in behavioral analysis owing to obesity [9,22,77]. Here we found that the WD impaired mouse performance in the wire test and rotarod, but not in the pole test. A lack of deficits in the muscle weight and strength in our experiments suggest that deficient coordination of mice housed on WD is unlikely to be related to a dysfunction of primary elements of the motor system, but are likely to be owing to cognitive and emotional changes under these conditions [9,21,78,79].

The inflammatory changes in the prefrontal cortex are likely to have been driven, as least in part by the molecular changes observed in the liver. It is becoming clear that perturbation of the gut-liver-brain axis can result in altered behavior. The animals displayed elevated serum AST and ALT, classic biomarkers of liver injury that is known to accompany obesity in humans [80] and rodents [81] and hepatitis in mice that is not associated with being over-weight [82,83]. There was

also a marked liver steatosis, which provides the evidence for the development of a NASH-like syndrome, which is characterized by pro-inflammatory changes of that is known to induce depressive-like and anxiety-like changes [43,46]. Increased blood levels of total cholesterol, LDL, HDL and remnant cholesterol in dietary challenged animals provide further evidence the development of systemic pro-inflammatory changes in our study. For example, high remnant cholesterol is associated with chronic inflammation, and is a strong predictor of myocardial infarction in people with normal weight [84,85]. Finally, insulin resistance and impaired glucose tolerance in mice housed on the WD suggest possible dysfunction in the central insulin receptor-mediated processes that play crucial in emotional regulation [86].

5. Conclusion

Taken together, these data suggest the importance of a WD in the development of neuropsychiatric symptoms, and, in particular, of ASD-like and ADHD-like behaviors in adulthood and highlight an early role for the prefrontal cortex in the development of these behaviors. Abnormal intake of highly caloric food and metabolic abnormalities are often observed in the ADHD patients where they might have additive or synergistic effects on symptoms [87-89]. Thus our results suggest that a WD is likely to impact on the pathogenesis of neuro-

developmentally acquired syndromes such as ADHD and ASD and that further investigation in human studies is warranted [90].

Declaration of competing interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Supplementary Material

Table 1. Diet composition for the Western diet and control diet used in the study. Western diet is characterized by increased content of fat, cholesterol and sucrose and high energy density.

Diet	Western diet	Control diet
Diet composition, %		
Fat	21	3.5
Saturated	12	0.6
Cholesterol	0.2	0
Carbohydrates	50	66
Sucrose	35	6
Protein	20	19
Fiber	5	5
Minerals and vitamins	4	6
Caloric Information, % kcal		
Fat	<i>43</i>	<i>9</i>
Carbohydrate	<i>40</i>	<i>67</i>
Protein	<i>17</i>	<i>24</i>
Energy Density, kcal/gm	4.67	3.23

Table 2. Sequences of the primers used in qPCR. Specific primer pairs were used in qPCR for measurement of serotonin transporter (*Sert*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene expression.

Gene	Forward primer	Reverse primer
<i>Sert</i>	TGCCTTTTATATCGCCTCCTAC	CAGTTGCCAGTGTCCAAGA
<i>Gapdh</i>	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC

Feeding with Western diet and testing in glucose and insulin tolerance tests

Cohort 1 was subjected to a glucose tolerance test, and thus food deprivation, on day 23. The glucose tolerance test was performed on day 22 in cohort 2, and, in cohort 3, the glucose tolerance test was performed on day 17 and then the insulin tolerance test was performed on day 22. Thus, total exposure of the groups to experimental diet was approximately 22 days.

Feeding with Western diet impairs motor coordination in rotarod test on Day 19

Mice fed with CD or WD were tested in rotarod test on day 19 of dietary challenge. After 6 training trials, they were tested in the 7th trial and the latency to fall was measured. The latency to fall was decreased in mice fed with WD compared to CD (U=5.5, P=0.0433, Mann-Whitney test, **Fig.S1**), suggesting impaired motor functions.

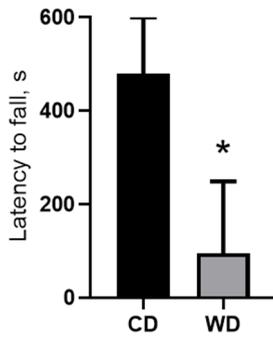


Figure S1. Feeding with Western diet impairs motor coordination in rotarod test on day 19. The latency to fall is decreased in the group fed with Western diet compared to control. CD – Control diet, WD – Western diet. * - $P < 0.05$ compared to CD (Mann-Whitney test). Data are presented as median and interquartile range.



Figure S2. Objects used in Object exploration / recognition test. Two objects were similar in size but had different surface texture and shape.

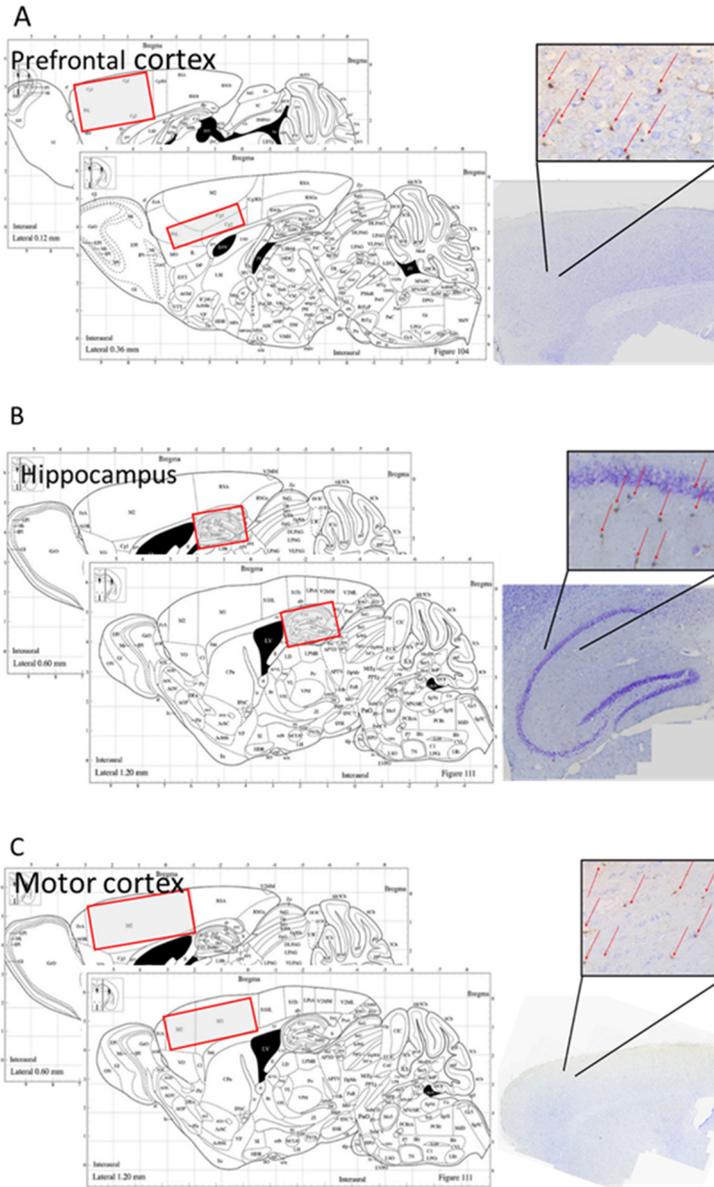


Figure S3. Coordinates of brain areas used for Iba-positive cell counting. (A) Prefrontal cortex, (B) hippocampus, (C) motor cortex were delineated according to the Paxinos and Franklin's the Mouse brain atlas.

Effects of the Western diet on mouse body weight, weights of organs and visceral fat and diet and water intake

In body weight, no significant effect of the diet was found (Cohort 1: $F=0.7261$, $P=0.4108$; Cohort 2: $F=0.005017$, $P=0.9458$; Cohort 3: $F=0.2193$, $P=0.6496$, two-way ANOVA). No differences between the groups fed WD or CD were found in body weight (**Figs.S4A-C**). No differences between the groups were found in the weights of liver, right and left kidneys, spleen, heart and visceral fat ($P>0.05$, unpaired t test, **Figs.S4D-I**). No differences were found in daily diet intake in calories normalized per body weight and in daily water intake ($P>0.05$, two-way ANOVA for repeated measures, **Fig.S4J-K**).

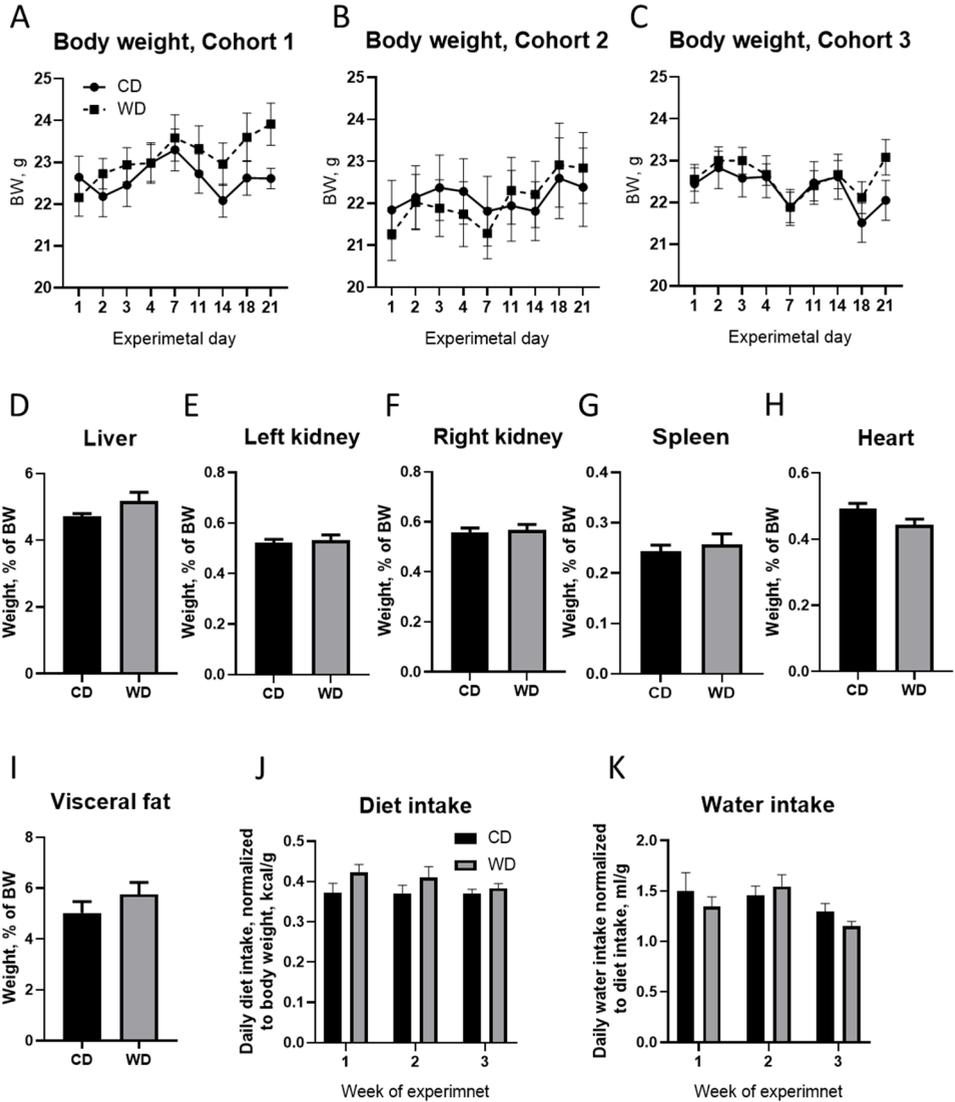


Figure S4. Effects of 3-week housing on Western diet on mouse body weight, weights of organs and visceral fat and diet and water intake. Western diet feeding does not alter mouse (A-C) body weight, (D-I) organ and visceral fat weight or (J-K) diet and water intake. CD – Control diet, WD – Western diet, BW – body weight. $P > 0.05$ compared to CD (two-way ANOVA or unpaired t test). Data are presented as Mean \pm SEM.

Effects of Western diet on fasting blood glucose and glucose tolerance on days 17 and 23 of dietary challenge

In glucose tolerance tested on experimental day 23 (Cohort 1), two-way ANOVA revealed significant effects of diet x time interaction, diet and the time of after glucose load ($F=3.905$, $P=0.0053$; $F=12.50$, $P=0.0166$ and $F=117.6$, $P<0.0001$, respectively, **Fig.S5A**). In comparison with control mice, WD-fed group showed a significant increase in blood glucose levels at the time point 10 min of the test ($P<0.0001$, Sidak's multiple comparisons test), suggesting an impairment in glucose tolerance in the latter group. No difference between the groups was found in blood glucose after 18h food deprivation on day 21 ($P>0.05$, unpaired t test, **Fig.S5B**). In the glucose blood level measured in glucose tolerance test on day 17, a two-way ANOVA for repeated measures revealed no diet x time after glucose load interaction or effect of the diet ($F=0.435$, $P=0.8514$ and $F=3.211$, $P=0.1476$, respectively, **Fig.S5C**). Basal glucose level after 5h food deprivation measured on Day 22 was increased in WD group ($t=2.869$, $P=0.0203$, unpaired t test, **Fig.S5D**).

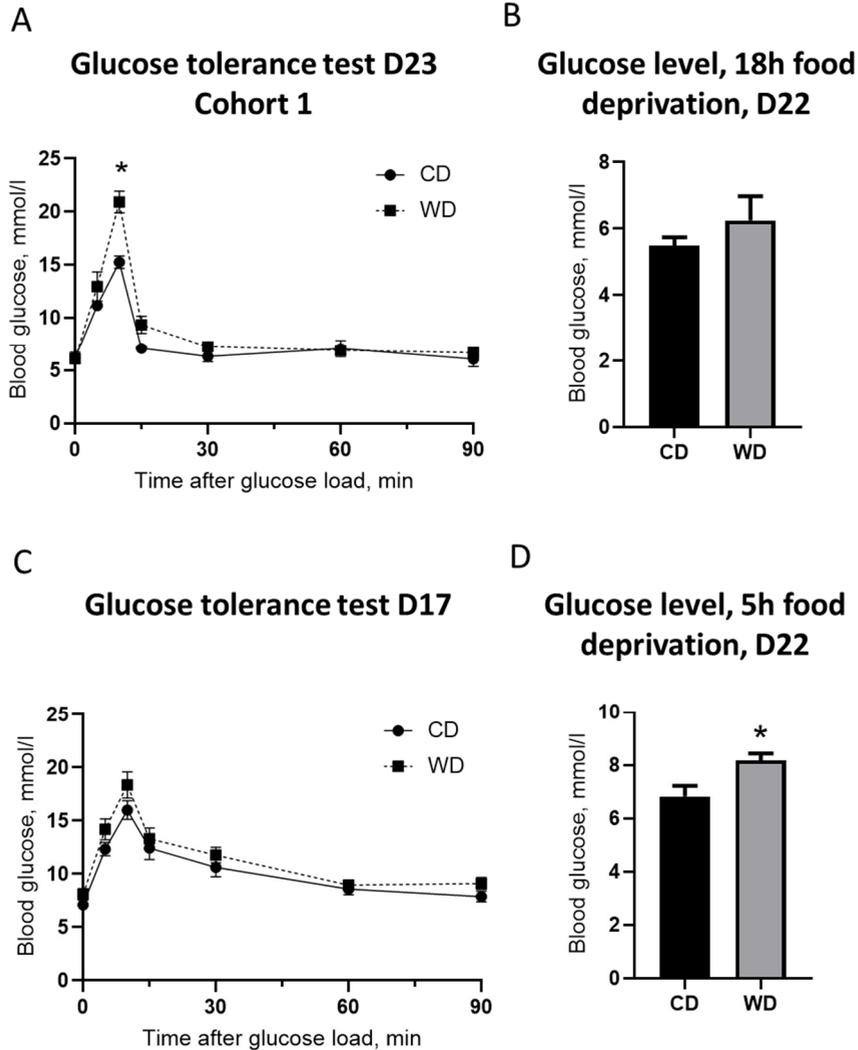


Figure S5. Effects of Western diet on fasting blood glucose and glucose tolerance on Day 23. (A) Glucose tolerance was decreased in WD group on day 23 in cohort 1. (B) Basal blood glucose level did not differ between the groups after 18h of food deprivation. (C) No difference in glucose tolerance was found after 16 days of WD feeding. (D) Basal blood glucose level was increased in WD-fed mice after 5h food deprivation. CD- Control diet, WD – Western diet. * - $P < 0.05$ compared to CD (unpaired t test or Sidak's multiple comparisons test). Data are presented as Mean \pm SEM.

Effects of Western diet on latency to fall in inverted screen and duration of object exploration

No difference was found in the latency to fall in inverted screen test between the groups ($P > 0.05$, Mann-Whitney test, **Fig.S6A**). In duration of object exploration, two-way ANOVA revealed significant diet x object interaction and effect of the object ($F = 14.40$, $P = 0.0016$; $F = 7.143$, $P = 0.0167$, respectively, **Fig.S6B**). Post hoc analysis revealed increase in duration of new object exploration compared to familiar object in control group ($q = 6.467$, $P = 0.0016$, Tukey's multiple comparisons test) but not in WD group ($P > 0.05$).

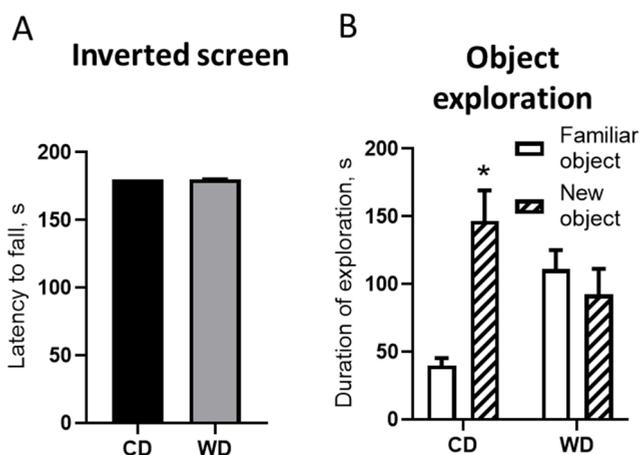


Figure S6. Effects of Western diet on latency to fall in inverted screen and duration of object exploration. (A) No difference between the groups was found in inverted screen test performance. (B) The duration of new object exploration compared to familiar object was increased in control group but not in WD group. CD – Control diet, WD – Western diet. * - $P < 0.05$ compared to CD (unpaired t test or Tukey's multiple comparisons test). Data are presented as Mean ± SEM.

Effects of Western diet on floating and climbing in forced swim test

On Day 1 of Forced swim test, latency to float, total duration of floating and duration of climbing behaviour did not differ between the groups ($P>0.05$, **Fig.S7A-C**). No difference was found in latency to float on day 2 ($t=1.837$, $P=0.0912$, unpaired t test, **Fig.S7D**). The total duration of floating on day 2 was increased in WD-fed mice compared to control ($t=2.924$, $P=0.0148$, **Fig.S7E**). Duration of climbing behaviour on day 2 did not differ between the groups ($P>0.05$, **Fig.S7F**).

Western diet does not alter locomotor activity in open field test

To rule out possible confounds of altered general locomotion in analysis of other behavior, possible effects of WD on locomotor activity were studied in open field test. Mice were fed with CD or WD, and one cohort (6 mice per group were used) was tested in open field on day 18 and another cohort (5 mice per group were used) was tested in open field on day 21. The open field apparatus consisted of a square arena (50 cm x 50 cm). Mice were put in the center of the open field arena, and their behavior was video recorded for 10 min under a stress-free 5 Lux lighting. Distance travelled was analyzed off-line using the EthoVision software (Noldus, Wageningen, The Netherlands). No significant differences between the groups were found in distance

travelled on either day 18 or day 21 ($t=0.7482$, $P=0.4837$ and $t=0.0124$, $P=0.9904$, respectively, unpaired t test, **Figs.S8A-B**). As same mice were tested on day 19 in a rotarod and showed signs of motor dyscoordination, taken together these data suggest that motor deficits induced by WD cannot be ascribed to altered locomotion (**Fig.S1**).

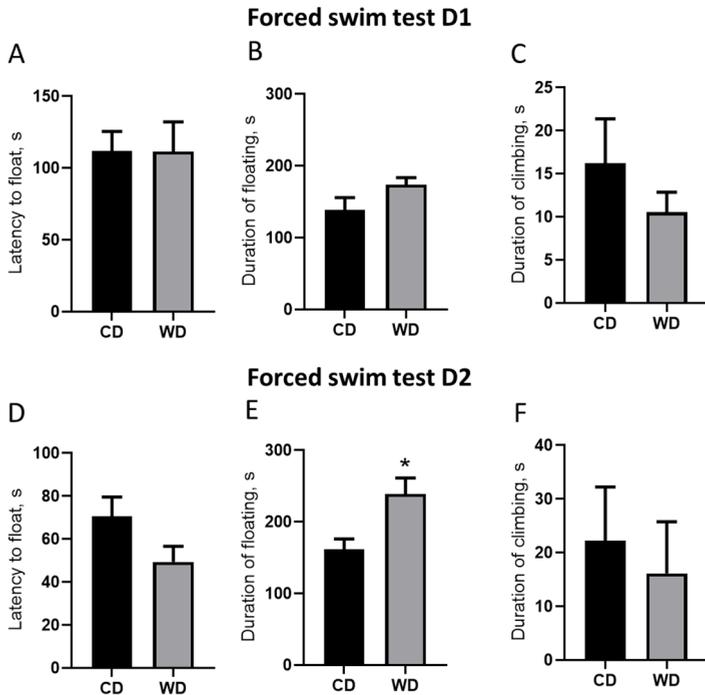


Figure S7. Effects of Western diet on floating and climbing in the swim test. On Day 1 of swim test, **(A)** latency to float, **(B)** total duration of floating and **(C)** duration of climbing behaviour did not differ between the groups. **(D)** On Day 2 of the swim test, latency to float did not differ between the groups. **(E)** Total duration of floating on Day 2 was increased in WD-fed mice compared to controls. **(F)** Duration of climbing behaviour on Day 2 did not differ between the groups. CD – Control diet, WD – Western diet. * - $P < 0.05$ compared to CD (unpaired t test or Tukey's multiple comparisons test). Data are presented as Mean \pm SEM.

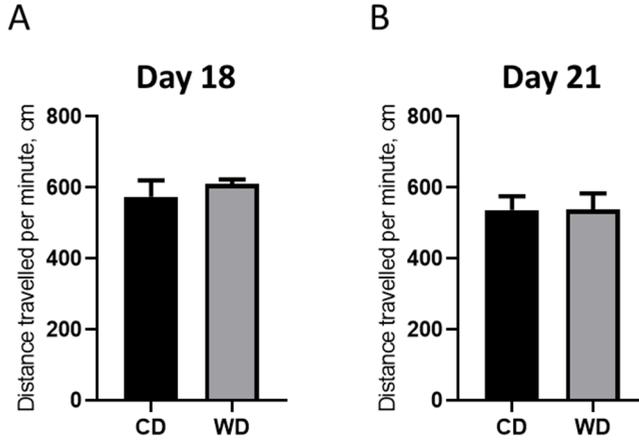


Figure S8. Western diet does not affect locomotor activity in open field test under 5 lux lighting. (A-B) No difference between the groups was found in distance travelled in open field on days 18 or 21 of dietary challenge. CD – Control diet, WD – Western diet. $P > 0.05$ (Unpaired t test). Data are presented as Mean \pm SEM.

Chapter 4

**Metabolic, molecular and
behavioural effects of
Western diet in serotonin
transporter-deficient mice:
rescue by heterozygosity?**

Chapter 4. Metabolic, molecular and behavioural effects of Western diet in serotonin transporter-deficient mice: rescue by heterozygosity?

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Veniaminova E, Cespuglio R, Chernukha I, Schmitt-Boehrer AG, Morozov S, Kalueff AV, Kuznetsova O, Lesch KP, Anthony DC, Strekalova T. Metabolic, molecular and behavioural effects of Western diet in serotonin transporter-deficient mice: rescue by heterozygosity? Frontiers in Neuroscience. Under revision.

Abstract

Reduced function of the serotonin transporter (SERT) is associated with increased susceptibility to anxiety and depression, and with type-2 diabetes, which is especially true in older women. Preference for a “Western diet” (WD), enriched with saturated fat, cholesterol and sugars, may aggravate these conditions. In previous studies, decreased glucose tolerance, central and peripheral inflammation, dyslipidaemia, emotional, cognitive and social abnormalities were reported in WD-fed young female mice. We investigated the metabolic, molecular, and behavioural changes associated with a three-week long dietary regime of either the WD or control diet in 12-month-old female mice with three different *Sert* genotypes: heterozygous (*Sert*^{+/-}: HET), homozygous (*Slc6a4*) gene knock out (*Sert*^{-/-}: KO) or wild-type mice (*Sert*^{+/+}: WT). In the WT-WD and KO-WD groups, but not in HET-WD-fed mice, most of changes induced by the WD paralleled those found in the younger mice, including brain overexpression of inflammatory marker toll-like receptor 4 (*Tlr4*), impaired hippocampus-dependent performance in the marble test. However, the 12-month-old female mice became obese. Control diet KO mice exhibited impaired hippocampal-dependent behaviours, increased brain expression of the serotonin receptors *Htr2c* and *Htr1b*, as well as increased *Tlr4* and mitochondrial regulator, peroxisome proliferator-activated receptor gamma-coactivator-1a

(*Ppargc1a*). Paradoxically, these, and other changes, were reversed in KO-WD mutants, suggesting a complex interplay between Sert deficiency and metabolic factors as well as potential compensatory molecular mechanisms that might be disrupted by the WD exposure. Most, but not all, of the changes in gene expression in the brain and liver of KO mice were not exhibited by the HET mice fed with either diet. Some of the WD-induced changes were similar in the KO-WD and HET-WD-fed mice, but the latter displayed a “rescued” phenotype in terms of diet-induced abnormalities in glucose tolerance, neuroinflammation and hippocampus-dependent performance. Thus, complete versus partial Sert inactivation in aged mice results in distinct metabolic, molecular and behavioural consequences in response to the WD. Our findings show that Sert^{+/-} mice are resilient to certain environmental challenges and support the concept of heterosis as evolutionary adaptive mechanism.

Keywords

Sert-deficient mice, Western diet, aging, glucose tolerance, Toll-like receptor 4 (TLR4), serotonin receptors, obesity, heterosis.

1. Introduction

Serotonin transporter (SERT), a key element of serotonergic neurotransmission (Collier et al., 1996; Murphy et al., 2004), is also involved in the regulation of metabolic processes (Giannaccini et al., 2013; Pomytkin et al., 2015, 2018; Stuart and Baune, 2012). In humans, a variant of the upstream regulatory region of the SERT (*SLC6A4*) gene, the so-called short (s) allele, in comparison with long (l) allele is associated with lower SERT activity and stressed-related vulnerability to anxiety and depression (Caspi et al., 2010; Greenberg et al., 2000; Lesch et al., 1996), and also with higher body mass index (BMI) (Fuemmeler et al., 2008; Sookoian et al., 2007) and incidence of type-2 diabetes (Iordanidou et al., 2010), which are typical for the female sex and aging (Batsis and Zagaria, 2018; Kautzky-Willer et al., 2016; Khabazkhoob et al., 2017).

Individuals with metabolic syndrome and obesity display decreased SERT expression in the brain and periphery (Giannaccini et al., 2013; Nam et al., 2018). Excessive intake of a hypercaloric diet, enriched with saturated fat and sugars, has been shown to suppress the binding of hypothalamic SERT in obese subjects and in insulin-resistant subjects, that is independent of body weight gain (Koopman et al., 2013; Versteeg et al., 2017). Thus, diminished SERT activity is believed to underlie negative changes associated with metabolic syndrome (Stuart and Baune, 2012) and in turn, metabolic

abnormalities resulting in reduced SERT function that can contribute to emotional disturbances (Pomytkin et al., 2015, 2018). However, the molecular mechanisms of the interplay between genetic SERT deficiency and metabolic dysregulation remain unclear.

Animal studies support the observations made in humans concerning the relationship between SERT deficiency and diabetes-like metabolic changes. Sert-deficient mice (*Sert*^{-/-}: KO) have been reported to display decreased glucose tolerance, increased deposition of white adipose tissue that increases with aging, and late-onset obesity; these changes were particularly marked in females (Chen et al., 2012; Murphy and Lesch, 2008; Üçeyler et al., 2010; Zha et al., 2017).

Aging is well known to be associated with compromised metabolic function (Boemi et al., 2016) and changes in the serotonergic regulation (Rodríguez et al., 2012). Abnormal distribution of fat in the elderly increases the risk or exacerbates the negative effects of obesity on metabolic function, including a decline in insulin sensitivity and glucose tolerance (Karakelides et al., 2010; Morita et al., 2006). In humans, each decade results in a 10% decrease in the density of SERT binding sites in the brain stem and thalamus (Yamamoto et al., 2002); this decrease is also found in monkeys and mice (Herrera-Pérez et al., 2013; Kakiuchi et al., 2001). Aging also results in a decrease in circulating serotonin and alterations in the densities of the brain serotonin

receptors 5-HT1A, 5-HT2A, 5-HT1B (Matuskey et al., 2012; Meltzer et al., 1998) that are more profound in women (Meltzer et al., 1998).

Despite the evidence of a relationship between genetic SERT deficiency and diabetes-like metabolic conditions, little is known about the underlining molecular mechanisms, and few studies have addressed this issue. In the human population, the combination of genetic SERT deficiency, increased intake of Western diet (WD), aging is a widespread, but the interactions are difficult to explore owing to the presence of so many potentially confounding factors. Mutant animals provide an opportunity to model the interactions between genotype and diet at different ages and in different sexes. The majority of studies addressing the impact of decreased SERT function on metabolic regulation during hypercaloric dietary challenge have been performed with young *Sert*-deficient mice. For example, Chen et al. (2012) reported the presence of elevated fasting glucose levels, impaired glucose tolerance and insulin resistance in *Sert*^{-/-} animals exposed to a high-fat diet for 3 months in male mice. *Sert*^{-/-} female rats, but not male animals, demonstrated increased abdominal fat when fed either standard chow or a diet with high in fat and sugar content (Homberg et al., 2010).

Concerning the effects of the Western diet and aging on the metabolic characteristics of *Sert* heterozygous (*Sert*^{+/-}: HET) animals, very limited literature is available. It has been reported that diet-

induced metabolic changes in young SERT heterozygous mice are intermediate in their magnitude, showing impaired glucose tolerance and insulin resistance, with respect to the changes in the wild type and knockout phenotypes (Chen et al., 2012). However, HET mice are considered to be qualitatively distinct from *Sert*^{-/-} animals and closer mimic of the short allele human condition, displaying allelic variation of SERT function. Substantial differences between two genotypes have been described in a maternal separation and other stress models, in which the HPA axis was suggested not to be effect in the same way in HET and KO animals (Jiang et al., 2009; van der Doelen et al., 2014). In a prenatal stress model, HET mice demonstrated signs of superior stress resilience compared to WT, displaying reduced scores of anxiety-like behaviour and improved cognitive performance (van den Hove et al., 2011), while other studies showed increased stress reactivity in KO (Bearer et al., 2018; Wellman et al., 2007).

In the present study we sought to investigate metabolic, molecular and behavioural changes induced by the WD in aged mice with complete or partial genetic SERT deficit. We employed a previously validated model that involves feeding mice with the WD for three weeks and we evaluated metabolic and neurobiological hallmarks of the WD-induced induced syndrome *in vitro* and *ex vitro* assays (Strekalova et al., 2015, 2016; Veniaminova et al., 2016, 2017, 2020). In this model, impaired glucose tolerance, increases in cholesterol and

leptin blood levels, brain and liver over-expression of toll-like receptor 4 (*Tlr4*), decreased expression of mitochondrial markers peroxisome proliferator-activated receptor gamma coactivator 1 (*Ppargc1*) a and b and decreased *Sert* expression in the brain are all features. These molecular changes are accompanied by depressive- and anxiety-like behaviours, signs of impulsivity, lowered sociability and cognitive deficits (Strekalova et al., 2015, 2016; Veniaminova et al., 2016, 2017, 2020). Here, in view of the changes observed in 5HT receptor densities in aging and in relation to SERT deficiency, we also studied gene expression of serotonin receptors *Htr1a*, *Htr1b* and *Htr2a* in the brain. The expression of the serotonin receptors 5-HT_{2C} and 5-HT₆ were also owing to their know role in the regulation of emotionality and metabolic function (Bickerdike, 2003; Heal et al., 2008; Millan, 2005; Wesółowska, 2010).

2 Methods

2.1 Animals

Experiments were performed using 12-month-old homozygous *Sert*^{-/-} and heterozygous *Sert*^{+/-} female mice and wild type littermates born from heterozygous mutants at the tenth generation (F10) of backcrossing with C57BL/6J mice, all genotypes were confirmed by PCR. Mice were housed 3-4 per cage during the study, under a reversed 12 h light-dark cycle (lights on: 21:00 h) with food and water ad libitum

and under controllable laboratory conditions ($22 \pm 1^\circ\text{C}$, 55% humidity). Laboratory housing conditions and experimental procedures were set up and maintained in accordance with the European Communities Council Directive for the care and use of laboratory animals (2010/63/EU) and approved by the local ethics committees of C. Bernard University and MSMU (#11-18).

2.2 Study design and diets

Mice were fed with a standard laboratory diet (control diet, CD) with an energy content of 3.8 kcal/g, 4.3% of fat (1.3 of saturated fat) (D18071801, Research Diet Inc., New Brunswick, NJ, USA) or with a diet containing 0.2% cholesterol, 21.3% of fat (10.5% of saturated fat), and an energy content of 4.6 kcal/g, Western diet (D11012302, Research Diet Inc., New Brunswick, NJ, USA) for three weeks as described elsewhere (Strekalova et al., 2015, 2016; Veniaminova et al., 2017). The content of the nutrients in calories and weight and the ingredients are indicated in Supplementary Table S1. Body weight and intake of diet and water were monitored weekly (on day 1, day 8, day 15 and day 21). Daily intake of calories and water was normalized to body weight.

After a three-week period of dietary challenge, a cohort of mice was studied in novel cage, O-maze test, depressive-like behaviours in tail suspension and forced swim tests, in the pellet displacement tube

(marble) test, a rodent paradigm for a hippocampus-dependent performance (Deacon et al., 2002; Strekalova and Steinbusch, 2010) and a glucose tolerance test (**Fig. 1A**). Another cohort of animals was exposed to the same dietary conditions and sacrificed and dissected for the analysis of gene expression (**Fig. 1B**). 6-7 mice per group were used in each study.

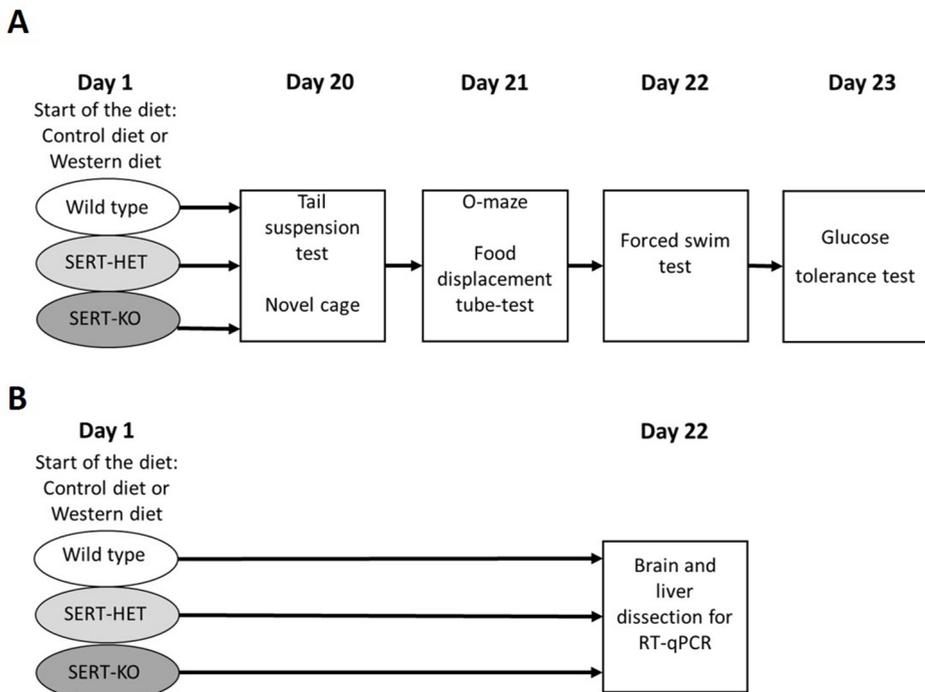


Figure 1. Experimental design. (A) Study of the effects of 3-week WD feeding of WT, HET and KO mice on behaviour in tail suspension, novel cage, O-maze, food displacement test, forced swim test, glucose tolerance test. **(B)** Study of the effects of 3-week WD feeding of WT, HET and KO mice on gene expression in the brain and liver.

2.3 Behavioural testing

All behavioural tests were carried out during an active period of animals' light cycle (09:00–21:00); behaviour was recorded and scored offline. The experimenter was blind for the diet used and the genotype.

2.3.1 Novel cage test

The novel cage test was performed to assess exploration of a new environment. Mice were introduced into a standard plastic cage (21×27×14 cm), under 5 Lux lighting. The number of exploratory rears was counted for the first minute of the test by visual observation, as described elsewhere (Costa-Nunes et al., 2015).

2.3.2 Elevated O-maze

The maze consisted of a black circular path (runway width 5.5 cm, diameter 46 cm) that was placed 20 cm above the floor. Illumination intensity was 5 Lux. Two opposing compartments were protected by the walls (height 10 cm). Mice were introduced to one of the two closed compartments. Latency to exit to the anxiety-related open compartments of the maze was scored, as described elsewhere (Strekalova et al., 2015).

2.3.3 Tail suspension test

Mice were subjected to the tail suspension by being hung by their tails with adhesive tape to a rod 50 cm above the floor for 6 min, as described elsewhere (Malatynska et al., 2012). The lighting intensity

on the height of the mouse position was 25 Lux. The trials were recorded by a video camera positioned directly in front of the mice while the experimenter observed the session from a distance in a dark area of the experimental room. The latency of the first episode of immobility was scored. In accordance with the commonly accepted criteria of immobility, the immobility behaviour was defined as the absence of any movements of the animals' head and body. The scoring method was previously validated using CleverSys software (CleverSys, Reston, VA, USA) and Noldus software (Wageningen, The Netherlands) (Malatynska et al., 2012).

2.3.4 Swim test

This test was carried out as described previously (Strekalova et al., 2015). Mice were placed into a plastic transparent cylinder ($\text{\O} 17 \text{ cm}$) filled with water ($+23^\circ\text{C}$, water height 13 cm, and height of cylinder 20 cm) under the red lighting. Total duration of floating behaviour, defined by the absence of any directed movements of animals' head and body, was scored offline during the 6-min period. Latency to float was evaluated as well. The scoring method was previously validated using CleverSys software (CleverSys, Reston, VA, USA) and Noldus software (Wageningen, The Netherlands) (Malatynska et al., 2012).

2.3.5 Pellet displacement tube (marble) test

All experimental groups were tested for pellet displacement in a tube test as described elsewhere (Deacon et al., 2002; Strekalova and Steinbusch, 2010). A tendency to displace small objects, for example, small stones or food pellets from a tube inside the cage, is species-specific in mice and has been demonstrated to depend on an intact hippocampal formation (Deacon et al., 2002). Using a paper tube (internal diameter 4 cm, length 10 cm), filled with 20 food pellets and placed in the cage (21×27×14 cm), the time required for 50%-emptying of tube was recorded.

2.4 Glucose Tolerance Test

Oral glucose tolerance test (OGTT) was performed as described elsewhere (Veniaminova et al., 2017). The test mice were fasted overnight for 18 h, beginning at 1600. Thereafter, a glucose solution (2 g/kg, 1.8 g/l) was delivered by oral gavage and blood was sampled from the tail vein. Samples were obtained prior to glucose administration at time point 0 and 5, 15, 30, 60, 90 min afterwards. The level of blood glucose was analyzed using the OneTouch UltraEasy glucometer and strips (LifeScan OneTouch, Dubai, UAE). Fasting blood glucose concentrations and the area under a curve (AUC) for the whole test period and for the period between 60 and 90 min after glucose load were analyzed.

2.5 Tissue dissection

Mice were terminally anaesthetized with isoflurane inhalation for a subsequent material collection. The brain of each mouse was perfused with saline and dissected, and dorsal raphe region zone, hypothalamus, hippocampus and prefrontal cortex were isolated and stored at -80°C until use as described elsewhere (Couch et al., 2013).

2.6 RNA extraction and qRT-PCR

Total mRNA was extracted by using RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). First-strand cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA); 1 µg total RNA was converted into cDNA. Quantitative PCR for the genes of interest (*Htr1a*, *Htr1b*, *Htr2a*, *Htr2c*, *Htr6*, *Ppargc1a*, *Ppargc1b*, *Tlr4*) and the reference genes (glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), beta-actin (*Actb*), beta-2 microglobulin (*B2m*)) was performed using the SYBR Green PCR Master Mix (Applied Biosystems) and QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). Sequences of primers used are indicated in Supplementary Table S2. Reference genes for normalization were tested for stability using RefFinder software. Results of qRT-PCR measurement were expressed as Ct values and the comparative Ct method was used. Data are given as expression

folds compared to the mean expression values in WT mice fed control diet as described elsewhere (Couch et al., 2013).

2.8 Statistics

Data were analyzed using GraphPad Prism version 8.01 (San Diego, CA, USA). For comparison of six groups, two-way ANOVA followed by Tukey's post-hoc test was used. One-way ANOVA followed by post-test for trend was used for analysis of genotype body weight data at baseline. For comparison of a group mean with 100%, one sample t test was performed. Three-way ANOVA analysis was performed in IBM SPSS Statistics 23 (Armonk, NY, USA). The level of significance was set at $p < 0.05$. Data were presented as Mean \pm SEM or Mean.

3 Results

3.1 Western diet and SERT deficiency affect metabolic parameters

There was a significant difference in body weight at baseline between the Sert genotypes ($F=4.547$, $p=0.016$, one-way ANOVA). A linear increase in body weight from WT to KO group ($p=0.005$, post-test for trend; **Fig. 2A**) was found. Two-way ANOVA revealed a significant effect of the diet type on the body weight measured both in absolute values and normalized to basal values ($p < 0.05$, **Table 1**) after three weeks of feeding as measured on day 21.

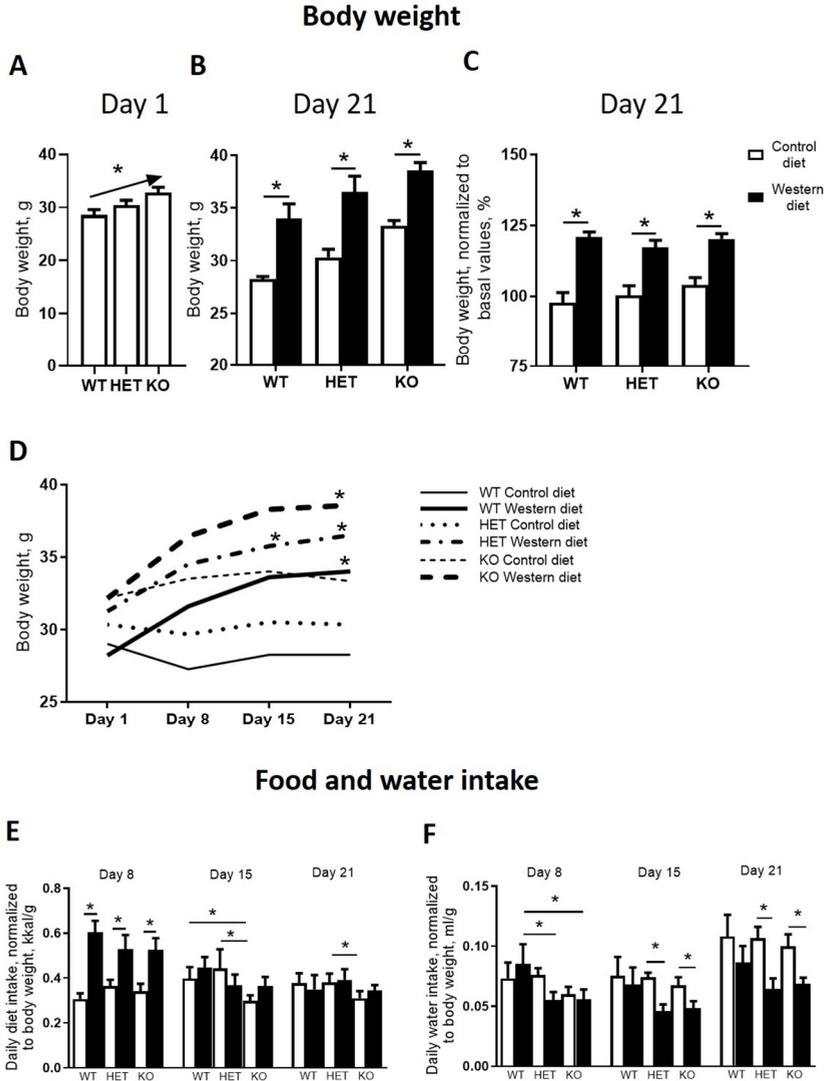


Figure 2. Effects of Western diet feeding on body weight and food and water intake. (A) Basal body weight of mice from different *Sert* genotypes: WT, HET, KO. (B-C) Body weight after 3-week housing on WD or CD in absolute values and normalized to basal body weight. (D) Dynamics of the mouse body weight measured every week during the experiment. Mice fed with WD demonstrated increase in body weight during the study. (E-F) Diet and water intake. * $p < 0.05$, one-way ANOVA and post-test for trend (A) or two-way ANOVA and Tukey's test (B-F), 6-7 animals per group were used. Data are shown as mean \pm SEM (A-C,E-F) or mean (D).

The body weight of WT, HET and KO mice fed with WD was increased compared with the respective genotype-matched control groups (absolute body weight values: $p=0.010$, $p=0.001$, and $p=0.005$, respectively, Tukey's test; normalized to basal values: $p<0.001$, $p<0.001$, and $p<0.001$, respectively, Tukey's test; **Figs. 2B-C**). A significant interaction between the diet type and the day of the experiment and a significant effect of the genotype were found for the dynamics of body weight ($F=6.452$, $p<0.001$ and $F=28.827$, $p<0.001$, three-way ANOVA). No differences between the groups were found on days 1 and 8. On day 15, body weight was increased in HET-WD mice compared to control HET mice ($p=0.014$, Tukey's test; **Fig. 2D**); on day 21, all Sert genotype groups fed with WD displayed increased body weight compared to controls ($p=0.044$ for WT-WD, $p=0.002$ for HET-WD, $p=0.020$ for KO-WD, Tukey's test). Thus, 3-week WD feeding resulted in body weight increase in all Sert genotypes.

As revealed by three-way ANOVA, there was a significant interaction between genotype, diet and experimental day for daily diet calorie intake ($F=5.944$, $p<0.001$). During the 1st week of the experiment, daily calorie intake was increased in WT, HET and KO mice fed with WD compared to mice fed with CD ($p<0.001$, $p<0.001$, and $p<0.001$, respectively, Tukey's test; **Fig. 2E**). Then, during the 2nd week, calorie intake was decreased in KO mice fed with CD compared to WT and HET fed with CD ($p=0.027$ and $p=0.030$,

respectively, Tukey's test). During the 3rd week, calorie intake was decreased in KO-CD mice compared to HET-CD (p=0.0231, Tukey's test). Similar results were obtained for daily diet intake measured in grams per kg of body weight (Supplementary Fig. S1). Significant interaction between genotype and diet and experimental day and diet was found for daily water intake (F=15.487, p<0.001 and F=20.020, p<0.001, respectively, three-way ANOVA). During the 1st week, water intake was decreased in HET-WD and KO-WD mice compared to WT-WD (p=0.016 and p=0.018, respectively, Tukey's test; **Fig. 2F**).

Table 1. Two-way ANOVA results for statistical analysis of metabolic parameters.

<i>Metabolic parameters</i>						
<i>Parameter</i>	<i>Interaction, F</i>	<i>Interaction, p</i>	<i>Genotype, F</i>	<i>Genotype, p</i>	<i>Diet, F</i>	<i>Diet, p</i>
Body weight on D21, absolute values	0.09742	0.9075	8.510	0.0012	37.98	<0.001
Body weight on D21, normalized to basal values	0.8320	0.4450	0.9820	0.3863	70.65	<0.001
Fasting blood glucose level	1.897	0.1710	7.369	0.0030	6.514	0.0172
AUC for glucose tolerance test	1.343	0.2808	1.053	0.3651	9.242	0.0058
AUC for glucose tolerance test, 60-90 min	2.392	0.1139	0.3612	0.7007	9.357	0.006

F and p values are shown for interaction between genotype and diet, for genotype effect and for diet effect.

During the 2nd and 3rd weeks, HET and KO groups fed with WD showed decreased water intake compared to the respective genotype-matched groups fed with CD (HET-WD: $p < 0.001$ and $p < 0.001$, KO-WD: $p = 0.0003$ and $p < 0.0001$, respectively, Tukey's test). That way, *Sert* deficiency resulted in a decreased diet intake in KO mice and decreased water intake in HET and KO after WD exposure.

We found significant effects of the diet type and the genotype (two-way ANOVA, $p < 0.05$, **Table 1**) on the fasting blood glucose levels. Glucose levels after 18 hours of food deprivation were decreased in KO mice fed with WD compared to KO fed with CD ($p = 0.024$, Tukey's test; **Fig. 3A**). Fasting blood glucose levels normalized to the respective genotype-matched CD group values were also decreased in KO-WD compared to 100% ($t = 3.528$, $p = 0.0243$, one sample t test; **Fig. 3B**). Two-way ANOVA analysis demonstrated a significant diet effect ($p < 0.05$, **Table 1**; **Fig. 3C**) on the area under the curve (AUC) calculated for glucose tolerance curve (**Fig. 3D**). Post-hoc analysis revealed a significant increase in AUC in WT and KO mice fed with WD compared to the respective control groups ($p = 0.021$ and $p = 0.028$, respectively, Tukey's test). No differences in AUC were found between HET-CD and HET-WD groups. There was a significant diet effect on AUC calculated for the period between 60 and 90 min after glucose load ($p < 0.05$, **Table 1**). This parameter was significantly increased in KO-WD compared to KO-CD group ($p = 0.021$, Tukey's test; **Fig. 3E**).

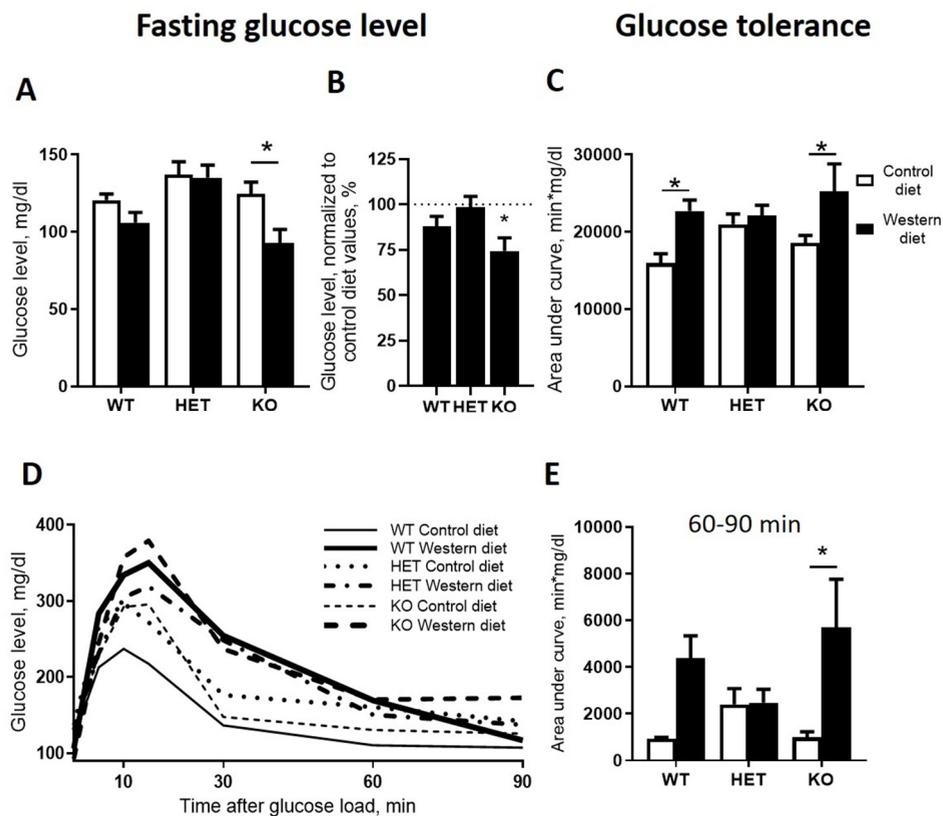


Figure 3. Effects of Western diet feeding on glucose tolerance. (A) Fasting blood glucose level after 18 hours of food deprivation. WD decreased fasting glucose level in KO mice (B) WD group basal glucose levels normalized to respective genotype group fed with CD values in comparison of with 100%. (C) Area under glucose tolerance curve. WD increased area under the curve in WT and KO mice. (D) Glucose tolerance curve. (E) Area under glucose tolerance curve for the period between 60 and 90 min. * $p < 0.05$, two-way ANOVA and Tukey's test (A,C,E) or one sample t test vs. 100% (B), 6-7 animals per group were used. Data are shown as mean \pm SEM (A-C,E) or mean (D).

These results indicate that the impairment in glucose tolerance due to feeding with WD was exacerbated in KO mice in comparison to WT but was absent in HET group.

3.2 Effects of Western diet and SERT deficiency on the expression of markers of mitochondrial activity and *Tlr4* in brain and liver

A significant interaction between the genotype and diet was shown in mRNA levels of *Ppargc1a* in the hypothalamus and the hippocampus (two-way ANOVA, $p < 0.05$, **Table 2**; Supplementary Fig. S2). The effect of genotype was significant in the dorsal raphe region and the prefrontal cortex ($p < 0.05$, **Table 2**). Post-hoc analysis revealed a significant increase of *Ppargc1a* expression levels in the hypothalamus and the prefrontal cortex of KO-CD in comparison to WT and HET mice fed with CD ($p = 0.039$ and $p = 0.045$ for hypothalamus and $p = 0.002$ and $p = 0.006$ for prefrontal cortex, Tukey's test; **Fig. 4A**). *Ppargc1a* expression in the hypothalamus of KO-WD was decreased in comparison to KO-CD ($p = 0.045$, Tukey's test).

Two-way ANOVA revealed a significant genotype effect on mRNA levels of *Ppargc1b* in the hypothalamus ($p < 0.05$, **Table 2**). In addition, there was a significant diet effect in the dorsal raphe region and the prefrontal cortex ($p < 0.05$, **Table 2**) on the expression of *Ppargc1b*. No differences were found in the hippocampus (Supplementary Fig. S2). *Ppargc1b* expression levels were decreased in the hypothalamus in KO and HET mice when compared to WT mice ($p = 0.0376$ and $p = 0.0095$, respectively, Tukey's test; **Fig. 4B**).

Table 2. Two-way ANOVA results for statistical analysis of *Ppargc1a* and *b* and *Tlr4* gene expression.

<i>Brain gene expression</i>							
<i>Gene</i>	<i>Brain structure</i>	<i>Interaction, F</i>	<i>Interaction, p</i>	<i>Genotype, F</i>	<i>Genotype, p</i>	<i>Diet, F</i>	<i>Diet, p</i>
Ppargc1a	HT	6.003	0.0054	1.799	0.1793	0.1123	0.7394
	DR	0.3308	0.7205	7.713	0.0017	3.750	0.0609
	HIP	3.455	0.0418	1.745	0.1883	2.065	0.1589
	PF	2.001	0.1492	10.46	0.0002	2.956	0.0937
Ppargc1b	HT	0.2827	0.7554	5.436	0.0085	0.0061	0.9380
	DR	0.1146	0.8921	0.9017	0.4166	5.301	0.0284
	HIP	0.1623	0.8508	2.629	0.0856	0.0159	0.9001
	PF	0.3177	0.7301	0.03676	0.9639	5.088	0.0313
Tlr4	HT	0.01194	0.9881	13.47	<0.0001	0.0408	0.8409
	DR	2.007	0.1509	4.994	0.0130	11.08	0.0022
	HIP	0.6018	0.5529	1.317	0.2800	0.2996	0.5874
	PF	1.370	0.2678	5.296	0.0100	2.930	0.0961
<i>Liver gene expression</i>							
<i>Gene</i>		<i>Interaction, F</i>	<i>Interaction, p</i>	<i>Genotype, F</i>	<i>Genotype, p</i>	<i>Diet, F</i>	<i>Diet, p</i>
Ppargc1a		6.052	0.0056	6.143	0.0053	1.733	0.1969
Ppargc1b		9.094	0.0007	10.46	0.0003	4.501	0.0412
Tlr4		1.696	0.1978	0.2381	0.7893	1.668	0.2048

F and p values are shown for interaction between genotype and diet, for genotype effect and for diet effect. HT – hypothalamus, DR – dorsal raphe region, HIP – hippocampus, PF – prefrontal cortex.

We found a significant effect of genotype on mRNA levels of *Tlr4* in the hypothalamus and the prefrontal cortex (two-way ANOVA,

$p < 0.05$, **Table 2**). Also, there was a significant diet effect in the dorsal raphe region ($p < 0.05$, **Table 2**). No differences were found in the hippocampus (Supplementary Fig. S2). Post-hoc analysis showed an increase of *Tlr4* expression levels in the hypothalamus of KO mice compared to WT and HET groups ($p < 0.001$ and $p = 0.005$, respectively, Tukey's test; **Fig. 4C**). In addition, a significant increase in *Tlr4* expression was detected in the dorsal raphe region in WT and KO mice fed with WD compared to groups fed with CD ($p = 0.042$ and $p = 0.040$, respectively, Tukey's test) but not in HET mice, and in the prefrontal cortex in KO-WD group compared to WT-WD group ($p = 0.0403$, Tukey's test). Thus, the most prominent increase in *Tlr4* expression was found in KO fed WD. No effect of WD on *Tlr4* expression was observed in HET mice.

In the liver, there was a significant interaction between genotype and diet on the expression of *Ppargc1a* and *Ppargc1b* (two-way ANOVA, $p < 0.05$, **Table 2**). *Ppargc1a* and *Ppargc1b* expression levels in liver were decreased in WT-WD compared to WT-CD ($p = 0.026$ and $p = 0.002$, respectively, Tukey's test; **Fig. 4D**), and in HET and KO mice fed with CD compared to WT mice fed with CD ($p = 0.011$, $p = 0.002$ and $p < 0.001$, $p < 0.001$, respectively, Tukey's test). No differences between the groups were found in *Tlr4* expression in the liver.

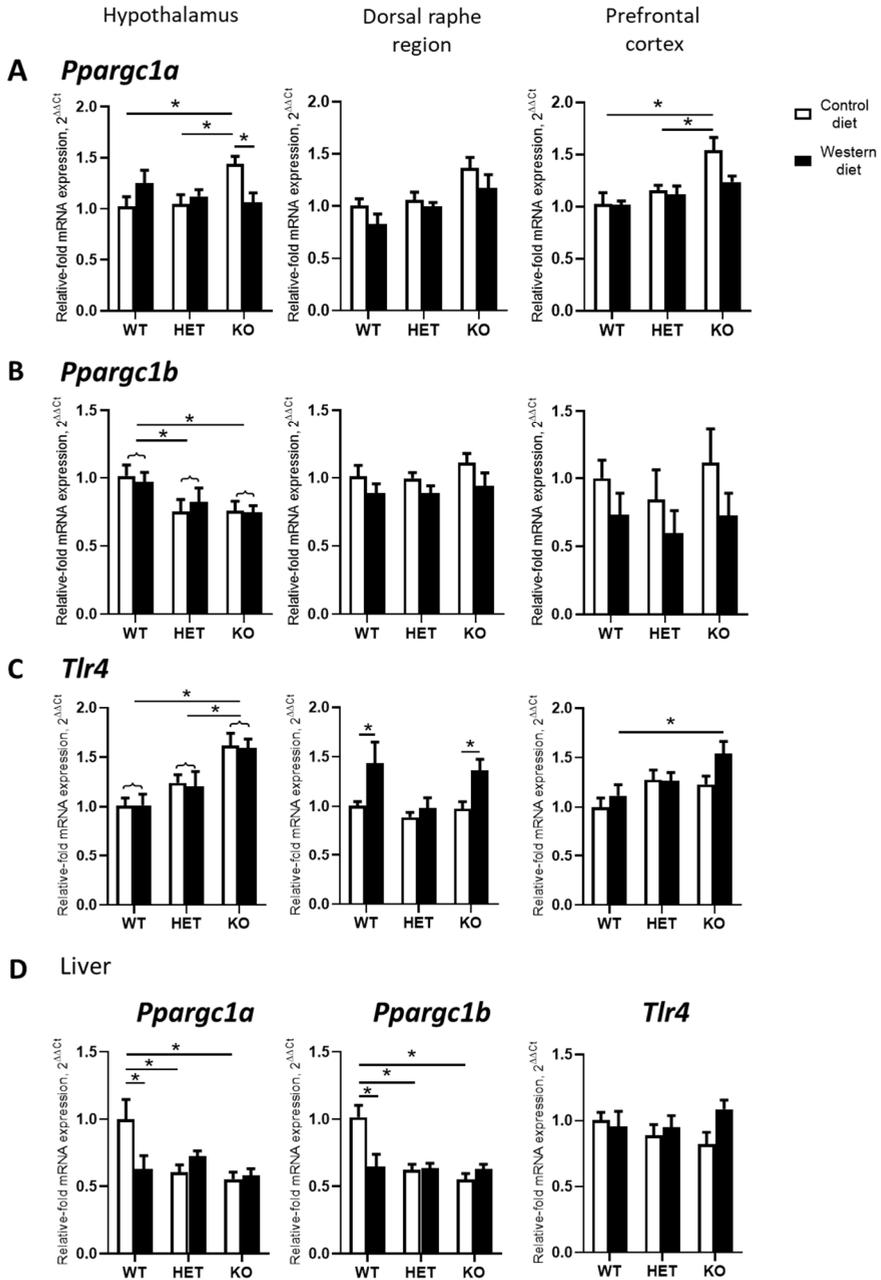


Figure 4. Effects of Western diet and SERT deficiency on brain and liver gene expression of markers of mitochondrial activity and *Tlr4*. (A-C) *Ppargc1a*, *Ppargc1b* and *Tlr4* expression in hypothalamus, dorsal raphe

region and prefrontal cortex. *Tlr4* expression in dorsal raphe region was significantly increased in WT and KO but not HET mice fed with WD compared to respective groups fed with CD. **(D)** *Ppargc1a*, *Ppargc1b* and *Tlr4* expression in the liver. * $p < 0.05$, two-way ANOVA and Tukey's test, } – main genotype effect, 6-7 animals per group were used. Data are shown as mean \pm SEM.

3.3 Changes in brain expression of serotonin receptors by Western diet and SERT deficiency

Two-way ANOVA analysis demonstrated a significant genotype effect on the *Htr1a* mRNA levels in the dorsal raphe region and the prefrontal cortex ($p < 0.05$, **Table 3**). In addition, a significant effect of diet was shown in prefrontal cortex ($p < 0.05$, **Table 3**). *Htr1a* expression levels in the dorsal raphe region were decreased in HET and KO mice compared to WT mice ($p < 0.001$ and $p = 0.011$, respectively, Tukey's test; **Fig. 5A**).

There was a significant interaction between genotype and diet in mRNA levels of *Htr2a* in the hippocampus (two-way ANOVA, $p < 0.05$, **Table 3**). *Htr2a* expression was elevated in the hippocampus of KO-CD mice compared to WT-CD and HET-CD mice ($p = 0.001$ and $p = 0.006$, Tukey's test; **Fig. 5B**).

A significant interaction between genotype and diet in mRNA levels of *Htr1b* receptor was found in the hypothalamus, the dorsal raphe region and the prefrontal cortex (two-way ANOVA, $p < 0.05$, **Table 3**) but not in hippocampus. Post-hoc analysis revealed a significant increase in *Htr1b* expression in the hypothalamus, the dorsal raphe region and the prefrontal cortex of KO-CD group compared to

WT-CD and HET-CD groups ($p=0.040$ and $p=0.020$ for hypothalamus, $p<0.001$ and $p<0.001$ for the dorsal raphe region, $p=0.001$ and $p=0.001$ for prefrontal cortex, Tukey's test; **Fig. 5C**).

Table 3. Two-way ANOVA results for statistical analysis of serotonin receptors gene expression.

<i>Brain gene expression</i>							
<i>Gene</i>	<i>Brain structure</i>	<i>Interaction, F</i>	<i>Interaction, p</i>	<i>Genotype, F</i>	<i>Genotype, p</i>	<i>Diet, F</i>	<i>Diet, p</i>
Htr1a	HT	0.07188	0.9308	2.915	0.0664	1.419	0.2409
	DR	0.3828	0.6850	8.898	0.0008	0.00130	0.9715
	HIP	0.5541	0.5792	2.066	0.1407	0.02632	0.8720
	PF	0.8123	0.4523	6.690	0.0035	9.665	0.0038
Htr2a	HT	0.4899	0.6165	0.8927	0.4180	0.8340	0.3669
	DR	0.5235	0.5971	2.932	0.0669	0.2083	0.6510
	HIP	3.485	0.0408	9.263	0.0005	1.188	0.2827
	PF	0.3957	0.6759	2.289	0.1152	0.2406	0.6266
Htr1b	HT	14.07	<0.0001	1.108	0.3407	0.7577	0.3895
	DR	8.429	0.0010	13.71	<0.0001	2.331	0.1358
	HIP	0.7293	0.4889	0.9170	0.4084	0.4561	0.5035
	PF	4.426	0.0189	10.70	0.0002	1.309	0.2600
Htr2c	HT	9.803	0.0004	10.14	0.0003	0.6533	0.4240
	DR	5.308	0.0097	10.55	0.0003	6.429	0.0158
	HIP	0.2895	0.7503	1.057	0.3574	0.6697	0.4183
	PF	6.560	0.0036	6.111	0.0050	0.4158	0.5229
Htr6	HT	0.2326	0.7936	0.3814	0.6856	0.07229	0.7895
	DR	0.05340	0.9481	5.580	0.0079	0.4079	0.5272
	HIP	0.2349	0.7918	4.290	0.0211	0.3645	0.5497
	PF	1.420	0.2546	0.2677	0.7666	0.09352	0.7615

F and p values are shown for interaction between genotype and diet, for genotype effect and for diet effect. HT – hypothalamus, DR – dorsal raphe region, HIP – hippocampus, PF – prefrontal cortex.

In addition, *Htr1b* expression levels were decreased in the hypothalamus and the dorsal raphe region of KO mice fed with WD in comparison to KO mice fed with CD (p=0.006 for hypothalamus, p=0.002 for the dorsal raphe region, Tukey's test).

We found a significant interaction between genotype and diet in mRNA levels of *Htr2c* receptor in the hypothalamus, the dorsal raphe region and the prefrontal cortex (two-way ANOVA, p<0.05, **Table 3**), but not in hippocampus. *Htr2c* expression levels in the hypothalamus, the dorsal raphe region and the prefrontal cortex of KO mice fed with CD were increased compared to WT and HET mice fed with CD (p<0.001 and p<0.001 for hypothalamus, p=0.003 and p<0.001 for the dorsal raphe region, p=0.003 and p=0.002 for prefrontal cortex, Tukey's test; **Fig. 5D**). Decreased *Htr2c* expression levels in the hypothalamus and the dorsal raphe region of KO-WD compared to KO-CD were also detected (p=0.002 for hypothalamus, p=0.003 for the dorsal raphe region, Tukey's test).

There was a significant effect of genotype on mRNA levels of *Htr6* receptor in the hippocampus and the dorsal raphe region (p<0.05, **Table 3**). In hippocampus, *Htr6* expression was increased in HET mice compared to WT (p=0.029, Tukey's test; **Fig. 5E**). *Htr6* expression levels were significantly decreased in the dorsal raphe region of HET and KO mice compared to WT mice (p=0.010 and p=0.023, Tukey's test).

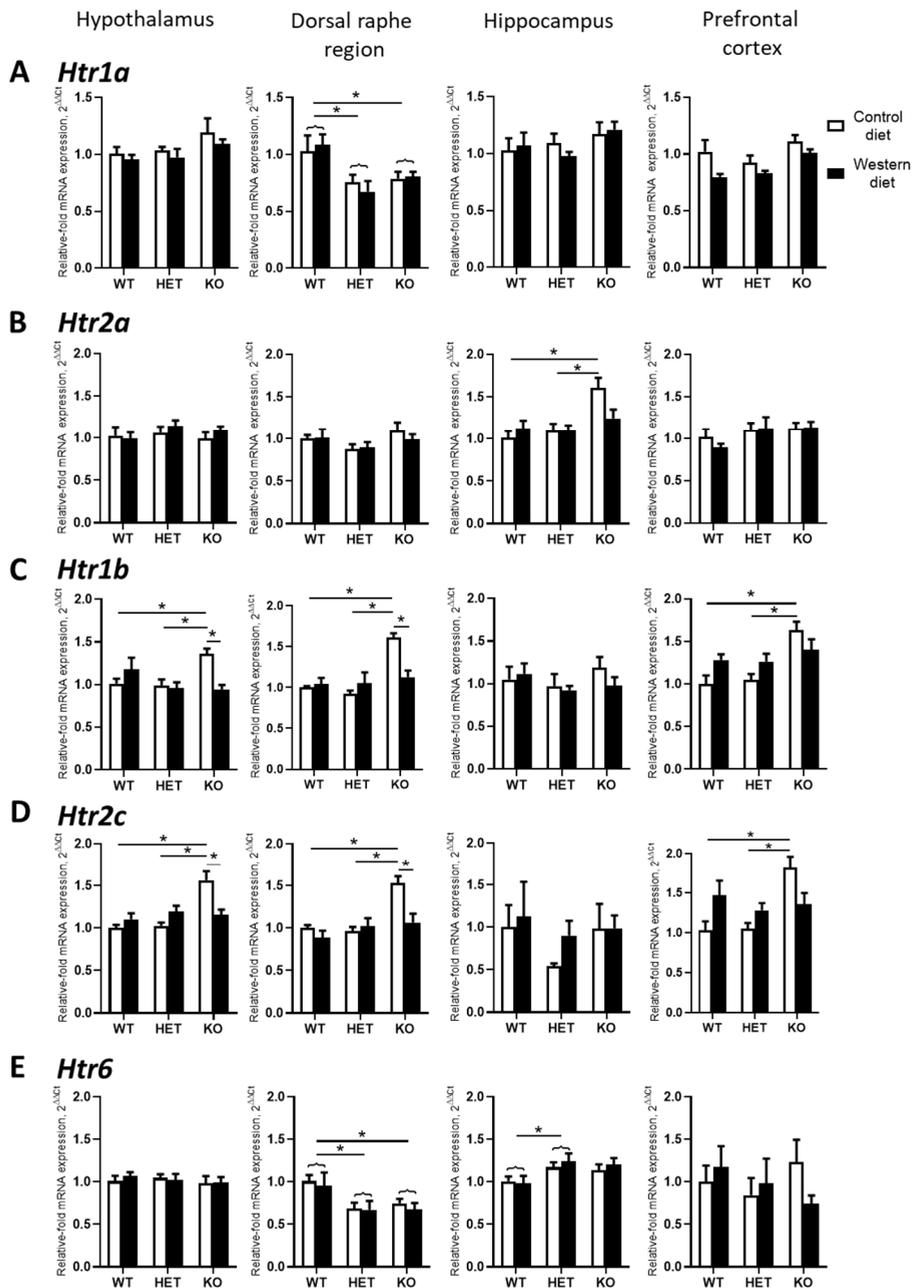


Figure 5. Changes in brain expression of serotonin receptors by Western diet and SERT deficiency. (A-B) *Htr1a* and *Htr2a* expression in hypothalamus, dorsal raphe region, hippocampus and prefrontal cortex.

(C-D) *Htr1b* and *Htr2c* expression in the brain. KO mice fed with CD compared to WT and HET mice fed with CD displayed significant increase of *Htr1b* and *Htr2c* expression levels in hypothalamus, dorsal raphe region and prefrontal cortex. Compared to KO mice fed with CD, KO mice fed with WD demonstrated significant decrease of *Htr1b* and *Htr2c* expression levels in hypothalamus and dorsal raphe region. (E) *Htr6* expression in hypothalamus, dorsal raphe region, hippocampus and prefrontal cortex. * $p < 0.05$, two-way ANOVA and Tukey's test, } – main genotype effect, 6-7 animals per group were used. Data are shown as mean \pm SEM.

That way, the expression of *Htr1b*, *Htr2a*, and *Htr2c* was increased in KO group fed with CD. Feeding with WD inversed these changes. *Sert*-deficient mice, irrespective of the diet, demonstrated a decreased *Htr1a* and *Htr6* expression in the dorsal raphe region.

3.4 Western diet and SERT deficiency affected emotionality and hippocampus-dependent performance

A comparison of exploratory rearing activity in the novel cage test during 1st minute of the test by two-way ANOVA showed a significant genotype effect on the number of rears ($p < 0.05$, **Table 4**). The number of rears was decreased in WD-fed KO group in comparison to WD-fed WT and HET mice ($p = 0.0034$ and $p = 0.0441$, respectively, Tukey's test; **Fig. 6A**), suggesting a reduced exploration in KO mice fed with WD.

In the O-maze test, there was a significant diet effect (two-way ANOVA, $p < 0.05$, **Table 4**) on the latency to exit to open arm. This parameter was decreased in KO mice fed with WD compared to KO

group fed with CD ($p=0.0095$, Tukey's test; **Fig. 6B**), which could be interpreted as a sign of increased impulsivity in KO-WD group.

Table 4. Two-way ANOVA results for statistical analysis of behavioural parameters.

<i>Behavioural parameters</i>						
<i>Parameter</i>	<i>Interaction, F</i>	<i>Interaction, p</i>	<i>Genotype, F</i>	<i>Genotype, p</i>	<i>Diet, F</i>	<i>Diet, p</i>
Number of rears, 1 st min	3.336	0.0502	5.034	0.0136	1.680	0.2055
Latency to exit lit arm	1.937	0.1652	1.888	0.1724	8.482	0.0074
Latency to float	1.080	0.3556	7.790	0.0025	13.34	0.0013
Duration of floating	1.303	0.2903	3.514	0.0459	33.47	<0.0001
Latency to immobility	0.08797	0.9161	1.601	0.2216	10.86	0.0029
Latency of 50% accomplishment in tube-test	4.210	0.0192	29.07	<0.0001	27.17	<0.0001

F and p values are shown for interaction between genotype and diet, for genotype effect and for diet effect.

In the tail suspension test, two-way ANOVA revealed a significant diet effect (two-way ANOVA, $p<0.05$, **Table 4**) on the latency to immobility (**Fig. 6C**). While no significant differences were observed between the groups, there was a trend of decreasing latency to immobility in groups fed with WD compared to the respective genotype-matched groups fed with CD. Significant genotype and diet effects ($p<0.05$, **Table 4**) were found in the latency to floating in the forced swim test. Post-hoc analysis revealed a significant decrease in the latency to floating in HET mice fed WD and KO mice fed CD in comparison to CD-fed HET group ($p=0.0276$ and $p=0.0356$,

respectively, Tukey's test, **Fig. 6D**). Total duration of floating was increased in WD-fed WT, HET, and KO groups compared to CD-fed mice (Supplementary Fig. S3). Correlation analysis revealed no significant correlation between mouse body weight and duration of floating, latency to floating, and latency to immobility ($p > 0.05$). This data suggests that WD feeding induces depressive-like behaviour in all *Sert* genotypes.

In the pellet-displacement test, there was a significant interaction between the genotype and diet type ($p < 0.05$, **Table 4**) in the latency of 50%-performance accomplishment. The latency of 50%-performance accomplishment was increased in WT-WD and KO-WD mice but not in HET-WD compared to mice fed with CD ($p < 0.001$ and $p = 0.037$, respectively, Tukey's test; **Fig. 6E**). In addition, this parameter was increased in KO group fed with CD compared to WT-CD and HET-CD ($p < 0.001$ and $p = 0.001$, respectively, Tukey's test) as well as in KO group fed with WD compared to WT-WD and HET-WD ($p < 0.001$ and $p < 0.001$, respectively, Tukey's test). Results of this test suggest that complete *Sert* deficiency impairs hippocampal-dependent performance. The same effect was observed in WT and KO but not HET mice fed with WD.

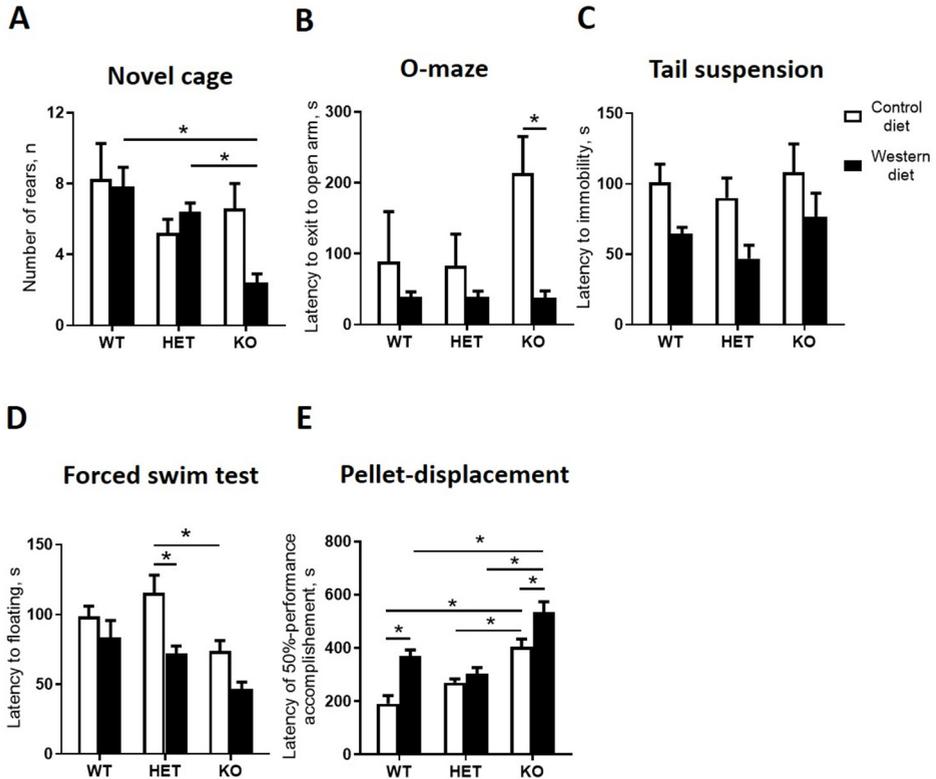


Figure 6. Western diet and SERT deficiency affected behavioural parameters, especially in KO mice. (A) Number of rears during the 1st minute in novel cage test. KO group fed with WD compared to WT and HET mice fed with WD showed decrease in the number of rears. (B) Latency to exit to open arm in O-maze. The latency to exit to open arm was decreased in KO mice fed with WD compared to KO group fed with CD. (C) Latency to immobility in tail suspension test. (D) Latency to floating in forced swim test. Compared to HET mice fed with CD, the latency to floating was decreased in HET mice fed with WD and KO mice fed with CD. (E) Latency of 50%-performance accomplishment in the pellet-displacement test. WT and KO mice fed with WD in comparison with mice fed with CD demonstrated increased latency of 50%-performance accomplishment. This parameter was also significantly increased in KO mice fed with CD and WD compared to respective diet WT and HET groups. * $p < 0.05$, two-way ANOVA and Tukey's test, 6-7 animals per group were used. Data are shown as mean \pm SEM.

4. Discussion

Our results have revealed that the effects of the WD are exacerbated in aged *Sert* KO mice on all our previously established metabolic, molecular and behavioural endpoints. Key hallmarks of the WD-induced syndrome were observed in the *Sert*^{-/-} mice and WT controls, including decreased glucose tolerance, brain expression of *Tlr4*, disrupted hippocampus-dependent performance, but these were not observed in *Sert*^{+/-} animals. However, all the genotypes challenged with WD displayed similar changes in weight gain, depressive-like behaviour, and suppressed expression of *Ppargc1a* and *Ppargc1b* in the liver. Most molecular changes that occurred in the two *Sert*-deficient genotypes developed regardless of diet. Overall, our data indicate distinct metabolic, molecular and behavioural effects of WD on aged mice with complete versus partial *Sert* inactivation and suggest resilience of *Sert*^{+/-} mice to several key negative effects of WD. Effects of WD on mice with different *Sert* genotype and comparison of HET and KO groups fed CD with WT mice are summarized in **Tables 5 and 6**.

Table 5. Comparison of WD and CD groups with different *Sert* genotype.

Parameter \ Group	WT-WD vs. WT-CD	HET-WD vs. HET-CD	KO-WD vs. KO-CD
<i>Metabolism</i>			
Body weight	↑	↑	↑
Food intake, week 1	↑	↑	↑
Water intake, weeks 2 and 3	=	↓	↓
Fasting blood glucose	=	=	↓
Glucose tolerance	↓	=	↓↓
<i>Liver gene expression</i>			
<i>Ppargc1a</i> and <i>b</i>	↓	=	=
<i>Brain gene expression</i>			
<i>Ppargc1a</i> , HT	=	=	↓
<i>Thr4</i> , DR	↑	=	↑
<i>Htr1b</i> , HT and DR	=	=	↓
<i>Htr2c</i> , HT and DR	=	=	↓
<i>Behaviour</i>			
Rearing in novel cage	=	=	↓
Latency to exit open arm in O-maze	=	=	↓
Duration of floating in swim test	↑	↑	↑
Latency to float in swim test	=	↓	=
Latency of 50%-performance accomplishment in pellet displacement test	↑	=	↑↑

Most of the metabolic, molecular and behavioural changes found in WT-WD mice were exacerbated in KO-WD. Some of the effects of the WD were absent in HET mice compared to WT and KO. HT – hypothalamus, DR – dorsal raphe region, HIP– hippocampus, PF – prefrontal cortex. Body weight was analyzed after 3 weeks of WD feeding. Food intake was measured in kkal/g of body weight, water intake – in ml/g of body weight.

Table 6. Comparison of HET and KO groups fed CD with WT mice.

Parameter \ Group	HET-CD vs. WT-CD	KO-CD vs. WT-CD
Metabolism		
Basal body weight	↑	↑↑
Food intake, weeks 2 and 3	=	↓
Liver gene expression		
<i>Ppargc1a</i> and <i>b</i>	↓	↓
Brain gene expression		
<i>Ppargc1a</i> , HT and PF	=	↑
<i>Ppargc1b</i> , HT	↓	↓
<i>Tlr4</i> , HT	=	↑
<i>Htr1a</i> , DR	↓	↓
<i>Htr2a</i> , HIP	=	↑
<i>Htr1b</i> , HT, DR and PF	=	↑
<i>Htr2c</i> , HT, DR and PF	=	↑
<i>Htr6</i> , DR	↓	↓
<i>Htr6</i> , HIP	↑	=
Behaviour		
Latency of 50%-performance accomplishment in pellet displacement test	=	↑

While some metabolic, molecular and behavioural differences with WT were similar in HET and KO, KO mice demonstrated a more distinguishable phenotype. HT – hypothalamus, DR – dorsal raphe region, HIP – hippocampus, PF – prefrontal cortex. Food intake was measured in kkal/g of body weight.

The present study on aged mice has replicated the principal findings reported for young mice fed the WD, such as impaired glucose tolerance, altered expression of *Tlr4*, *Ppargc1a* and *Ppargc1b* (Strekalova et al., 2015, 2016) and signs of emotional and cognitive

abnormalities (Strekalova et al., 2015, 2016; Veniaminova et al., 2016, 2017, 2020). In comparison with young mice, aged animals exposed to WD gained weight and exhibited less profound changes in the expression of markers of inflammation and mitochondrial function. This is likely due to age-related alterations in the expression of these genes (Burgueño et al., 2013; Letiembre et al., 2007; Scarpulla, 2002). The aged mice displayed a prolonged increase of intake in the amount of calories after the switch to the highly caloric WD lasting, which lasted for at least one week instead of three days in young mice (Strekalova et al., 2015). This is in accordance with the well-described age-related dysregulation of consummatory behaviour and metabolic processes (Gill et al., 2015) in the WD model.

Sert^{-/-} mice fed a WD displayed greater changes in most diet-induced abnormalities that were found in the WT controls, including impairment of glucose tolerance, behavioural despair, suppressed novelty exploration and hippocampus-dependent performance, impulsivity in the O-maze and brain over-expression of *Tlr4*. At the end of the experiment, in comparison with other genotypes, *Sert*^{-/-} mice displayed a significant decrease in the intake of calories and lowered blood levels of fasting glucose after being fed the WD. Similar findings were obtained after a 24-week exposure of hamsters to a high-fat diet (Guo et al., 2016) and seemed to be underpinned by the facilitated conversion of blood glucose to lipids, in corollary to the present study,

and *Sert*^{-/-} mice revealed increased glucose absorption in a bowel (Greig et al., 2017). Previous work with WD-fed six-month-old male *Sert*^{-/-} mice reported the opposite effects (Chen et al., 2012), but this appears to be due to a sex-related bias, and further supported by the dietary conditions used here (Comhair et al., 2011). Notably, the finding that there was reduced energy intake in *Sert*^{-/-} mutants rules out the possibility that their weight gain was due to increased diet intake. Instead, the metabolic changes described in *Sert*^{-/-} genotype are likely to be caused by functional disturbances in hypothalamic regulation of intake, which might be related to the over-expression of *Tlr4* that was observed in this brain structure. The activation of TLR4 by long-chain saturated fatty acids is considered as a major trigger of inflammatory mechanisms during excessive consumption of WD-like diets (Lancaster et al., 2018). Generalized brain over-expression of TLR4 on gene and protein levels was shown in WD-exposed young mice (Strekalova et al., 2015). Pro-inflammatory changes, and particularly, elevated expression of *Tlr4* in the hypothalamus, a primary brain area regulating metabolism (Kahn and Flier, 2000) were shown to affect insulin receptor signaling (Benomar et al., 2013; Wellen and Hotamisligil, 2003; Zhao et al., 2017). Accumulating evidence indicates that neuroinflammatory processes of various cause markedly affects insulin receptor sensitivity (Olefsky and Glass, 2010; Savage et al., 2001). The dysregulation of insulin receptor-mediated signaling can result in a

suppression of mitochondrial functions and decreased expression of *Ppargca1* and *PPargcb1* that is reported in the present study and in other experiments (Burgueño et al., 2013; Savage et al., 2001; Scarpulla, 2002; Strekalova et al., 2016). The latter results in impaired glucose tolerance, decreased metabolic rate and obesity (Kahn and Flier, 2000; Wellen and Hotamisligil, 2003). A close functional relationship between decreased SERT functions, pro-inflammatory changes and insulin resistance is well supported by the literature (Haub et al., 2010; Pomytkin et al., 2015, 2018).

Naïve *Sert*^{-/-} mice displayed increased brain expression of *Ppargc1a*, *Htr2a*, *Htr1b* and *Htr2c* that were not found in other genotypes and were “reversed” in WD-fed animals. Given the previously demonstrated association between most of these receptors with obesity and aging (Lee et al., 1998; Meltzer et al., 1998; Nonogaki et al., 2006; Ridderstråle et al., 2006; Simansky and Nicklous, 2002) it can be hypothesized that these changes may be adaptive, and WD disrupts these compensatory changes resulting in aggravation of metabolic and behavioural abnormalities in *Sert*^{-/-} mutants.

Expression of several genes was found to be similarly altered in both *Sert*^{-/-} and *Sert*^{+/-} genotypes, regardless dietary conditions. Decreases in expression of *Ppargc1b*, *Htr1a*, *Htr6* in the brain, and of *Ppargc1a* and *Ppargc1b* in the liver were similar in both genotypes and were not affected by WD, suggesting ceiling effects of complete or

partial Sert deficiency on these receptors. Earlier studies revealed lowered *Htr1a* receptor expression in the dorsal raphe region of female *Sert^{-/-}* and *Sert^{+/-}* mice that was not found in the hypothalamus or hippocampus (Li et al., 2000). Altered function of 5-HT1A and 5-HT6 are known to underlie depressive-like behaviours (Savitz et al., 2009; Wesołowska, 2010), cognitive (Mitchell and Neumaier, 2005; Ögren et al., 2008) and social (Meneses, 2001; Wang et al., 2013) abnormalities, and likely to be implicated in the behavioural changes observed here.

The greater reduction of brain *Ppargc1b* expression in mutants than in controls could be associated with more pronounced obesity in the former groups. Similar to the changes in expression of serotonin receptors induced by WD reported here have been previously reported. For example, cafeteria and high-energy diets decreased brain expression of *Htr2c* receptor in rat (Beilharz et al., 2018; Lopez-Esparza et al., 2015). Mice exposed to high-fat diet displayed changes in expression of *Htr2a* in the olfactory nucleus and of *Htr2c* in the medial amygdaloid nucleus (Huang et al., 2004). 5-HT2C and 5-HT1B receptors were shown to play an inhibitory role in the regulation of calories intake, while elevated gene expression in the hypothalamus was proposed as a compensatory mechanism of hyperphagia in Ay mice (Nonogaki et al., 2006). 5-HT1B receptor was found to regulate food intake and insulin receptor sensitivity in mice with genetic inactivation of 5-HT2C receptor (Lee et al., 1998; Simansky and Nicklous, 2002).

Similar to our results, compromised expression of 5-HT_{2C} receptor was associated with obesity, abnormal feeding behaviour (Heisler et al., 1998) as well as insulin resistance and elevated blood glucose concentrations (Zhou et al., 2007). Finally, 5-HT₆ receptor ligands have been demonstrated to improve insulin receptor sensitivity and regulate blood insulin, glucose concentrations, mechanisms of satiety and body weight (Heal et al., 2008).

Remarkably, unlike WT controls and *Sert*^{-/-} mice *Sert*^{+/-} mice did not reveal critical hallmarks of the WD-induced syndrome, such as decreases in glucose tolerance, brain expression of *Tlr4* and disrupted hippocampus-dependent performance. In contrast to *Sert*^{-/-} they showed no changes in the brain expression of *Ppargc1a*, *Htr2a*, *Htr1b* and *Htr1c* receptors, decreases in calories and water intake, fasting glucose concentration, as well as novelty hypoexploration and impulsive-like behaviour in the elevated O-maze. Thus, *Sert*^{+/-} mice were different from the *Sert*^{-/-} genotype consequences following WD challenge and exhibited partial resilience to its negative effects on the metabolic parameters and associated changes. This is in line with previous findings suggesting distinct physiology of two genotypes as has been shown for the expression of HPA regulatory protein binding protein 5 (FK506) in the pituitary of mice exposed to early life stress model (van der Doelen et al., 2014) and reduced basal corticosterone

plasma levels and improved memory performance in the object recognition test in *Sert*^{+/-} mice (van den Hove et al., 2011).

Relative resilience of *Sert*^{+/-} mice to the diet might be viewed as an improved ability to adjust to environmental changes associated with this genotype (Belsky et al., 2009). It is also observed in clinical studies that showed beneficial effects of heterozygosity of SERT, such as higher cognitive function in elderly adults (Fiedorowicz et al., 2007) and other differences (Malmberg et al., 2008; van Dyck et al., 2005) and generally greater fitness in heterozygotes, because they show a broader range of gene expression than both homozygotes (Comings and MacMurray, 2000; Homberg and Lesch, 2011). This phenomenon is also discussed in framework of heterosis (Sonuga-Barke et al., 2011), or outbreeding enhancement, i.e. the improved or increased function of any biological quality in a hybrid offspring (Shull, 1948).

Conclusions

The comparison of the effects of the WD in *Sert*^{-/-} mice *Sert*^{+/-} reveals an intricate interplay between SERT deficiency and regulation of metabolism during aging. Thus, complete versus partial genetic SERT deficiency in aged mice is associated with distinct metabolic, molecular and behavioural consequences following the WD-challenge. While some diet-induced changes were similar in KO-WD and HET-WD mice, the latter displayed a “rescued” phenotype in terms of

dietary-induced decrease in glucose tolerance, neuroinflammation and hippocampus-dependent behaviour. Sert deficiency was found to enhance inflammatory processes (Haub et al., 2010) and null-mutant Sert mice demonstrate higher susceptibility to the effects of WD on *Tlr4* expression.

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Author Contributions Statement

KPL and TS conceived the study. TS, AS, OK, DA designed the experiments. EV, SM, TS, DA carried out the animal experiments. EV performed the molecular analyses. EV, RC, SM performed data analysis. IC, AK, DA, TS supervised the project. IC, OK, KPL, TS got the funding. EV, TS wrote the initial draft of the manuscript and all other authors (RC, IC, AS, SM, OK, AK, DA, KPL) revised it.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Supplementary tables

Supplementary table S1. Composition of diets used in the study.

Diet	Western diet		Control diet		
	%	gm	<i>kcal</i>	gm	<i>kcal</i>
Protein	16.8	16	15	14.3	15
Carbohydrate	50.5	50	43	72.0	75
Fat	21.3	21	42	4.3	10
Total			100		100
kcal/gm	4.61			3.84	
Ingredient	gm	<i>kcal</i>	gm	<i>kcal</i>	
Casein, 30 Mesh	106	424	106	424	
L-Cystine	1.6	6.4	1.6	6.4	
Sucrose	150	600	150	600	
Maltodextrin 10	100	400	150	600	
Corn Starch	216	864	481	1924	
Cellulose, BW200	50	0	50	0	
Soybean Oil	0	0	25	225	
Palm Oil	185	1665	20	180	
Mineral Mix S10026	10	0	10	0	
DiCalcium Phosphate	13	0	13	0	
Calcium Carbonate	5.5	0	5.5	0	
Potassium Citrate, 1 H ₂ O	16.5	0	16.5	0	
Vitamin Mix V10001	10	40	10	40	
Choline Bitartrate	2	0	2	0	
Cholesterol, NF	1.8	0	0	0	
Total	867.45	3999	1040.65	3999	

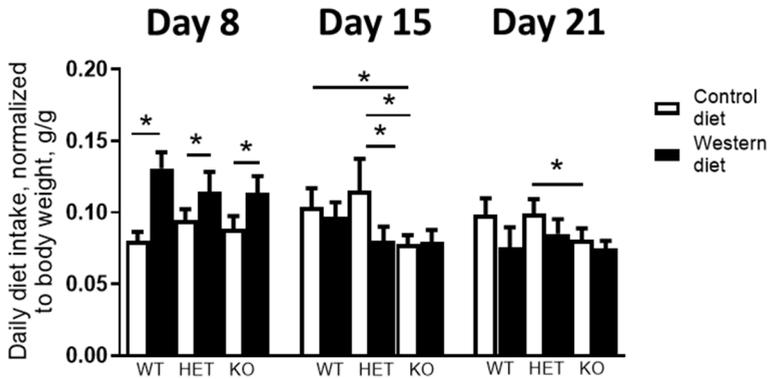
Compared to control diet, Western diet is characterized by increased content of fat, cholesterol and sucrose and high energy density.

Supplementary Table S2. Sequences for primers used in qPCR.

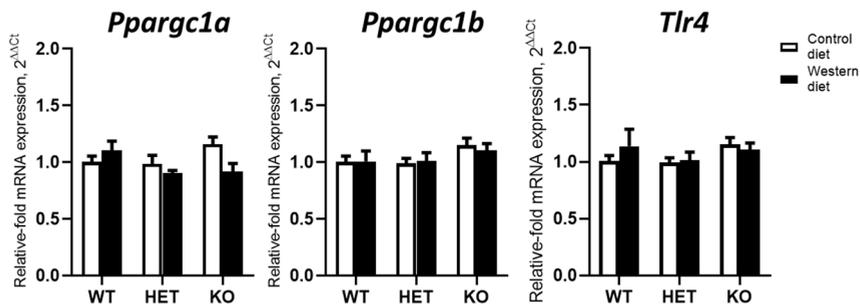
Gene	Gene ID	Forward primer	Reverse primer
<i>Gapdh</i>	14433	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC
<i>Actb</i>	11461	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
<i>B2m</i>	12010	QuantiTect Primer Assays Cat. No. QT01149547 (Qiagen, Netherlands)	
<i>Htr1a</i>	15550	AACCAGTTTTGTGTCTCTCA	AGCACCTAAATAATTTTCTCTC
<i>Htr1b</i>	15551	CGCCGACGGCTACATTTAC	TAGCTTCCGGGTCCGATACA
<i>Htr2a</i>	15558	CAGGCAAGTCACAGGATAGC	TTAAGCAGAAAGAAAATCCCA CA
<i>Htr2c</i>	15560	CTAATTGGCCTATTGGTTTG GCA	CGGGAATTGAAACAAGCGTCC
<i>Htr6</i>	15565	GCATAGCTCAGGCCGTATGT	CACCACTGTGAGAGGTCCAC
<i>Tlr4</i>	21898	CTGGCTAGGACTCTGATCAT G	GCATTGGTAGGTAATATTAGG AACTA
<i>Ppargc1a</i>	19017	CTCCAGTTCCGGCTCCTC	CCCTGTGCTCTCACGTCTG
<i>Ppargc1b</i>	170826	CTCCAGTTCCGGCTCCTC	CCCTGTGCTCTCACGTCTG

Specific primer pairs were used in qPCR for gene expression measurement.

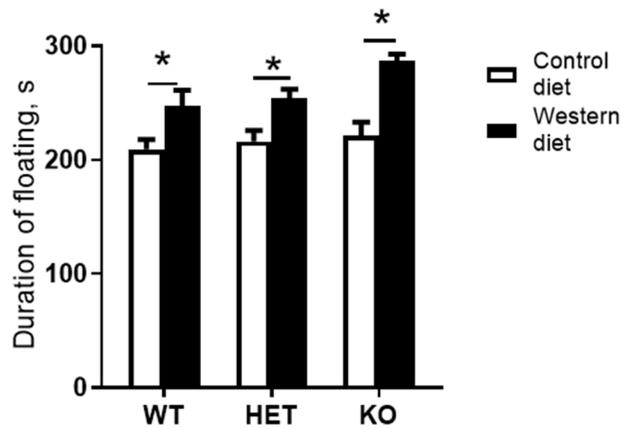
Supplementary Figures



Supplementary Fig. S1. Daily diet intake measured in grams of diet per gram of body weight. During the 1st week of the experiment, housing on WD led to increased daily diet intake in WT, HET and KO mice compared to mice fed with CD ($p < 0.001$, $p = 0.044$, and $p = 0.003$, respectively). During the 2nd week, diet intake was decreased in KO mice fed with CD compared to WT and HET fed with CD ($p = 0.027$ and $p = 0.030$, respectively) and in HET WD compared to HET CD ($p = 0.038$, Tukey's). During the 3rd week, calorie intake was decreased in KO-CD mice compared to HET-CD ($p = 0.023$, Tukey's test). * $p < 0.05$, two-way ANOVA and Tukey's test, 6-7 animals per group were used. Data are shown as mean \pm SEM.



Supplementary Fig. S2. *Ppargc1a*, *Ppargc1b* and *Tlr4* expression in the hippocampus. No differences in gene expression of *Ppargc1a*, *Ppargc1b* and *Tlr4* in the hippocampus were found between the groups. $p > 0.05$, two-way ANOVA and Tukey's test, 6-7 animals per group were used. Data are shown as mean \pm SEM.



Supplementary Fig. S3. Duration of floating in forced swim test. Groups fed with WD demonstrated increased duration of floating compared to controls ($p=0.049$, $p=0.030$, $p<0.001$, respectively). $*p<0.05$, two-way ANOVA and Tukey's test, 6-7 animals per group were used. Data are shown as mean \pm SEM.

Chapter 5

General discussion

Chapter 5. General discussion

5.1 Environmental factors aggravating autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) symptoms: focus on diet

Autism spectrum disorder (ASD) refers to a broad spectrum of conditions characterized by impaired social behaviour, communication and language, and a narrow range of interests and activities (WHO). Attention-deficit/hyperactivity disorder (ADHD) is a disorder defined by a persistent pattern of inattention and/or hyperactivity-impulsivity that interferes with functioning or development (National Institutes of Health). Both ASD and ADHD are considered to be highly heritable, however, exposure to specific environmental factors might be necessary to trigger development of the disorder (Rommelse et al., 2010). Studies have shown that there is an increased risk of ASD and ADHD in offspring due to particular prenatal and perinatal environmental exposures, such as stress, medications, chemical exposure, alcohol and tobacco exposure, maternal metabolic status, and diet (Froehlich et al., 2011; Peretti et al., 2018). The influence of maternal diet during the prenatal period on the neurodevelopment of the offspring was demonstrated both in humans and animal models (Lyall et al., 2014; Sullivan et al., 2014). ASD and ADHD demonstrate significant diagnostic overlap and shared characteristics (Taurines et al., 2012; Visser et al., 2016). These disorders co-occur with a high frequency: in

20–50% of children with ADHD meeting criteria for ASD and in 30-80% of ASD children meeting criteria for ADHD (Rommelse et al., 2010), with the co-occurrence found to increase with age (Rommelse et al., 2011).

While social dysfunction is a core symptom in ASD (Leitner, 2014), social difficulties are also a common feature of ADHD (Carpenter Rich et al., 2009). We found that adult female mice fed with Western diet spent less time in social contact both in a home cage and when encountered with an unfamiliar mouse (Veniaminova et al., 2017) (**Chapter 2**). Previously, maternal high-fat diet was shown to negatively impact offspring social behaviour in mice (Buffington et al., 2016a). Later, our findings of altered diet-induced social behaviour in the postnatal period were supported by other studies. It was reported that high-fat diet impairs social memory in adolescent rats (Reichelt et al., 2019) and reduces sociability in adult male mice (Hassan et al., 2019). Finally, Western diet, when fed from weaning to adulthood, was shown to induce increased cognitive rigidity and diminished preference for social novelty in a BTBR T+tf/J mice, which present a common model for autism (Zilkha et al., 2017).

In our model, mice fed with the Western diet were found to display behavioural abnormalities reminiscent of certain key ADHD-like symptoms: locomotor hyperactivity (Veniaminova et al., 2017) (**Chapter 2**), increased impulsivity (Strekalova et al., 2015),

and emotional dysregulation in classic tests for anxiety and behavioural despair (Strekalova et al., 2016; Veniaminova et al., 2020, *under revision*) (**Chapters 3 and 4**). In addition, Western diet feeding led to impaired cognitive functions and motor coordination (Veniaminova et al., 2017, 2020) (**Chapters 2 and 3**). Motor coordination deficits were independent of changes in muscle weight, strength, or body weight. Problems with motor coordination and cognitive impairment, including difficulties with learning, have also been reported for ASD (Fournier et al., 2010; O'Brien and Pearson, 2004) and ADHD patients (Fliers et al., 2008; Mayes et al., 2000).

Thus, in our experiments, we found phenomenological similarity between the behaviours observed in mice fed with the Western diet and behaviours associated with human ASD and ADHD. Up to date, the impact of diet on ASD and ADHD in adulthood has inadequately been investigated. Several studies have reported beneficial effects of dietary interventions in children with ASD or ADHD (Heilskov Rytter et al., 2015; Pelsler et al., 2017; Sanctuary et al., 2018). Abnormally high intestinal permeability was shown in nearly 40% of autistic patients whereas this excessive permeability was present in less than 5% of normal control subjects (D'Eufemia et al., 1996). An altered gut microbiome, which can increase gut permeability, was demonstrated in ADHD (Ly et al., 2017). High intestinal permeability, which is associated with proinflammatory processes (Ding et al., 2010; Yiu et

al., 2017), can contribute to exacerbated reactions to food components in the intestinal tract of people with ASD and ADHD. Thus, it may be hypothesized that adherence to Western dietary pattern might aggravate ADHD and ASD symptoms in adulthood, however, clinical studies are needed to investigate this hypothesis.

5.2 Toll-like receptor 4 (Tlr4) and microglia activation in ASD, ADHD and depression

In our model, Western diet induced ASD/ADHD-like behaviours and altered emotionality in mice, and this was accompanied by signs of neuroinflammation such as increased microglial density in prefrontal cortex (Veniaminova et al., 2020) (**Chapter 3**) and elevated brain expression of *Tlr4* (Strekalova et al., 2015, 2016; Veniaminova et al., *under revision*) (**Chapter 4**). A growing body of evidence suggests that neuroimmune responses play an important role in the pathophysiology of mental disorders including ASD, ADHD and depression (Bennett and Molofsky, 2019).

The only way to confirm the involvement of microglia in the above-mentioned disorders in humans is to perform postmortem tissue examination or imaging studies. Assessment of microglia in postmortem brains of autism cases demonstrated cortical and cerebellum microglial activation (Morgan et al., 2010; Vargas et al., 2005). Strong activation of microglia specifically in the dorsolateral prefrontal cortex was evident, however neither microglial soma volume

nor density showed significant correlation with ASD patient age. This suggests that microglial activation may be a long-running alteration present in developing and established ASD (Morgan et al., 2010). The early presence of microglial activation during the period of brain development indicated that it may play a central role in the pathogenesis of autism. In addition, positron emission tomography (PET) imaging, which is regarded to be the only valid method to evaluate microglial activation in the brain of living subjects, indicated excessive microglial activation in multiple brain regions in young adult patients with ASD (Suzuki et al., 2013).

When activated, microglial cells release proinflammatory cytokines and other factors contributing to neuroinflammation. Cytokine profiling indicated that MCP-1 and tumor growth factor- β 1, derived from neuroglia, were the most prevalent cytokines in ASD brain tissues (Vargas et al., 2005). CSF showed a unique proinflammatory profile of cytokines in ASD, including a marked increase in MCP-1 (Vargas et al., 2005). Also, a recent meta-analysis identified higher concentrations of IL-1 β , IL-6, IL-8, interferon-gamma, eotaxin and MCP-1 in the blood of participants with ASD compared to healthy controls (Masi et al., 2015).

So far, no direct study in postmortem brain or in living humans has investigated microglial activation associated with ADHD (Anand et al., 2017). However, there is indirect evidence that activation of

microglia might play a role in ADHD development. ADHD symptoms were associated with increase in serum IL-16, which is found to promote recruitment of microglial cells to injured axons, and higher levels of IFN γ , which has been shown to change the protective response of microglia and macrophages into a proinflammatory response (Oades et al., 2010). The results of other peripheral cytokines levels were complex and inconsistent, but overall, they are suggestive of a low-grade inflammatory profile in patients with ADHD (Anand et al., 2017).

PET imaging revealed increased measure of microglial activation in the prefrontal cortex, anterior cingulate cortex and insula of patients with major depressive disorder (MDD) during a major depressive episode compared to healthy controls (Setiawan et al., 2015). Microglial activation measured by PET was greater in patients with chronologically advanced MDD with long periods of no antidepressant treatment than in patients with MDD with short periods of no antidepressant treatment, which is strongly suggestive of a different illness phase. Consistent with this, the yearly increase in microglial activation was no longer evident when antidepressant treatment was given (Setiawan et al., 2018). Another PET imaging study confirmed the evidence for increased microglial activation during major depressive episode, especially in patients with suicidal thoughts (Holmes et al., 2018). Postmortem studies were mainly focused on suicide victims with

depression, demonstrating increased microglia activation in multiple brain areas (Schnieder et al., 2014; Steiner et al., 2008; Torres-Platas et al., 2014). Numerous studies have reported increases in circulating proinflammatory cytokines, IL-1, IL-6, TNF, CRP and MCP-1, in patients with major depression (Felger and Lotrich, 2013; Young et al., 2014). Some studies also observed increased proinflammatory cytokine concentrations in the CSF of depressed patients compared to controls or correlations between CSF cytokines and depression severity (Levine et al., 1999; Lindqvist et al., 2009; Martinez et al., 2012).

Microglia cells express numerous members of the TLR family, including TLR4 (Kielian, 2006). TLR4 has been reported to induce microglial activation and cytokine production, promoting inflammation, which in turn may positively regulate increased expression of TLR4 (Kielian, 2006). Mice fed with the Western diet demonstrated increased TLR4 expression together with impairment in social, cognitive, motor, and emotionality-related behaviours (Strekalova et al., 2016; (Veniaminova et al., 2017, 2020, *under revision*) (**Chapters 2, 3 and 4**). In agreement with our results, other animal studies suggested involvement of TLR4-dependent signaling in the development of ASD/ADHD-like behaviours. Embryos and newborns of BTBR T+tf/J mice demonstrated increased Tlr4 and proinflammatory cytokine expression both in blood and in the brains compared to wild type animals (Cipriani et al., 2018). Improvement of

ASD-like behaviour in adult BTBR T+tf/J mice after treatment with natural phenol resveratrol was accompanied by decreased Tlrs, including Tlr4, and NF- α β expression (Ahmad et al., 2018). *Tlr4* knockout mice displayed altered emotional and social behaviour, further supporting that TLR4 signaling is involved in emotional regulation (Femenia et al., 2018)

Moreover, ASD individuals demonstrated increased TLR4 expression on T cells (Nadeem et al., 2017), and higher impairment in social behaviours as well as non-verbal communication were associated with increased production of IL-1 β and IL-6 after TLR4 stimulation (Enstrom et al., 2010). Altered TLR responses, including increased production of proinflammatory cytokine IL-23 in blood mononuclear cells in response to TLR4 agonist LPS, were found in a subset of ASD children characterized by frequent infections and by worsening behavioural symptoms following infection (Jyonouchi et al., 2008). NF- α B upregulation had been found in the postmortem prefrontal cortex in ASD and depression patients (Malki et al., 2015; Young et al., 2011). Upregulated TLR4 expression was demonstrated in peripheral blood mononuclear cells in newly diagnosed patients with MDD (Kéri et al., 2014) and in brain tissue of depressed subjects, compared with controls (Pandey et al., 2014). While the involvement of TLR4-dependent signaling was not directly investigated in ADHD patients, a genome-wide association study found an increased burden

of genetic variation in the genes involved in TLR signaling to be associated with ADHD (O’Dushlaine et al., 2015). Exploration of the possible role of TLR4 in pathogenesis of ADHD requires further clinical studies.

In summary, based on clinical studies and postmortem patient examination, it can be suggested that microglial activation and TLR4 signaling play a significant role in the mechanisms of ASD, depression and, likely, ADHD (**Table. 5.1**). Our results in an animal model of the Western diet feeding are in line with these data, further supporting the hypothesis of a poor diet as a key environmental factor contributing to the development of mental disorders.

Table 5.1. Clinical data supporting the involvement of TLR and microglia activation in mechanisms of ASD, ADHD and depression.

Disorder	Microglial activation	TLR activation
ASD	Postmortem examination PET imaging	Altered TLR responses ↑ TLR4 on T cells, ↑ NF- κ B
ADHD	No direct examination ↑ serum IL-16 and INF γ	No direct examination Polymorphisms in genes involved in TLR signaling
Depression	Postmortem examination PET imaging	↑ TLR4 in brain and blood ↑ NF- κ B

In patients with ASD and depression, postmortem brain examination and PET imaging revealed marked microglia activation. To date, there is only indirect evidence that activation of microglia might play a role in ADHD development, such as increased levels of IL-16 and INF γ , as no direct study has investigated microglial activation in ADHD patients. Patients with ASD demonstrate altered TLR responses and both in ASD and depression TLR and NF- κ B are upregulated. Polymorphisms in genes related to TLR signaling were detected in ADHD subjects.

5.3 Oxidative stress and mitochondrial dysfunction in ASD, ADHD and depression

An increase in oxidative stress and a decrease in the antioxidant capacity of the brain are suggested as key factors involved in the mechanisms of neuropsychiatric diseases (Popa-Wagner et al., 2013). ROS can produce membrane damage, affect the structure and function in the inner proteins, cause denaturation of lipids and structural damage to DNA. Mitochondria is the most important source of ROS. The brain, being the major metabolizer of oxygen and containing a large amount of polyunsaturated peroxidizable fatty acids, is especially vulnerable to oxidative stress (Popa-Wagner et al., 2013). Oxidative stress can impair neuronal proliferation and mediate apoptosis, and therefore lead to progressive neuronal damage and disturbance of normal brain functions (Kannan and Jain, 2000; Klein and Ackerman, 2003). The levels of several metabolites and activity of enzymes altered in brain tissue and serum can be used as potential biomarkers of central and peripheral oxidative stress.

The Western diet in our model induced an increase in the level of marker for lipid peroxidation malondialdehyde (MDA) (Veniaminova et al., 2020) (**Chapter 3**) and decrease in the expression of mitochondrial biogenesis regulators peroxisome proliferator-activated receptor gamma coactivator (Ppargc) 1a and b in mouse brain (Strekalova et al., 2016; Veniaminova et al., 2017, *under revision*)

(**Chapters 2 and 4**). These changes are the signs of oxidative stress and impaired mitochondrial functioning in brain. Western diet feeding in mice also induced behaviours reminiscent of several symptoms of ASD, ADHD and depression. Taken together, the data on the involvement of mitochondrial dysfunction and oxidative stress in ASD, ADHD and depression would strengthen our hypothesis on the role of a dietary factor in the mechanisms of these disorders.

Reports indicate increased levels of lipid peroxidation markers in ASD patients, thus confirming an increased level of oxidative stress in autism. Compared to healthy controls, autistic subjects demonstrated elevated MDA in plasma (Chauhan et al., 2004) and erythrocytes (Zoroglu et al., 2004). Increased levels of NO, a toxic free radical that reacts with the superoxide anion and generates cytotoxic peroxynitrate anions (ONOO⁻), have been reported in red blood cells of patients with autism and have suggested that nitric oxide synthase (NOS) may be activated in autism (Söğüt et al., 2003). Elevated plasma levels of nitrite and nitrate were also shown in ASD patients (Sweeten et al., 2004; Zoroglu et al., 2003). Increased activity of xanthine oxidase (XO), which is an endogenous prooxidant, has been reported in the erythrocytes of patients with ASD (Zoroglu et al., 2004).

Several studies have suggested alterations in the enzymes that play an important role in the defense mechanism against damage by ROS in autism. Compared to healthy control, ASD patients exhibited

significantly lower total antioxidant status (TAS), which represents the cumulative effect of all antioxidants, in urine (Yui et al., 2017) and serum (Ozturk et al., 2016). Patients with autism showed decreased activity of glutathione peroxidase in plasma and erythrocytes (Paşca et al., 2006; Yorbik et al., 2002), as well as reduced levels of total glutathione and lower redox ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) in plasma (James et al., 2004). Decreased catalase and superoxide dismutase (SOD) activity in erythrocytes has also been indicated in individuals with autism (Yorbik et al., 2002). Consistent with studies on plasma and immune cells, GSSG and GSH/GSSG were significantly decreased in both cerebellum and temporal cortex of ASD patients (Rose et al., 2012). There was also a significant increase in biomarkers of oxidative protein damage, 3-nitrotyrosine (3-NT), and oxidative DNA damage, 8-oxo-deoxyguanosine (8-oxo-dG), in the ASD brain (Rose et al., 2012).

Mitochondrial DNA (mtDNA) copy number is a strong biomarker for mitochondrial dysfunction, since it may be increased with mtDNA damage or mitochondrial dysregulation to compensate for impaired mitochondrial energy metabolism. In several reports, the mtDNA copy number was increased in the peripheral blood and prefrontal cortex of ASD patients (Gu et al., 2013; Yoo et al., 2017). Likewise, activities of electron transport chain complexes and pyruvate dehydrogenase (PDH) were significantly reduced in muscle tissue, blood and brain,

including cerebellum and prefrontal cortex (Chauhan et al., 2011; Gu et al., 2013; Guevara-Campos et al., 2010; Oliveira et al., 2005).

PPARGC1a and b are important regulators of mitochondrial fatty acid oxidation, which is impaired in ASD. These co-activators bind and activate a number of transcription factors, including the nuclear peroxisome proliferator-activated receptors (PPARs), and, accordingly, mediate a variety of stimulatory effects on mitochondrial bioenergetics across multiple organs, including the brain (Lin et al., 2005; Nierenberg et al., 2018). Various studies document the presence of PPARs in neurons and glial brain cells and support the role of these nuclear receptors in neuroprotection (Agarwal et al., 2017; Tontonoz and Spiegelman, 2008). Evidence suggests the effectiveness of PPAR α activation in amelioration of ASD-like symptoms in BTBR T+tf/J mice (D'Agostino et al., 2015). In addition, PPAR α -deficient mice have a distinct cognitive and behavioural phenotype characterized by reduced spatial information processing and cognitive flexibility, along with repetitive behaviour reminiscent of that seen in preclinical models of ASD (D'Agostino et al., 2015). Finally, the use of PPAR γ activator pioglitazone was effective in the amelioration of behavioural symptoms of ASD in clinical trials (Ghaleiha et al., 2015).

Various studies demonstrated elevated levels of peripheral oxidative damage markers in ADHD. For example, increased lipid peroxidation was shown in ADHD reflected by raised urinary acrolein-

lysine levels and by breath ethane levels (Kawatani et al., 2011; Ross et al., 2003). Results on MDA are inconsistent, with higher (Bulut et al., 2007; Ceylan et al., 2010) as well as lower plasma levels found in ADHD patients compared to controls (Oztop et al., 2012). Higher serum xanthine oxidase (XO) activity was observed in paediatric ADHD patients (Ceylan et al., 2012). In addition, NO levels and NOS activity were higher in ADHD patients than in controls, but while NOS activity was positively correlated with teacher hyperactivity ratings, ADHD subtype did not correlate with NO level or NOS activity (Ceylan et al., 2010, 2012). Total DNA damage in ADHD as compared to controls was increased as determined by the concentration of 8-oxo-7,8-dihydroguanine (8-oxoG), the oxidized product of a free guanine base, in lymphocytes (Chovanová et al., 2006). Finally, higher total oxidant status (TOS) representing the cumulative effect of all oxidants present in the investigated sample and oxidative stress index (OSI) were found in ADHD (Guney et al., 2015; Kul et al., 2015; Sezen et al., 2016).

Plasma and saliva activity levels of the antioxidant enzymes glutathione-S-transferase (GST), SOD and catalase (CAT) were decreased in children and adolescents with ADHD as compared to controls, indicating lower antioxidant activity (Ceylan et al., 2010, 2012; Ruchi et al., 2011; Russo, 2010). Evidence concerning thiol levels is conflicting, as lower and higher levels were reported in plasma and

serum of ADHD patients compared to controls (Alpak et al., 2014; Avcil et al., 2017; Guney et al., 2015). Finally, many studies have reported lower TAS in ADHD as compared to healthy participants (Kul et al., 2015; Ruchi et al., 2011; Sezen et al., 2016). Higher TAS and no difference have also been reported (Guney et al., 2015). It should be mentioned that higher antioxidant levels in some cases might be attributed to a reactive, compensatory increase due to protection against increased oxidative stress (Guney et al., 2015; Oztop et al., 2012).

Several recent studies showed mitochondrial dysfunction in ADHD pathogenesis. Lower mitochondrial respiration, lower ATPase 6/8 transcripts levels, reduced mitochondrial complex V activity and loss of mitochondrial membrane potential were reported in cybrids from ADHD patients compared to healthy controls (Verma et al., 2016). Another study showed a significant association of mtDNA 10398 A/G polymorphism with ADHD in children (Hwang et al., 2017). Relative mtDNA copy number was significantly higher and mtDNA methylation ratio of PPARGC1A was decreased in ADHD patients compared to healthy volunteers (Kim et al., 2019).

Oxidative stress-related damage has been described extensively in depression and, unlike the data on ASD and ADHD obtained mostly from blood or peripheral tissue, many studies were done using brain tissue. Membrane damage was shown in the blood of patients with

depression by elevated lipid peroxidation products (Peet et al., 1998; Sarandol et al., 2007). In patients with depression, elevated levels of MDA were found to adversely affect cognitive function and were associated with increased severity of depressive symptoms (Talarowska et al., 2012). Antidepressant administration decreased MDA levels (Gałecki et al., 2009; Khanzode et al., 2003). In addition, serum levels of a marker of lipid peroxidation 8-isoprostane-F2alpha were shown to be higher in patients with MDD compared to healthy controls (Yager et al., 2010). Cerebral membrane abnormalities and altered membrane phospholipids have been suggested by an increased choline-containing compound seen in the putamen of patients with depression which has been interpreted as a result of increased oxidative stress (Ende et al., 2007).

One report of unmedicated patients with depression found decreased TAS in plasma as well as increased total plasma peroxide and OSI compared to healthy control subjects (Yanik et al., 2004). The activity of antioxidant enzymes in patients with depression is controversial. Both increased and decreased activity of SOD has been described (Kodydková et al., 2009; Lukic et al., 2014). In the frontal cortex, Cu/Zn-SOD concentration was increased in patients with depression compared to matched controls (Michel et al., 2007). Cu/Zn-SOD is mainly located in glial cells, which are more vulnerable to oxidative damage (Papadopoulos et al., 1997). There was also a

significant increase in XO activity in some brain areas of patients with MDD compared to non-affected controls (Michel et al., 2010). Lowered activity of glutathione peroxidase, which causes accumulation of ROS, was detected in patients with depression (Maes et al., 2011). Some studies on the other hand did not find significantly different level of glutathione peroxidase in depressed patients (Gałecki et al., 2009; Lukic et al., 2014). In urine of depressed patients, elevated levels of 8-OxoG were detected compared to healthy controls (Maes et al., 2009), suggestive of oxidative DNA damage.

Depression is accompanied by mitochondrial disturbances, such as lower activities of respiratory chain enzymes and ATP production (Gardner, 2003). Findings in brains of depressed patients demonstrated altered expression of mitochondrial-located proteins in the anterior cingulate cortex (Beasley et al., 2006) and complex I subunits in the cerebellum (Ben-Shachar and Karry, 2008). MtDNA copy number was increased in patients with depression and anxiety and the change in mtDNA copy number after treatment was associated with the treatment response (Wang et al., 2017). In addition, PPAR γ and PPAR α activation was demonstrated as a promising therapy in affective disorders (Colle et al., 2016; Nisbett and Pinna, 2018).

In summary, patients with depression, ASD and ADHD demonstrate similar oxidative-stress related impairments both in peripheral tissue and the brain, suggesting shared pathophysiological

mechanism associated with mitochondrial dysfunction. Specifically, the mitochondria in individuals with these disorders may produce elevated quantities of ROS, increasing brain vulnerability to oxidative damage. When the ability of the antioxidant machinery to balance ROS production fails, oxidative stress predominates and leads to further mitochondrial (and cellular) dysfunction (**Fig. 5.1**). Our findings in the model of Western diet were similar to the clinically derived associations between increased lipid peroxidation, decreased mitochondrial biogenesis and ASD/ADHD-like and depressive-like behaviours. This suggests the Western diet as a potential contributor to the development of these disorders.

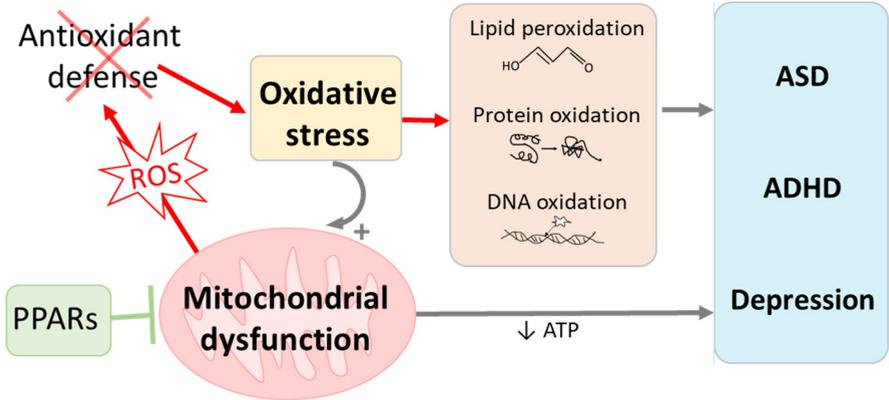


Figure 5.1. Oxidative stress and mitochondria dysfunction as factors contributing to ASD, ADHD and depression development. Mitochondria produce elevated quantities of ROS increasing brain vulnerability to oxidative damage. When the ability of the antioxidant machinery to balance ROS production fails, oxidative stress predominates and leads to further mitochondrial dysfunction. Increased lipid peroxidation, protein and DNA oxidation results in neuronal damage and disturbance of normal brain functions contributing to the development of mental disorders, including ASD, ADHD and depression.

5.4 Interplay between peroxisome proliferator-activated receptors (PPARs), Tlr4, serotonin transporter and insulin receptors activity

The Western diet in our mouse model induces increases in *Tlr4* expression and microglial activation, elevated MDA level (Veniaminova et al., 2020, *under revision*; Strekalova et al., 2015, 2016), decreased expression of *Ppargc1a* and *Ppargc1b* (Strekalova et al., 2016; Veniaminova et al., 2017) as well as a decrease in serotonin transporter expression (*Sert*) (Veniaminova et al., 2020) in the brain (**Chapters 2-4**). Importantly, increased protein level of Tlr4, microglial activation, elevated MDA content and decreased Sert mRNA level were detected in the prefrontal cortex of young mice fed with the Western diet (Veniaminova et al., 2020; Strekalova et al., 2015). These data led us to suggest that these brain changes did not happen independently but are interconnected, influencing each other and resulting in the alteration of mouse behaviour.

Both TLRs and PPARs are involved in modulating the inflammation responses effecting the same molecular pathways (Dana et al., 2019). Signaling pathways of most of the TLRs, including TLR4, operate through MyD88 and are accompanied by a TIR domain-containing adaptor protein (TIRAP), which results in activation of transcription factors including NF- κ B and activator protein-1 (AP-1) (Troutman et al., 2012). NF- κ B and AP-1 pathways regulate many inflammation-related genes. PPARs can attenuate the expression of

inflammatory response genes by inactivation of p65 complexes or through inducing I κ B α which is the main inhibitor of NF- κ B signaling pathway. It was also shown that PPARs can regulate AP-1 activity (Delerive et al., 2001). Studies in cell culture and animal models demonstrated that there is bidirectional regulation between TLRs and PPARs (Dana et al., 2019). One study described a regulatory feedback loop in which in unstimulated macrophages, PPAR γ inhibited NF- κ B-mediated inflammatory signaling, while in LPS-stimulated macrophages, when the TLR4 was activated, the PPAR γ expression was attenuated by NF- κ B and as a result, any further potential anti-inflammatory effects of PPAR γ were terminated (Necela et al., 2008). Moreover, PPAR γ agonists were shown to inhibit TLR-mediated activation of dendritic cells via the MAP kinase and NF- κ B pathways (Appel et al., 2005). In a murine model of sepsis, PPAR γ activation by an agonist resulted in attenuated proinflammatory cytokine production and increased level of IL-10. These effects were associated with a decrease in STAT-1-dependent expression of MyD88. Blocking the IL-10 receptor was able to eliminate PPAR γ -mediated inhibition of MyD88 expression (Ferreira et al., 2014).

Glial PPAR α and γ play an important role in inflammatory brain pathologies. PPAR γ agonists were shown to attenuate microglia activation, TLR4 and cytokines expression (Jia et al., 2016) and TLR ligand-induced production of IL-12 p40 (Gurley et al., 2008). Also,

agonists of TLR4, TLR1/2 and TLR5 in astrocytes demonstrated NF- κ B-dependent inhibitory activity against gene expression, protein translation and the activity of PPAR α and PPAR γ (Chistyakov et al., 2015). Protective anti-inflammatory efficiency of PPAR β/δ agonists was shown in animal models (Aleshin and Reiser, 2013). However, the fact that PPAR β/δ expression levels were found to be upregulated after TLR4 stimulation through NF- κ B and MAPK-dependent pathways, raised a question of whether PPAR β/δ has a dual pro- and anti-inflammatory role (Chistyakov et al., 2014).

Inflammation-related regulation was demonstrated for serotonin transporter (SERT) expression. Thus, in cell culture SERT expression and activity were inhibited by activation of TLR2 via MAPK and protein kinase A (PKA) pathways (Latorre et al., 2016) and by activation of TLR4 mediated by protein kinase C (PKC) (Mendoza et al., 2009). On the other hand, individuals carrying two short (ss) versus long (ll) 5-HTTLPR alleles showed a higher ratio of proinflammatory to anti-inflammatory cytokines IL-6/IL-10 at baseline and during stress (Fredericks et al., 2010). In the rat brain, both inhibition of SERT by fluoxetine (Macgillivray et al., 2011) and genetic *Sert* deficiency (Macchi et al., 2013) resulted in microglia activation. However, in the other study, systemic LPS led to increased *Sert* function in male rats (Korte-Bouws et al., 2018). In addition, serotonin metabolites, 5-methoxyindole-3-acetic acid and 5-hydroxyindoleacetic acid (5-HIAA),

can directly activate PPAR γ and this process was inhibited by blocking of endogenous SERT (Waku et al., 2010). Treatment with a PPAR β/δ agonist was shown to enhance the expression of PPAR β/δ and *Sert* in the hippocampus of rats with stress-induced depression (Liu et al., 2017a). Metabolic endotoxemia induced by decreased SERT in the gut might lead to inhibition of peripheral and brain IRS1/PI3K/Akt regulatory cascades which is a key mechanism in the regulation of glucose metabolism (Pomytkin et al., 2015).

Both chronic inflammation and alterations in mitochondrial biogenesis are implicated in the mechanisms of insulin resistance (Pomytkin et al., 2015; Zand et al., 2017). TLR4 is involved in insulin resistance-associated inflammation via activating proinflammatory kinases JNK, IKK, and p38 that impair insulin signal transduction directly through inhibitory phosphorylation of IRS on serine residues. (Kim and Sears, 2010; Yin et al., 2014). In addition, activation of TLR4 leads to phenotypic change of macrophage from M2 to M1. The proinflammatory phenotype of M1 macrophages further promote insulin resistance (Orr et al., 2012). Some studies found increased TLR4 mRNA expression and signaling in type 2 diabetic patients (Creely et al., 2007; Jialal et al., 2012; Lee et al., 2013). Also, increased NF- κ B activation may lead to decreased insulin receptor-facilitated insulin uptake by the brain (Gray et al., 2017).

PPARGC1a-responsive genes involved in oxidative phosphorylation were shown to be downregulated in human diabetes (Mootha et al., 2003). PPARs can affect insulin signaling by decreasing production of proinflammatory mediators and NF- κ B transcriptional activities and upregulation of IRS proteins (McArdle et al., 2013; Wahli and Michalik, 2012). In addition, PPAR γ was demonstrated to promote alteration of immune cells phenotypes to anti-inflammatory M2 macrophage polarization (Patel et al., 2013), thus antagonizing TLR4-dependent effects.

It is important to mention that previous studies conducted in our model revealed ameliorative effects of insulin receptor sensitizer dicholine succinate on Western diet-induced elevation of *Tlr4* and decrease of *Ppargc1b* brain expression in mice (Strekalova et al., 2016). Recent unpublished data demonstrate that in *Sert* knockout mice, Western diet induces a decrease in the expression of both isoforms of insulin receptor, IRA and IRB, in the brain (Veniaminova et al., unpublished data; **Fig. 5.2**). Interestingly, in naïve *Sert* knockout mice, IRA and IRB expression was elevated compared to wild type and *Sert* heterozygous mice, possibly demonstrating adaptive changes due to the absence of *Sert*.

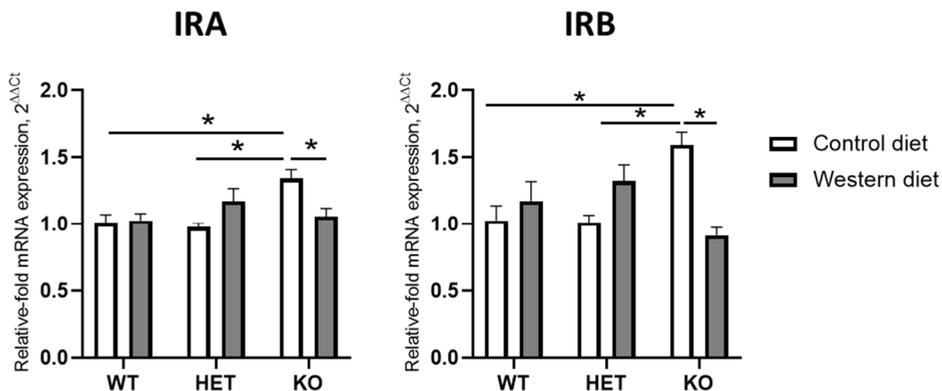


Figure 5.2. IRA and IRB expression in Sert knockout mice fed Western or control diet. Sert knockout mice fed with the Western diet compared to control diet demonstrated decreased expression of IRA and IRB in hypothalamus. Naïve Sert knockout mice compared to wild type and heterozygous showed increase in IRA and IRB expression. Similar results were obtained for dorsal raphe region, hippocampus and prefrontal cortex (not shown). WT, wild type; HET, Sert heterozygous, KO, Sert knockout mice. * $p < 0.05$, two-way ANOVA and Tukey's test, 6-7 animals per group were used. Data are shown as mean \pm SEM.

Thus, there is a complicated crosstalk involving TLRs, PPARs, SERT and insulin receptor signaling. TLR4 and PPARs have shared signaling pathways, antagonizing each other. Experimental data suggest that activation of TLR and inhibition of PPARs and insulin receptor-mediated signaling may lead to impaired functioning both in the periphery and in the brain.

5.5 Direct and peripheral inflammation-mediated effects of Western diet components on neuroinflammation and brain mitochondrial activity

In our model, Western diet enriched with fats, especially saturated fats, and sugar, was shown to induce changes both in peripheral metabolism and in the brain in female mice. Among the peripheral changes, we found impaired glucose tolerance and insulin resistance, dyslipidemia, and signs of liver steatosis and inflammation in mice fed with the Western diet compared to controls. In the brain, Western diet induced signs of neuroinflammation and impaired mitochondrial activity (Veniaminova et al., 2017, 2020, *under revision*) (**Chapters 2-4**). While we did not investigate exact molecular cascades connecting Western diet consumption with brain changes, there are two pathways that possibly contribute to the diet effects on the brain.

First, components of the Western diet can induce peripheral inflammation-related metabolic states, which in turn may lead to neuroinflammation (*discussed in section 1.2*). In human, consumption of excess sugar or fat can cause overeating, increased body weight and fat gain (Stinson et al., 2018). Enlarged adipocytes of obese individuals are known to promote inflammation and predispose toward insulin resistance (Greenberg and Obin, 2006). Some studies have found an association between the consumption of added sugar or fat and development of insulin resistance and type 2 diabetes, independent of

body weight gain or total energy intake (Marshall and Bessesen, 2002; Stanhope, 2016). On the contrary, others argue that much of the association between sugars and type 2 diabetes is eliminated by adjusting data for body mass index (Lean and Te Morenga, 2016; Marshall and Bessesen, 2002), which makes this topic controversial. In our study, Western diet feeding induced impairment in glucose tolerance both in the presence of weight gain in aged mice (Veniaminova et al., *under revision*) (**Chapter 4**) and in young mice without a change in body weight (Veniaminova et al., 2017, 2020) (**Chapters 2 and 3**).

Altered diet can lead to rapid, short-term changes in hepatic glucose metabolism. In healthy people, fructose and glucose overfeeding for 6 days increased liver fat content and reduced hepatic insulin sensitivity (Lecoultre et al., 2013). Sucrose stimulates de novo lipogenesis which leads to the alteration of the fatty acid composition in very-low-density lipoprotein (Hoekstra et al., 1993). Dietary intake of lipids, the lipolysis of adipose tissue, and de novo lipogenesis contribute to the pool of lipids stored in the liver and to the development of non-alcoholic fatty liver disease (Marchesini et al., 2016). Western diet feeding resulted in liver steatosis and inflammation, demonstrated in our study (Veniaminova et al., 2020) (**Chapter 3**) and has been previously shown to decrease *Ppargc1b* liver expression (Strekalova et al., 2016). In line with our findings,

PPAR δ was shown to suppress hepatic lipogenesis and reduce the hepatic expression of proinflammatory genes (Tong et al., 2019). In addition, increased fructose or SFA levels can lead to a higher gut permeability via disruption of the tight junctions (de Velasco et al., 2018; Johnson et al., 2013). The resulting endotoxemia leads to further systemic low-grade inflammation.

Secondly, circulating fatty acids and glucose may also directly affect the brain. Two main types of transporters facilitate glucose transport across the BBB: glucose transporters (GLUTs) and sodium-dependent glucose transporters (SGLTs) (Patching, 2017). There is no consistency in reported effects of blood hyperglycemia on activity and expression of glucose transporters, as upregulation, downregulation and no changes have been shown (Patching, 2017). No major adaptational changes in the maximal transport velocity or affinity to BBB glucose transporters were found under conditions of acute hyperglycemia in normal human subjects (Hasselbalch et al., 2001). In rats, chronic hyperglycemia was shown to elevate brain extracellular fluid glucose, suggesting no protective adaptation in BBB (Jacob et al., 2002). Hence, the brain tissue may be exposed to elevated levels of glucose. In addition, glucose influx in the brain may be increased due to increased BBB permeability, induced by peripheral inflammation (Van Dyken and Lacoste, 2018). Once in the brain, glucose in extracellular fluid is rapidly taken up by astrocytes, microglia and neurons. Elevated

glucose concentrations were shown to increase secretion of IL-6 and IL-8 in cultured human astrocytes and increased the susceptibility of human neuronal cells to injury by hydrogen peroxide (Bahniwal et al., 2017). In mouse microglial cells, a shift from normal glucose to high glucose induced oxidative and inflammatory stress, microglial activation and increased LPS-induced inflammation (Hsieh et al., 2019). Changes in glucose level activated MAPK/JNK and NF- κ B signaling pathways (**Fig. 5.3**). This way, acute glucose fluctuation altered microglial activity, inducing oxidative and proinflammatory changes (Hsieh et al., 2019). This study is in line with our findings, as consumption of the Western diet in mice, which might lead to fluctuations in glucose levels, also induced microglial activation and oxidative stress (Veniaminova et al., 2020) (**Chapter 3**).

Numerous studies have discussed the transport of both saturated fatty acids (SFA) and unsaturated fatty acids across the BBB. Fatty acids can enter the brain in a number of ways. They can be transported via lipoproteins or bound to albumin in esterified or non-esterified form. Some may enter via passive diffusion, through a flip-flop mechanism or through protein transporters (Chen et al., 2015; Hachem et al., 2016; Murphy, 2015; Nguyen et al., 2014; Umhau et al., 2009). Several studies have reported that SFAs, such as palmitic acid, can induce activation of TLR4 receptors and stimulate cytokine release (Caesar et al., 2015; Nguyen et al., 2007; Valdearcos et al., 2014).

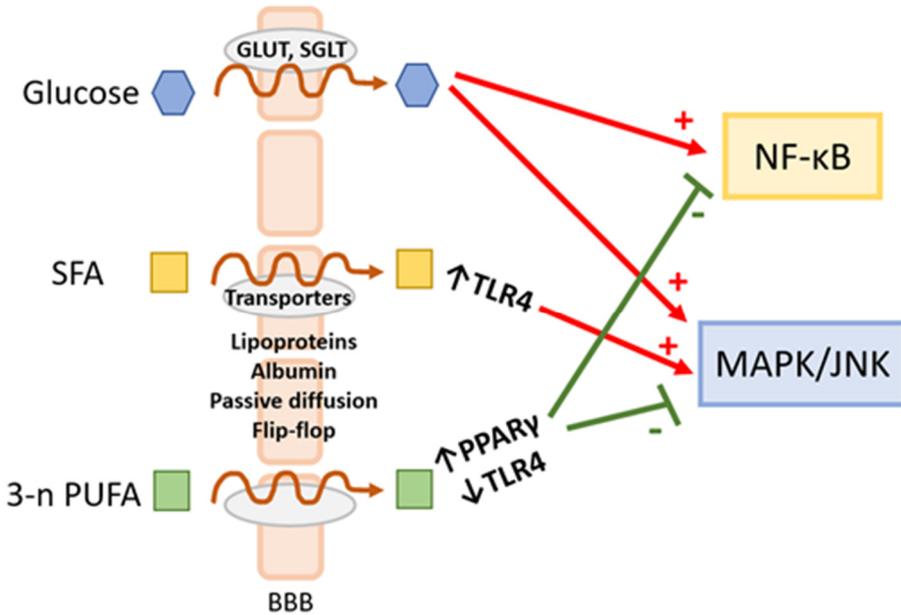


Figure 5.3. Direct effects of fatty acids and glucose on signaling in the brain. Glucose is transported across the BBB by GLUTs and SGLTs and activates MAPK/JNK and NF- κ B signaling pathways. Fatty acids enter the brain via lipoproteins or bound to albumin, via passive diffusion, through a flip-flop mechanism or through protein transporters. SFAs induce JNK signaling activation and increased inflammation, which is regulated by TLR4. n-3 PUFAs can inhibit TLR4 presentation and induce PPAR γ nuclear translocation, which prevents NF- κ B and MAPK phosphorylation

It has been previously hypothesized that long-chain SFAs are TLR4 agonists (Osborn and Olefsky, 2012). The principal component of LPS responsible for its immunostimulatory activity is the lipid A region, which contains numerous saturated fatty acyl chains that are required for binding to and activating TLR4 (Park and Lee, 2013). However, it was demonstrated that long-chain SFAs take several hours to initiate inflammatory signaling, while LPS activates inflammatory

signaling within minutes (Hernandez et al., 2014; Holzer et al., 2011; Nguyen et al., 2007). Recently, Lancaster et al. (2018) demonstrated that SFA palmitic acid is not a TLR4 agonist. Rather, they provided evidence that TLR4 indirectly regulates SFA-induced inflammation by altering macrophage lipid metabolism. Specifically, metabolic reprogramming facilitates SFA effects on endoplasmic reticulum, which leads to JNK signaling activation and increased inflammation (Lancaster et al., 2018). Nevertheless, as SFA-induced inflammation is regulated by TLR4, it provides a link between increased brain expression of *Tlr4* and high content of saturated fat in the Western diet in our study.

On the contrary, n-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), typically low in the Western diet, were shown to decrease circulating inflammatory markers and reduce oxidative stress (Freund-Levi et al., 2006; Kiecolt-Glaser et al., 2012). When incorporated into microglial membranes, n-3 PUFAs can prevent LPS-induced NF- κ B activation by inhibiting TLR4 presentation and reduce NO production, inducible nitric oxide synthase expression and the overall level of oxidative stress (Antonietta Ajmone-Cat et al., 2012; Fourrier et al., 2017; Inoue et al., 2017). Moreover, DHA might induce PPAR γ nuclear translocation and prevent LPS-induced MAPK phosphorylation (Antonietta Ajmone-Cat et al., 2012). It was proposed that DHA also attenuates the

inflammatory response in LPS-activated microglia by being incorporated into and remodeling lipid bodies, dynamic organelles that serve many functions, including to assist in preserving cell membrane and mitochondrial integrity (Tremblay et al., 2016). EPA also was shown to inhibit NF- κ B phosphorylation via sirtuin-1 (Inoue et al., 2017; Moon et al., 2007). In addition, DHA and EPA are able to enhance myelin or amyloid β peptide phagocytosis that is associated with a shift in microglial polarization toward the beneficial M2 phenotype, with a concomitant decrease in proinflammatory cytokine production (Chen et al., 2015; Hjorth et al., 2013). PUFA metabolites, such as resolvins, can limit the activation of microglial cells and inhibit the TLR4/NF κ B pathway (Tian et al., 2015).

Based on this evidence from prior research, it can be suggested that the effects of Western diet in mice could be mediated both by peripheral inflammation and associated systemic metabolic changes, as well as the direct interaction between diet components and brain molecular pathways related to TLR4 and PPARs.

5.6 The role of Sert deficiency in the interaction between genetic predisposition to psychiatric disorders and diet

Gene-environment interaction plays an important role in the development of psychiatric disorders (Schmitt et al., 2014). Dietary factors, such as Western-pattern diet consumption, are proposed among those environmental factors which could interact with genetic

susceptibility. Genetic Sert deficiency in human induced by s variant in the 5-HTTLPR of the *SERT (SLC6A4)* gene, was extensively discussed in a context of association with anxiety, depression and other psychiatric conditions (Homberg and Lesch, 2011; Murphy et al., 2008). In our study, behavioural consequences of a 3-week Western diet feeding in aging female mice were exacerbated by knockout of the *Sert* gene (Veniaminova et al., *under revision*) (**Chapter 4**). Thus, Western diet-fed mice with complete *Sert* knockout (Sert-/-) demonstrated increased compared to wild type mice (WT) suppression in novelty exploration and hippocampus-dependent cognitive performance. Intensified effects of other environmental factors were shown in Sert-/- mice. For example, after mild postnatal foot-shock stress experiences or exposure to predator odors, anxiety-like behaviours were increased in Sert-/- but not WT mice (Adamec et al., 2006; Carroll et al., 2007). In response to chronic mild stress, Sert-/- mice exhibited elevated plasma corticosterone levels (Lanfumeu et al., 2000), and adrenocorticotrophic hormone elevations were found in response to the placement of Sert-/- mice on the elevated plus maze (Li, 2006).

We found elevated *Tlr4* and decreased *Ppargc1b* expression in the hypothalamus of naïve Sert-/- mice (Veniaminova et al., submitted), suggesting the presence of proinflammatory status at the baseline in these mice which can contribute to their susceptibility to

environmental factors. Further, we demonstrated Western diet-induced increase in Tlr4 expression in dorsal raphe and prefrontal cortex of *Sert*^{-/-} mice, while in WT mice it was present only in dorsal raphe region (Veniaminova et al., submitted). In line with our findings, rats with a genetic decrease of *Sert* showed altered brain cytokine expression, exacerbated cytokine response to the LPS challenge and microglia activation (Macchi et al., 2013).

Naïve *Sert*^{-/-} mice compared to WT displayed increased brain expression of *Ppargc1a*, serotonin receptors *Htr1b* and *Htr2c* (Veniaminova et al., submitted) and insulin receptor isoforms IRA and IRB (Veniaminova et al., unpublished; Fig. 5.3). These changes were “reversed” in animals fed with the Western diet. Changes in the expression of serotonin receptors were previously reported for *Sert*^{-/-} mice and discussed as being adaptive due to the compensatory rewiring of brain circuits and neurotransmitter systems in the life-long and developmental absence of *Sert* (Murphy et al., 2008). Decreased expression levels of *Ppargc1a*, *Htr1b*, *Htr2c*, IRA and IRB compared to naïve *Sert*^{-/-} mice are associated with inflammation, impairment of metabolism and insulin receptor-mediated signaling (Handschin and Spiegelman, 2008; Nonogaki et al., 2006; Pomytkin et al., 2018).

Enhanced intestinal permeability and LPS leakage were shown in *Sert*^{-/-} mice (El Aidy et al., 2017), which may have additive effect with diet-induced increases in gut permeability (*see section 1.2*) and

inflammation. In addition, we demonstrated increased body weight and impaired glucose tolerance in Sert^{-/-} mice housed on the Western diet (Veniaminova et al., submitted). These peripheral metabolic conditions might also contribute to neuroinflammation (*discussed in section 5.4*) resulting in aggravation of behavioural abnormalities seen in Sert^{-/-} mice.

Remarkably, unlike WT and SERT^{-/-} mice, those heterozygous for the Sert gene (Sert^{+/-}) did not show impairment in glucose tolerance, elevated brain expression of Tlr4, or disrupted hippocampus-dependent performance (Veniaminova et al., submitted). While Sert^{+/-} as well as WT and Sert^{-/-} demonstrated increased Western diet-induced body weight gain and depressive-like behaviour, Sert^{+/-} mice exhibited partial resilience to the diet with regard to metabolic parameters.

While in the majority of reports, Sert^{+/-} rodents demonstrated an intermediate phenotype between WT and Sert^{-/-} (Murphy et al., 2008), some findings suggest distinct physiology of the two Sert-deficient genotypes. Sert^{+/-} differed from WT and Sert^{-/-} by expression of HPA regulatory protein binding protein 5 (FK506) in the pituitary of mice exposed to early life stress model (van der Doelen et al., 2014) and reduced basal corticosterone plasma levels (Houwing et al., 2017). However, in the other studies, Sert^{+/-} animals demonstrate

higher than WT or even *Sert*^{-/-} vulnerability to environmental stimuli (Macchi et al., 2013; van den Hove et al., 2011).

It is also observed in clinical and animal studies that heterozygosity of *Sert* or *s* allele may lead to a beneficial effect on cognition (Fiedorowicz et al., 2007; Homberg and Lesch, 2011; van den Hove et al., 2011). In this case it is important to highlight again that in our study in *Sert*^{+/-} mice, unlike WT and *Sert*^{-/-}, Western diet did not impair performance in the test for hippocampus-dependent performance (Veniaminova et al., *under revision*) (**Chapter 4**).

Together, based on the data discussed in the sections 5.1-5.5 and our results presented in chapters 2-4, it can be proposed that Western diet may induce neuroinflammation, oxidative stress and mitochondrial dysfunction mediated both by peripheral inflammation and direct interaction between diet components and brain molecular pathways related to TLR4 and PPARs, which interact with functioning of serotonergic system and activity of insulin receptors. The molecular brain changes are associated with depression, and generate in mice ASD-like and ADHD-like behaviours that could lead to aggravation of the symptoms if replicated in man (**Fig. 5.4**).

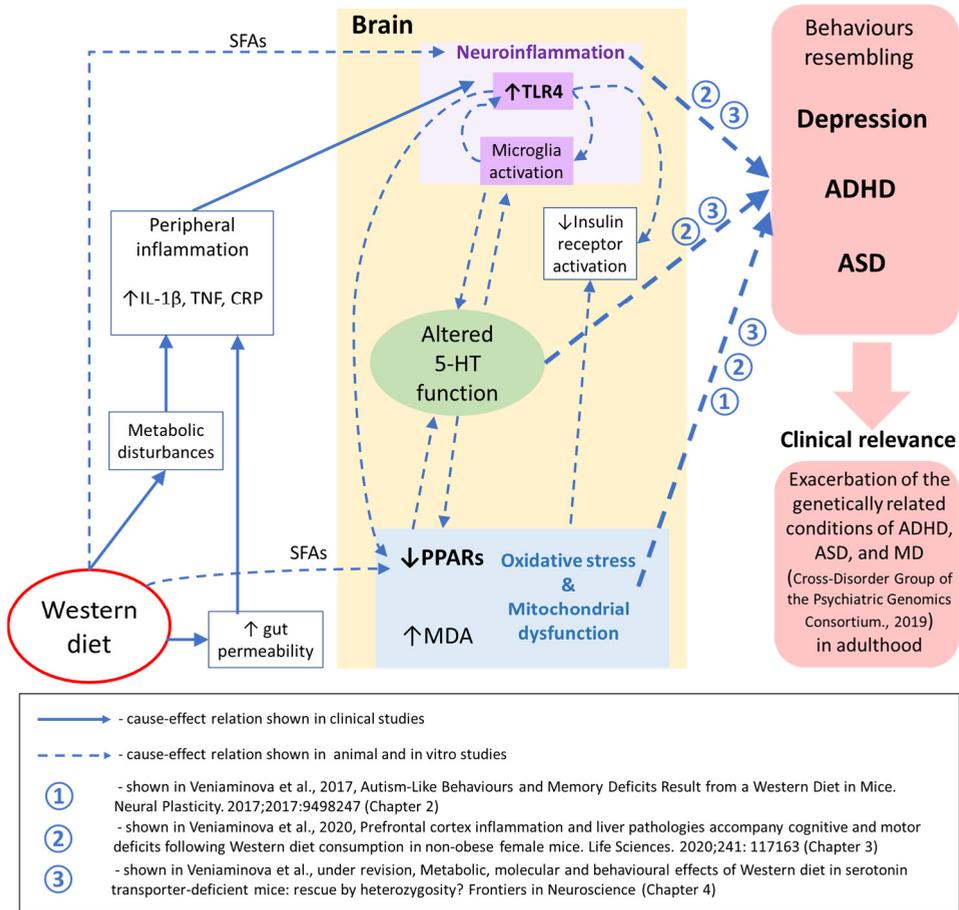


Figure 5.4. Proposed mechanism of the Western diet-induced aggravation of depression, ADHD and ASD symptoms. The effects of the Western diet effects are mediated both by peripheral inflammation and the associated systemic metabolic changes, increased gut permeability, as well as a direct interaction between diet components and brain molecular pathways related to TLR4 and PPARs. This leads to neuroinflammation, oxidative stress and mitochondrial dysfunction, altered function of the serotonergic system and decreased activity of insulin receptors. The molecular brain changes are associated with depression, and generate ASD-like and ADHD-like behaviours that could lead to aggravation of the symptoms if replicated in man. SFA's – saturated fatty acids, IL-1 β – interleukin 1 β , TNF – tumor necrosis factor, CRP – C-reactive protein, MDA – malondialdehyde, 5-HT – serotonin, TLR4 – toll-like receptor 4, PPARs – peroxisome proliferator-activated receptors, ASD – autism spectrum disorder, ADHD – attention deficit/hyperactivity disorder, MD – major depression

5.7 Limitations of animal models of Western diet and Sert deficiency when studying human pathologies

Rodents are widely used model organisms in human disease research. Mouse and rat models have been used extensively to provide insight into the mechanisms underlying many diseases, to explore the efficacy of candidate drugs, and to predict patient response to treatment. Development of methods for the creation of transgenic, knockout and knockin mice have provided powerful tools for research and have made mice the most commonly used model organism (Rosenthal and Brown, 2007). However, the use of mice in biomedical research must take into consideration both the differences and similarities between mice and humans.

First, given that mice have a greater surface area to volume ratio than humans, their metabolic rate per gram of tissue is roughly seven times higher (Kleiber, 1961). Differences in metabolic rate between mice and humans are correlated with many anatomic, physiologic and biochemical differences. Mice have relatively larger metabolically active tissues, such as the liver and kidney, and larger deposits of brown fat. Mouse cells differ from human cells not only in mitochondrial density and metabolic rate, but also in the fatty acid composition of their membrane phospholipids (Hulbert, 2008). Mice have higher rates of production of reactive oxygen species and suffer higher rates of oxidative damage than do humans.

Importantly to nutrition research, mice have different microbiomes (Nguyen et al., 2015) and have coevolved with different pathogens than humans. The anatomy of the gastrointestinal track differs between the two species, as mice have a prominent cecum and no appendix (Nguyen et al., 2015). Thus, the two species provide different environments that apparently support the growth of different gastrointestinal microbiota. Despite this fact, the trend of microbiota shift due to Western diet consumption observed in mouse studies agrees with that found in human studies (Nguyen et al., 2015). While main features of the immune system are similar in mice and humans, there are also significant differences due to coevolution with different pathogens and the microbiome (Bailey et al., 2013). Moreover, mice exhibit differences in lipid metabolism versus humans. For example, they lack cholesteryl ester transfer protein (Guyard-Dangremont et al., 1998) which shuttles cholesteryl esters from high density lipoproteins (HDL) to low (LDL) and very low density lipoproteins in humans. Thus, mice carry the majority of plasma cholesterol in HDL while humans carry much of it in LDL (Camus et al., 1983).

Due to discussed differences in metabolism and the immune system, dietary effects will not be the same in mouse and human. Moreover, a large number of diets used for studies in rodents may not appropriately represent the general human diet, particularly the Western diet. Typical Western diet in human on average consists of

30-40% dietary fat (Ford and Dietz, 2013). Therefore, using, for example, 60% fat in rodent dietary interventions is too extreme compared with the average Western diet. In our study we used the diet which as closely as possible mimics fat percentage of human Western diet and contains around 40% of calories from fat. Human Western diet can be also associated with decreased intake of vitamins, lack of certain nutrients and excessive alcohol and sodium (Koliaki and Katsilambros, 2013), which are not found in standard rodent Western diet feed and which effects are investigated distinctly in separate studies. In addition, although both mice and humans are omnivores, standard control diet in rodents is mainly grain based and differs significantly to human diet (Perlman, 2016). While in humans diet-induced obesity and associated disease occurs over months, years, and decades, it takes only several weeks of Western diet feeding to induce obesity in mouse models, making it impossible to translate the relevance of duration of metabolic disease development to humans (Lai et al., 2014). For example, in our study, 3-week Western diet induced impaired glucose tolerance both in young and aging female mice, while weight gain was found only in aging animals (Veniaminova et al., 2017, 2020, *under revision*) (**Chapters 2-4**). Because of the rapid disease induction, mouse models often lack vascular complications associated with metabolic disorders (Wu et al., 2006).

Nevertheless, using the Western diet model in mice provides an opportunity to reproduce general features of metabolic disorders and further investigate molecular mechanisms of their effects on the brain. Thus, in young female mice fed with the Western diet we demonstrated metabolic alterations as well as behavioural phenotypes reminiscent of ASD/ADHD-like symptoms and proposed that neuroinflammation and oxidative stress may undelay these changes (Veniaminova et al., 2017, 2020) (**Chapters 2 and 3**). While our results cannot be directly translated to a human context, they highlight the importance of future clinical studies in investigating the effects of dietary pattern in patients with ASD or ADHD and in groups of high risk for developing these disorders.

Manipulations of the mouse genome provide a tool to investigate the role of certain genes and to model the effects of a combination of environmental factors and allele gene variants on pathology. While mice do not have 5-HTTLPR polymorphism in the *SERT* gene found in human, *Sert*^{+/-} mouse closely resemble human 5-HTTLPR ss genotype in regard to levels of SERT expression and associated functional consequences (Murphy et al., 2008). As it was reviewed previously, *Sert*^{-/-} rodents, like s-allele carriers, demonstrate increased stress response in behavioural tests for anxiety-like behaviour (Kalueff et al., 2010). Several studies showed that *Sert*^{+/-} mice develop corresponding behavioural changes when exposed to psychosocial stress

in early life or adulthood (Bartolomucci et al., 2010; Jansen et al., 2010; Lewejohann et al., 2010), which is also associated with ss genotype. However, one study demonstrated signs of reduced anxiety and enhanced memory performance in Sert+/- compared to WT (van den Hove et al., 2011). Depressive-like behaviour however is less consistent for Sert-/- rodents and highly dependent on genetic background (Kalueff et al., 2010) which suggests an interaction between the genes, a factor that is not translatable to human variability. Consistent with humans, however, improved cognitive performance was reported in Sert-/- rodents (Brigman et al., 2010). In summary, some of the traits of the ss genotype are more closely associated with mouse Sert+/- genotype, while others with Sert-/- genotype.

While the s allele in human was associated with obesity and type 2 diabetes (Fuemmeler et al., 2008; Iordanidou et al., 2010; Sookoian et al., 2007), no clinical studies investigated the susceptibility of s allele carriers to excessive intake of fat and sugar. In our study, some behavioural, metabolic and molecular changes induced in Sert-/- mice by the Western diet were not induced in Sert+/- (Veniaminova et al., *under revision*) (**Chapter 4**). Thus, opposite to Sert-/- and WT, Sert+/- mice did not develop impairment in glucose tolerance after three weeks of Western diet feeding. Translation of this finding into human situation is limited, suggesting, however, that 5-HTTLPR polymorphism may play an important role in the susceptibility to the

effects of the Western diet. Future studies in this mouse model with a longer period of dietary feeding, as well as retrospective studies in humans, will be important to clarify these findings.

5.8 Future perspectives

The next step for our project is to run additional studies on the brain tissue of mice with different *Sert* genotype fed either with the Western or control diet. First, high performance liquid chromatography (HPLC) analysis of 5-HT and 5-HIAA brain levels will show the diet-induced changes of tryptophan-serotonin metabolism. Then, the measurement of Sert level in the brain of *Sert*^{+/-} mice after Western diet feeding might provide a key to the mechanisms of their resilience to the effects of the diet. Further, the examination of metabolome of brain, liver and blood is essential for complex understanding of the difference in diet effects in mice with partial and complete *Sert* deficiency, which underly different behavioural and metabolic outcomes. Data is already obtained for the blood metabolome and the paper is in preparation.

As we hypothesized significant role of the TLR4 in the negative effects of the Western diet, the use of TLR4 antagonists in our model, in case of its protective effect, would support our hypothesis. It could be also suggested to perform a study with the Western diet feeding on mice, which were treated with SSRI starting from an early age, and

see if it would mimic the situation of Sert^{+/-} or Sert^{-/-} mice in respect to their susceptibility to the Western diet.

In order to further expand our understanding of the interaction between Sert and the Western diet, studies with the same experimental protocol can be conducted on male mice, young mice with Sert deficiency, and longer period of dietary intervention can be applied.

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Summary

In my work I investigated the behavioural, metabolic and molecular effects of a ‘Western diet’, a diet enriched with saturated fat, cholesterol and sugars, in wild type and mutant mice with genetic deficiency of the serotonin transporter (Sert). In the applied model, female mice were housed for 3 weeks on a diet containing 21% fat (including 12% saturated fat), 35% sugar, and 0.2% cholesterol. We found that young C57BL/6 mice fed with this diet displayed reduced social interactions, impairment in cognitive functions and motor coordination as well as depressive-like behaviour. These mice also showed decreased glucose tolerance and insulin resistance, dyslipidemia, and signs of non-alcoholic steatohepatitis-like syndrome. As SERT deficiency in humans is associated with increased risk of metabolic syndrome, especially during aging, we studied effects of the Western diet on mice heterozygous (HET) or complete knock-out (KO) for the gene encoding Sert.

We demonstrated that young mice housed on the Western diet display reduced scores of social interaction, increased dominant-like behaviours and signs of hyperactivity in a context of social interactions (**Chapter 2**). These changes were accompanied by decreased scores of fear conditioning and performance in the marble test, two paradigms for hippocampus-dependent behaviour, novel object recognition

memory and increased depressive-like behaviour in the forced swim test. In addition, feeding with the Western diet impaired motor coordination in the rotarod and wire test, while no changes in muscle weight and strength or general locomotion were found. Behavioural changes in mice fed with the Western diet were accompanied by increased concentration of a marker of oxidative stress, malondialdehyde, signs of microglia activation, and decreased *Sert* gene expression in the prefrontal cortex (**Chapters 2 and 3**).

Based on the latter finding and well-known relationship between the variation in polymorphic region in the SERT gene (5-HTTLPR) in humans leading to its decreased activity and susceptibility to metabolic and mental alterations, we next studied how dietary challenge with Western diet interferes with *Sert* deficiency in mice (**Chapter 4**). We showed that complete genetic *Sert* inactivation in KO mice exacerbated consequences of the Western diet challenge in one-year-old female mice. These mice demonstrated greater decrease in glucose tolerance, increase in the expression of inflammation marker toll-like receptor 4 (*Tlr4*) and adverse behavioural changes compared to wild type mice housed on the Western diet. Remarkably, unlike wild type and KO, mice HET for the *Sert* gene did not reveal hallmarks of the Western diet-induced syndrome, such as decrease in glucose tolerance, increased brain expression of *Tlr4* and disrupted hippocampus-dependent performance. Yet, all *Sert* genotypes challenged with Western diet

displayed similar changes in weight gain and increased scores of depressive-like behaviour in the swim test. Overall, our data indicated distinct effects of the Western diet on aging mice with complete versus partial *Sert* inactivation and suggested the importance of the interaction between Sert function and Western dietary pattern in the development of adverse metabolic and behavioural changes. Our data has led us to hypothesize that Western diet consumption can be an environmental factor that may interact with genetic risk factors of ASD and ADHD, increasing the risk for these disorders to manifest in adulthood.

Samenvatting

Het effect van het “westers dieet” op emotioneel, sociaal en cognitief gedrag, aangetoond in een studie met normale en serotonine transporter-deficiënte muizen

In mijn werk onderzocht ik gedrags-, metabole en moleculaire effecten van het zogenaamde 'westerse dieet', een dieet verrijkt met verzadigd vet, cholesterol en suikers, bij muizen, inclusief mutanten met een genetische deficiëntie van serotonine transporter (Sert). In het toegepaste model werden vrouwelijke muizen gedurende 3 weken gehuisvest op een dieet dat 21% vet bevat, waaronder 12% verzadigd vet, 35% suiker en 0,2% cholesterol. We vonden bij jonge C57BL / 6 met dit dieet, verminderde sociale interacties, verminderde cognitieve functies en motorische coördinatie en verhoogd depressief gedrag, evenals een verminderde glucosetolerantie en insulineresistentie, dyslipidemie en tekenen van niet-alcoholische steatohepatitis-achtige syndroom. Aangezien Sert-deficiëntie bij mensen wordt geassocieerd met een verhoogd risico op het metabool syndroom, vooral bij vrouwen tijdens veroudering, hebben we de effecten bestudeerd van het westerse dieet op heterozygote muizen (HET) of op muizen met een volledige knock-out (KO) voor het gen dat voor Sert codeert.

We toonden aan dat jonge muizen met dit westers dieet lager scoorden op sociale interactie, dominant gedrag en tekenen van hyperactiviteit in de context van sociale interacties vertoonden (**Hoofdstuk 2**). Deze veranderingen gingen gepaard met verminderde scores van angstconditionering en verminderde prestaties gemeten met de knikkertest, de twee paradigma's voor hippocampus afhankelijk gedrag, het herkennen van nieuwe voorwerpen en een verhoogd depressief gedrag in de geforceerde zwentest. Bovendien verminderde het westerse dieet de motorische coördinatie gemeten met de rotarod- en de draadtest, terwijl er geen veranderingen werden waargenomen in het spiergewicht en de spierkracht of in de algemene motoriek. Gedragsveranderingen bij muizen die met een westers dieet werden gevoed, gingen gepaard met een verhoogde concentratie van malondialdehyde, een marker van oxidatieve stress, en tekenen van microglia-activering en verminderde Sert-genexpressie in de prefrontale cortex (**Hoofdstukken 2 en 3**).

Op basis van deze bevindingen en de bekende relatie tussen de variatie in polymorfe regio in het Sert-gen (5-HTTLPR) bij mensen wat aanleiding geeft tot verminderde activiteit en gevoeligheid voor metabole en affectieve tekorten, hebben we vervolgens naar de interferentie van het westers dieet op Sert-deficientie bij de onderzochte muizen (**Hoofdstuk 4**). We toonden aan dat de volledige genetische inactivering van Sert bij KO-muizen de gevolgen van de

westerse voedingsproblematiek bij vrouwelijke muizen van één jaar oud verergerde. Deze muizen vertoonden een grotere afname in glucosetolerantie, een toename in de hypothalamische expressie van de ontstekingsmarker tol-like receptor 4 (Tlr4) en gedragsveranderingen in vergelijking met wildtype muizen op een westers dieet.

In tegenstelling tot de wildtype en KO-muizen, vertoonden HET muizen niet de kenmerken van het door het westerse dieet-geïnduceerde syndroom, zoals afname van de glucosetolerantie, een verhoogde hersenexpressie van Tlr4 en verstoorde hippocampus-afhankelijke prestaties. Toch vertoonden alle Sert-genotypen met een westers dieet vergelijkbare veranderingen in gewichtstoename en verhoogde scores van depressief gedrag in de zwemtest. Over het algemeen wezen onze gegevens op duidelijke effecten van het westers dieet op de verouderende muizen met een volledige versus gedeeltelijke Sert inactivering en suggereert het belang van de interactie tussen de Sert-functie en het westerse voedingspatroon bij de ontwikkeling van metabole en gedragveranderingen. Onze resultaten veronderstellen dat het westerse dieet een milieurisicofactor kan zijn welke kan interfereren met genetische factoren van een autisme-spectrum-stoornis (ASS) en een aandachts-tekort-stoornis met hyperactiviteit (ADHD) op volwassen leeftijd.

Valorization

Relevance for society

Western-style foods consumption has been increasing steadily since the 1950s, especially in Western-culture countries. Due to a growing economy, higher income and faster pace of life, rates of Western diet consumption are also increasing nowadays in countries where such foods were never part of the traditional culture. Western diet is associated with metabolic disorders, such as obesity, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD). Worldwide, the estimated prevalence for obesity and NAFLD is around 650 million and for type 2 diabetes more than 500 million people. The WHO anticipates that worldwide deaths from diabetes will double by 2030.

Metabolic disorders are also often associated with increased risk of psychiatric disorders, including mood disorders, attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorder (ASD). ASD and ADHD are complex neurodevelopmental disorders with the prevalence estimated 7% and 2% respectively, thus affecting more than 100 million people in the world. ASD may significantly limit the capacity of an individual to participate in society. Those affected may not be able to conduct day-to-day activities and require life-long care and support. Both ASD and ADHD negatively influence the person's educational and social attainments. These disorders cooccur

with a high frequency with each other as well as with affective disorders such as depression.

Increasing prevalence of ASD and ADHD suggests an important role of environmental factors, including nutrition, in the development of these disorders. While adverse reactions to foods have often been reported in children with ASD or ADHD, the impact of diet on ASD/ADHD associated behaviours in adulthood is much less well understood. Our study showed that in adult female mice, feeding with the Western diet induced behaviours phenomenologically similar to the behaviours associated with human ADHD and ASD. Thus, this work provides a rationale for future clinical studies on the effects of nutrition on ADHD and ASD syndromes in adulthood. Based on our results, it can be suggested that dietary interventions may lead to symptom relief in patients with ASD or ADHD, which will decrease costs associated with disability and health management.

We also found metabolic changes and signs of neuroinflammation and oxidative stress in the brains of mice fed with the Western diet. These data suggest that compounds with anti-inflammatory and antioxidative properties might be useful to ameliorate symptoms in cases ASD or ADHD that cooccur with metabolic disorders such as type 2 diabetes or NAFLD. The main molecular targets reported in this study are toll-like receptor 4 (Tlr4) and peroxisome proliferator-activated receptor gamma (PPAR γ). Agonists and

antagonists of these receptors are now being studied in relation to diabetes, obesity, inflammatory disease treatment, and our work may further increase the interest of the industry in these compounds by proposing new applications.

Decreased activity of serotonin transporter (SERT), which occurs in a large percentage of people due to the 5-HTTLPR polymorphism in the SERT gene, was extensively discussed as a factor of genetic predisposition to affective and neurodevelopmental disorders. Current work demonstrated that mice with partial Sert deficiency displayed a resilience to dietary-induced abnormalities in glucose tolerance, Tlr4 brain expression and hippocampus-dependent behaviour, aberrations that were present in mice with complete Sert deficiency and wild type animals after feeding with the Western diet. Based on our findings, it seems important to further study the consequences of interaction between adherence to a certain dietary pattern and the 5-HTTLPR polymorphism in humans, which might increase the need for genotyping services. Based on the genotype, further dietary counseling services as well as pharmacotherapy, such as use of compounds targeting TLR4, PPAR γ or SERT might be advised.

Target groups

We consider our target groups could be 1) individuals with high adherence to the Western dietary pattern or patients with metabolic

disorders, 2) adult patients with ASD, ADHD and depression as well individuals with high genetic risk for these disorders.

Activity / Products

Our findings highlight the importance of future clinical investigation of the possible effects of the Western diet in those with a high susceptibility to, or having been diagnosed with, ASD, ADHD and depression. Dietary recommendations in combination with 5-HTTLPR polymorphism genotyping might be suggested for these groups. In some cases, pharmacotherapy using inhibitors/antagonists of TLR4 and agonists of PPAR γ might be used.

Innovation

The work presented herein has been innovative in various regards. First, we have shown that the Western diet mice can evoke behaviours reminiscent of ASD and ADHD symptoms, as well as impairment of emotionality in a mouse model. In particular, our studies were among the first to show that in adult mice, Western diet alters social behaviour, which is known to be affected both in ASD and ADHD. This result suggests dietary interventions as a promising treatment approach in adult patients with ASD and ADHD. Then, our model of the three-week Western diet feeding provides an opportunity to study behavioural and brain changes associated with metabolic syndrome as well as possible pharmacotherapy at low labor and time

costs. Much longer periods of diet feeding have been employed routinely elsewhere in similar studies, leading to serious obesity. Further, we extended the knowledge of the Western diet consequences on behavioural and brain molecular parameters in female mice, while in the past, predominately male rodents were used for this purpose. We also suggested important contributions of neuroinflammation, oxidative stress and altered Sert functioning in the discussed behavioural changes, which may direct pharmacotherapy development. And, finally, we investigated effects of the Western diet on metabolic, behavioural and molecular parameters in female mice with complete and partial deficiency of Sert. This has not been shown before, but is relevant to the human context regarding the 5-HTTLPR polymorphism, which also results in decreased SERT activity. We found that complete genetic Sert inactivation exacerbated metabolic alterations, neuroinflammation and behavioural consequences of the Western diet feeding in aging mice. On the contrary, mice with partial genetic Sert deficit displayed a “rescued” phenotype in dietary-induced abnormalities in glucose tolerance, Tlr4 brain expression and hippocampus-dependent behaviour, that was shown for the first time.

Implementation

Results of our study are relevant for scientific and medical communities and for the general public. From an academic perspective,

results were presented at national and international conferences (8 oral and 3 poster presentations at the conferences including “12th Goettingen Meeting of the German Neuroscience Society”, “20th EURON PhD Days”, and “19th WPA World Congress of Psychiatry”) and were or will be published in peer-reviewed international journals. Being a part of Eat2beNice project (Horizon 2020 EU Research and Innovation programme, grant No. 728018), the study was presented in the project reports and in the New Brain Nutrition Blog (newbrainnutrition.com/blog, blogs: “Why do we use mouse models in diet research?”, “Nutrition and Psychiatry: experience of attending the 19th WPA World Congress of Psychiatry”). Our model of the Western diet feeding in mice is currently being used by colleagues from the food industry and for investigating a ‘healthy’ meat product.

Acknowledgments

First of all, I would like to thank Prof. Klaus-Peter Lesch for the general supervision of my PhD project that was a joint research project between Maastricht University, Department of Neuroscience and I.M. Sechenov First Moscow State Medical University. Prof. Lesch provided me a unique opportunity to join the project supported by ‘Eat2beNICE’ grant as a part of Horizon 2020 EU Research and Innovation programme and ‘5-100 Russian Excellence Project’. During my PhD years I had a rare chance to participate in numerous studies, which resulted in several important publications, and this all would not be possible without irreplaceable contribution of Prof. Lesch. I am very thankful for his warm welcome in his laboratory and in his house in Wuerzburg where I had training at the very beginning of my PhD. I’m grateful for Prof. Lesch for his willingness to establish high standards of research practice in the laboratory, for his criticism, attention, encouragement and support across the years 2014-2019.

I would like to express my gratitude to the head of School for Mental Health and Neuroscience Prof. David Linden, the head of Division III Prof. Jos Prickaerts, and administration of Maastricht University for the opportunity to defend my thesis and for general support.

I express my special gratitude to Prof. Tatyana Strekalova, my immediate supervisor, who coached me across the years of my PhD. Prof. Strekalova provided me with unique research and training opportunities through her collaborations in the Universities of Oxford, Wuerzburg, Lisbon, Liege and Hong Kong. With her invaluable organizational help and support, I was lucky to attend international conferences and undergo trainings in the most prominent laboratories in the field of Neuroscience. During activities and traineeships arranged by Prof. Strekalova, I was able to learn important methods and carried out experiments for my Thesis. Prof. Strekalova gave me priceless experience of planning, study design, and organization of my experiments, communication with colleagues and presenting my data. She personally taught me many behavioural testing paradigms in mice, paying much attention to quality control and consistency of testing environment. Her attention to details and criticism helped me to improve my writing and reporting skills and to contribute to 7 peer-review papers over the period of PhD studies. I am very thankful for her constant support and for encouraging me to be productive and effective in my work. I trust it is impossible to overestimate the role of Prof. Tatyana Strekalova in the defense of my PhD Thesis.

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of Oxford and acquired the best research standards. Prof. Anthony's advice, discussions we had, his mentoring and help with manuscript writing are impossible to overestimate. I would like to especially thank Prof. Anthony, members of his laboratory and his family for their warm welcome in Oxford, where I am always glad to come back.

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I thank my dearest friends for their sensitive attention and for being tactful not to ask me each time we met if I am going to finish my PhD thesis soon.

I would like to address my warmest thanks to my relatives: my parents, my grandparents, my brother and his family for encouraging my education throughout my life and my relatives-in-law for their support. I especially thank my beloved husband Ilya for helping me to find a right decision when I was hesitating and for his infinite patience.

I am thankful to all the people whom I encountered during these years, who even not knowing about it has led me to the moment I am in now.

About the author



Ekaterina Veniaminova was born on November 17th, 1990 in Leningrad, USSR (now St. Petersburg, Russia). In 2008-2014, Ekaterina was studying at St. Petersburg State Polytechnical University, graduating first as a Bachelor of Science in physics in 2012 and then as a Master of Science in biophysics in 2014.

During her undergraduate studies, Ekaterina did an internship (under the supervision of Prof. Olga Zubareva) and was further employed as a laboratory assistant at the Department of Physiology, Institute of Experimental Medicine, a place where Ivan Pavlov was doing his groundbreaking work on the digestive systems of dogs. Starting with *in vitro* experiments, Ekaterina then became also interested in inflammation-induced behavioural changes in rodents and did her master research project in a rat model of the CNS neonatal pathology.

In 2014, after graduating from St. Petersburg State Polytechnical University, Ekaterina moved to Moscow and started to work as a junior researcher under the supervision of Prof. Tatyana Strekalova, first in the Institute of General Pathology and Pathophysiology and then, when in 2016 a new laboratory directed by Prof. Klaus-Peter Lesch

was established, in the I.M. Sechenov First Moscow State Medical University (Sechenov University).

Ekaterina became appointed as an external PhD student in Maastricht University in April 2015. She was doing her PhD research in a mouse model of the Western diet feeding under the immediate supervision of Prof. Tatyana Strekalova. Her work was also supervised by Prof. Klaus-Peter Lesch (University of Wuerzburg and University of Maastricht) and Prof. Daniel C. Anthony (University of Oxford). Ekaterina's research has been focusing on the metabolic, behavioural and molecular consequences of the Western diet feeding and the role of serotonin transporter deficiency in the diet-induced changes.

Ekaterina has acquired numerous techniques in behaviour testing, including tests for anxiety and depressive-like behaviour, social, motor and cognitive functions and stress models, brain dissection and surgery, metabolism assessment, histology and immunohistochemistry, as well as molecular biology through a number of local and international internships and collaborative research projects. During the internships in the New University of Lisbon (Portugal), Ekaterina underwent intensive training in behavioural methods under the supervision of Prof. Strekalova and got her first experience in working with transgenic mice. There, she was also trained in glucose tolerance assessment by Dr. Brandon Cline (Strasbourg University) and brain dissection by Prof. Anthony. She was studying immunohistochemistry, brain cutting

and microscopy first in the laboratory of Prof. Lesch in University of Wuerzburg (Germany) and then in the laboratory of Prof. Anthony in University of Oxford (UK), where she investigated microglial activation in the brains of mice fed with the Western diet. In Liege University (Belgium) she studied fluorometric assays of oxidative stress measurement under the supervision of Prof. Lucien Bettendorff. Ekaterina spent almost a year in Hong Kong, participating in the laboratory activities in University of Hong Kong (Prof. Chi Wai Cheung) and Chinese University of Hong Kong (Prof. Eugene Ponomarev), where she was involved in behavioural phenotyping of new transgenic mouse lines. Recently, she took a part in a collaborative project with V.M. Gorbatov Federal Research Center for Food Systems of Russian Academy of Sciences (Moscow, Russia, Dr. Liliya Fedulova and Prof. Irina Chernukha) dedicated to the testing of a new 'healthy' meat product in a mouse model of the Western diet feeding.

Ekaterina is a coauthor of 11 research papers in peer-review journals, including "CNS Neuroscience & Therapeutics", "Brain, Behavior, and Immunity" and "Neural Plasticity". Her H-index is currently 4. She actively participates in local and international conferences and, among other conferences, she has presented her work at "12th Goettingen Meeting of the German Neuroscience Society" in 2017, "20th EURON PhD Days" and "Forum of European Neuroscience Societies" in 2018, and "19th WPA World Congress of Psychiatry" in

2019. During her PhD studies she gave talks on her project in Universities of Maastricht, Wuerzburg, Liege, Oxford, and Hong Kong and in Sechenov University in Moscow. Ekaterina is a lab manager of the Laboratory of Psychiatric Neurobiology in Sechenov University, she supervises undergraduate students and writes blogs for the Eat2beNice project (newbrainnutrition.com/blog).

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Employment

Since 11/2016 – Junior researcher, Laboratory of Psychiatric Neurobiology, Institute of Molecular Medicine, I.M. Sechenov First Moscow State Medical University, Moscow, Russia (1 fte).

Since 4/2015 – External PhD student, School for Mental Health and Neuroscience, European Graduate School of Neuroscience, Maastricht University, Maastricht, Netherlands (0 fte).

10/2014 – 10/2016 – Junior researcher, Laboratory of Cognitive Dysfunctions, Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences, Moscow, Russia (1 fte).

9/2010 – 10/2014 – Laboratory assistant, Laboratory of Neurobiology of Integrative Functions of the Brain, Department of Physiology (Pavlov's), Institute of Experimental Medicine, Russian Academy of Medical Sciences, St. Petersburg, Russia (0.5 fte).

Training experiences in other research Institutes

2/2019 (3 weeks). GIGA Neuroscience, Centre de Neurobiologie Cellulaire et Moléculaire, University of Liege, Liege, Belgium. Supervisor: Prof. L.Bettendorff. Training in Malondialdehyde assay. *Project: Oxidative stress in the brain structures of mice fed the Western diet*

9/2018 (2 weeks). Department of Pharmacology, Oxford University, Oxford, UK. Supervisor: Prof. D.Anthony. Behavioural testing and qPCR analysis. *Project: Effects of the Western diet on behaviour and gene expression of mice with genetic deficiency of serotonin transporter*

3/2017, 12/2017 (4 weeks). School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong SAR. Supervisor: Prof. E.Ponomarev. Behavioural testing. *Projects: Behavioural assessment of mice with genetic deficiency of ST3GAL5, Behavioural assessment of APP transgenic mice*

1/2017, 10/2017, 3/2018 (10 weeks). Department of Pharmacology, Oxford University, Oxford, UK. Supervisor: Prof. D.Anthony. Training in histochemical and immunohistochemical staining. *Project: The effects of Western diet on microglia activation in mouse brain*

9/2016 (2 weeks). School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong SAR. Supervisor: Prof. E.Ponomarev. Training in flow cytometry. *Project: Effects of the Western diet on platelet activation in mice*

3/2015 (3 weeks). Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Wuerzburg, Germany. Supervisor: Prof. K.-P.Lesch. Training in immunohistochemical staining, brain cutting, microscopy. *Project: Study of potential protective effects of BDNF-1 fragment on stress-induced suppression of the hippocampal neurogenesis in a mouse*

10/2014, 12/2014 (4 weeks). Institute of Hygiene and Tropical Medicine, New University of Lisbon, Lisbon, Portugal. Supervisors: Prof. T.Strekalova, Dr. B.Cline, Prof. D.Anthony. Training in behavioural methods, glucose tolerance test, brain perfusion and dissection, blood collection. *Projects: Study of glucose tolerance, memory anxiety- and depressive-like behaviour in aged SERT-het mice, TPH2 mutants and aggressive behaviour in stressed male and female mice: genetic correlates*

Conferences attended, given presentations and seminars

1. **Veniaminova E**, Oplatchikova M, Gorlova A, Hebert J, Radford-Smith D, Cespuglio R, Schmitt-Boehrer A, Lesch K-P, Anthony D, Strekalova T. The role of genetic serotonin transporter deficiency in consequences of exposure to the Western diet: a study in mice // 19th WPA World Congress of Psychiatry, Lisbon, Portugal, 23-24.08.2019 (poster presentation)
2. **Veniaminova E**. Mouse model of 'Western diet', pro-oxidative changes in the brain and impact of genetic serotonin transporter deficiency // "Inflammation and oxidative stress in translational research: new models and treatments", Estoril, Portugal, 20.08.2019 (oral presentation)
3. **Veniaminova E**. Behavioural and molecular effects of the Western diet in mice with genetic serotonin transporter (Sert) deficiency // UK-Russia Young Medic Conference, I.M. Sechenov First Moscow State Medical University, Moscow, Russia, 01.03.2019 (oral presentation)
4. **Veniaminova E**, Gorlova A, Lesh K-P, Anthony D, Strekalova T. The role of serotonin transporter deficiency in the consequences of Western diet in mice // Poster session at the Department of Normal Physiology, I.M. Sechenov First Moscow State Medical University, Moscow, Russia, 24.01.2019 (poster presentation)

5. **Veniaminova E.** Behavioural, molecular and metabolic consequences of Western diet: a study on mice // Talk at Gorbатов Federal Research Center for Food Systems of RAS, Moscow, Russia, 26.12.2018 (oral presentation)
6. **Veniaminova E.** Behavioural, molecular and metabolic consequences of Western diet in mice and the role of genetic deficiency of the serotonin transporter // Talk at I.M. Sechenov First Moscow State Medical University, Moscow, Russia, 20.11.2018 (oral presentation)
7. **Veniaminova E,** Oplatchikova M, Gorlova A, Pomytkin I, Lesch K-P, Anthony D, Strekalova T. Behavioural, molecular and metabolic consequences of Western diet in mice and the role of genetic deficiency of the serotonin transporter // 20th EURON PhD Days, Brussels, Belgium, 13-14.09.2018 (oral presentation)
8. **Veniaminova E,** Kopeikina E, Zheng Kai S T, Khairuddin Sh, Lim L W, Ponomarev E, Strekalova T. Behavioural characteristics of KO mice lacking ST3GAL5, key enzyme in sialated ganglioside synthesis // Forum of European Neuroscience Societies, Berlin, Germany, 7-11.07.2018 (poster presentation)
9. **Veniaminova E,** Kopeikina E, Kai Tan SZ, Khairuddin Sh, Lesch K-P, Lim LW, Ponomarev E, Strekalova T. Behavioural characteristics of mice with genetic deficiency of ST3GAL5, the key enzyme in brain-specific ganglioside synthesis // Sechenov International

Biomedical Summit, Moscow, Russia, 21.05.2018 (poster presentation)

10. **Veniaminova E**, Oplatchikova M, Gorlova A, Pavlov D, Bazhenova N, Pomytkin I, Lesch KP, Anthony D, Strekalova T. Increased impulsivity and microglia activation in the prefrontal cortex in mice housed on Western diet are associated with multiple behavioural abnormalities // 25th Annual International “Stress and Behavior” Neuroscience and Biopsychiatry Conference, St. Petersburg, Russia, 16-20.05.2018 (oral presentation)
11. **Veniaminova E**, Shevtsova E, Markova N, Gorlova A, Pavlov D, Morozova A, Chekhonin V, Lesch K-P, Anthony D, Strekalova T. Behavioural, molecular and metabolic consequences of Western diet and ameliorating effect of dicholine succinate // 12th Goettingen Meeting of the German Neuroscience Society, Goettingen, Germany, 22-25.04.2017 (poster presentation)
12. **Veniaminova E**. Behavioural, molecular and metabolic consequences of The effects of Western diet on behaviour and molecular parameters in mice // International Evaluation Conference of the Laboratory of Psychiatric Neurobiology, I.M. Sechenov First Moscow State Medical University, Moscow, Russia, 06.04.2017 (oral presentation)
13. **Veniaminova E**. Dietary cholesterol as a pathogenetic factor of depressive-like syndrome: a study on mice // Talk at Laboratory

- and Clinical Research Institute for Pain, The University of Hong Kong, 13.05.2016 (oral presentation)
14. 22nd Annual International “Stress and Behavior” Neuroscience and Biopsychiatry Conference, St. Petersburg, Russia, 16-19.05.2015 (attended)
 15. 11th Goettingen Meeting of the German Neuroscience Society, Goettingen, Germany, 18-21.03.2015 (attended)
 16. **Veniaminova E.** Dietary cholesterol and genetically decreased SERT function in mice: potential interaction in the pathogenesis of depressive-like syndrome // Division of Molecular Psychiatry, Laboratory of Translational Neuroscience, University Clinic of Wuerzburg, Wuerzburg, Germany, Laboratory Seminar, 12.03.2015 (oral presentation)
 17. Zubareva OE, **Veniaminova EA**, Kalemenev SV, Schwarz AP, Fomalont KJ. Altered cognitive dysfunction and changes in NMDA and AMPA receptor subunit gene expression in rat brain after LPS injection in early postnatal ontogenesis // All-Russian Conference with International Participation “Neurochemical mechanisms of adaptive and pathological brain states”, St. Petersburg, Russia, 24-26.06.2014 (poster presentation)
 18. Fomalont KJ, **Veniaminova EA**, Kalemenev SV, Trofimov AN, Schwarz AP, Zubareva OE. Early-life LPS administrations induce cognitive decline and changes in NMDA receptor subunit gene

- expression in the rodent brain // 21st Annual PNIRS Scientific Meeting, Philadelphia, USA, 28-31.05.2014
19. 21st Annual International “Stress and Behavior” Neuroscience and Biopsychiatry Conference, St. Petersburg, Russia, 16-19.05.2014 (attended)
20. **Veniaminova EA**, Rotov AYu. LPS effects on NMDA receptor subunit gene expression in brain structures of juvenile rats // International Scientific Conference of Students and Young Scientists “Lomonosov – 2014”, Moscow, Russia, 7-11.04.2014 (poster presentation)
21. **Veniaminova EA**, Fomalont KJ, Manyukhina VO, Zubareva OE. The influence of bacterial lipopolysaccharide on NMDA receptor subunit gene expression in juvenile rat brain. All-Russian Conference of Young Scientists “Neurobiology of Integrative Brain Functions”, St. Petersburg, Russia, 12-14.11.2013 (poster presentation)
22. **Veniaminova EA**, Trofimov AN, Schwarz AP, Zubareva OE. The influence of lipopolysaccharide injections in early postnatal ontogenesis on expression of NMDA receptor subunits in rat brain structures // The XLI theoretical and practical conference with international participation «The week of science and academic research at SPBSPU», St. Petersburg, Russia, 3-8.12.2012 (oral presentation)

23. **Veniaminova EA**, Trofimov AN, Schwarz AP. Changes in NMDA receptor subunit gene expression in the rat hippocampus caused by increased level of interleukin-1 β in early postnatal ontogenesis // 17th International Pushchino School Conference of Young Scientists “Biology - The Science of the XXI Century”, Pushchino, Russia, 22-26.04.2013 (oral presentation)
24. **Veniaminova EA**, Zubareva OE. The influence of interleukin-1 β injections in early postnatal ontogenesis on mRNA expression of NMDA-receptor subunits in rat hippocampus. The XLII theoretical and practical conference with international participation «The week of science and academic research at SPBSPU», St. Petersburg, Russia, 1-6.12.2012 (oral presentation)

List of publications

1. **Veniaminova E**, Oplatchikova M, Bettendorff L, Kotenkova E, Lysko A, Vasilevskaya E, Kalueff A, Fedulova L, Umriukhin A, Lesch KP, Anthony DC, Strekalova T. Prefrontal cortex inflammation and liver pathologies accompany cognitive and motor deficits following Western diet consumption in non-obese female mice. *Life Sciences*. 2020; 241:117163.
2. de Munter J, Shafarevich I, Liundup A, Pavlov D, Wolters E, Gorlova A, **Veniaminova E**, Umrukhin A, Kalueff A, Svistunov A, Kramer BW, Lesch KP, Strekalova T. Neuro-Cell therapy improves motor outcomes and suppresses inflammation during experimental syndrome of amyotrophic lateral sclerosis in mice. *CNS Neurosci Ther*. 2019; 00:1–14
3. Dukhinova M, Kuznetsova I, Kopeikina E, **Veniaminova E**, Yung AWY, Veremeyko T, Levchuk K, Barteneva NS, Wing-Ho KK, Yung WH, Liu JYH, Rudd J, Yau SSY, Anthony DC, Strekalova T, Ponomarev ED. Platelets mediate protective neuroinflammation and promote neuronal plasticity at the site of neuronal injury. *Brain Behav Immun*. 2018;74:7-27.
4. Pomytkin I, Costa-Nunes JP, Kasatkin V, **Veniaminova E**, Demchenko A, Lyundup A, Lesch KP, Ponomarev ED, Strekalova T. Insulin receptor in the brain: Mechanisms of activation and the

- role in the CNS pathology and treatment. *CNS Neurosci Ther.* 2018;24(9):763-774.
5. Amakhin DV, Malkin SL, Ergina JL, Kryukov KA, **Veniaminova EA**, Zubareva OE, Zaitsev AV. Alterations in Properties of Glutamatergic Transmission in the Temporal Cortex and Hippocampus Following Pilocarpine-Induced Acute Seizures in Wistar Rats. *Front Cell Neurosci.* 2017;11:264.
 6. **Veniaminova E**, Cespuglio R, Cheung CW, Umriukhin A, Markova N, Shevtsova E, Lesch KP, Anthony DC, Strekalova T. Autism-Like Behaviours and Memory Deficits Result from a Western Diet in Mice. *Neural Plast.* 2017;2017:9498247.
 7. **Veniaminova E**, Cespuglio R, Markova N, Mortimer N, Cheung CW, Steinbusch HW, Lesch KP, Strekalova T. Behavioral features of mice fed with a cholesterol-enriched diet: deficient novelty exploration and unaltered aggressive behavior. *Transl. Neurosci. Clin.* 2016;2(2):87-95.
 8. Malkin SL, Amakhin DV, **Veniaminova EA**, Kim KK, Zubareva OE, Magazanik LG, Zaitsev AV. Changes of AMPA receptor properties in the neocortex and hippocampus following pilocarpine-induced status epilepticus in rats. *Neuroscience.* 2016;327:146-55.
 9. Strekalova T, Costa-Nunes JP, **Veniaminova E**, Kubatiev A, Lesch KP, Chekhonin VP, Evans MC, Steinbusch HW. Insulin receptor sensitizer, dicholine succinate, prevents both Toll-like

receptor 4 (TLR4) upregulation and affective changes induced by a high-cholesterol diet in mice. *J Affect Disord.* 2016;196:109-16.

10. **Veniaminova E**, Strekalova T. High fat and cholesterol consumption as pathogenetic factor of depression: possible molecular mechanisms. *Neurochemical journal.* 2015, 2016;1:33-41
11. **Veniaminova E**, Zubareva O. Delayed changes in exploratory behavior and fgf2 gene expression in rat brain cells after neonatal lipopolysaccharide administration. *Neurochemical journal.* 2015, 32 (2) 153-160

Paper under revision

Veniaminova E, Cespuglio R, Chernukha I, Schmitt-Boehrer AG, Morozov S, Kalueff AV, Kuznetsova O, Anthony DC, Lesch KP, Strekalova T. Metabolic, molecular and behavioural effects of Western diet in serotonin transporter-deficient mice: rescue by heterozygosity? *Frontiers in Neuroscience.*

Papers in preparation

1. **Veniaminova E**, Hebert J, Probert F, Radford-Smith D, Morozov S, Kalueff A, Cespuglio R, Schmitt-Boehrer AG, Lesch KP, Anthony DC, Strekalova T. In mice the absence of the serotonin transporter exacerbates the behavioural and metabolic phenotype induced by a Western diet.

2. Mostert J, Arteaga G, Becerra N, Bierens M, Bloemendaal M, Bosch A, Cabana J, Fernández N, Koch E, Konstanti P, Grimstvedt Kvalvik L, Li L, Niemeyer L, van Rooij D, Schwaren L, Shi Y, **Veniaminova E**, Willemse Y, Belzer C, Buitelaar J, Bullo M, Cormand B, Dietrich A, Ebner-Priemer U, Franke B, Haavik J, Haege A, Hartman C, Hoekstra P, Faraone S, Klunzler K, Larsson H, Lesch KP, Ramos-Quiroga A, Reif A, Réthelyi J, Rommelse N, Rucklidge J, Strekalova T, Salas J, de Weerth C, Arias-Vasquez A. The role of diet, exercise and gut-microbiome in aggressive, impulsive and compulsive behaviour: the Eat2beNICE project.