The interplay between a dietary preference for fat and sugar, gene expression in the dopaminergic system and executive cognition in humans

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1. Abbreviations

A	adenine
ACC	anterior cingulate cortex
ADP	adenosine diphosphate
ANCOVA	analysis of covariance
ANKK1	ankyrin repeat and kinase domain containing 1
ANOVA	analysis of variance
ATP	adenosine triphosphate
BDI	Beck Depression Inventory
BIS/BAS	Behavioral approach and avoidance system questionnaire
BIS15	Barrat Impulsiveness Scale
BMI	body mass index
Bp	base pairs
C	cytosine
cAMP	3′-5′-cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
COMT	catechol-O-methyltransferase
CREB	cAMP response element-binding protein
CRP	C-reactive protein
cT	threshold cycle
DA	dopamine
DARPP-32	dopamine-and-cAMP-regulated neuronal phosphoprotein 32
DAT	dopamine (active) transporter
DDC	DOPA-decarboxylase
DFS	Dietary Fat and free Sugar – Short Questionnaire
dlPFC	dorsolateral prefrontal cortex
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DRD 2/3/5	dopamine receptors D2/D3/D5
DS	Digit span test
DSST	Digit symbol substitution test
EDE-Q	Eating Disorder Examination Questionnaire
FCQ-T	Food Craving Questionnaire-Trait
FEV	Three-Factor Eating Questionnaire
fMRI	functional magnetic resonance imaging
fT3	free triiodothyronine
fT4	free thyroxine
FTO	fat mass and obesity-associated protein
g	gram
G	guanine
GABA	gamma-aminobutyric acid
GPi	globus pallidus internus
h	hour
HbA1c	glycated hemoglobin
HDL	high-density lipoprotein
HFD	high fat diet
HFS	high fat and sugar
HKG	housekeeping gene
HOMA-IR	Homeostasis model assessment
HPRT1	hypoxanthine phosphoribosyl
hsCRP	highly sensitive C-reactive protein
HSD	high sugar diet
HVA	homovanillic acid
IL-6	interleukin 6
iNOS	inducible nitric oxide synthases
IPAQ	International Physical Activity Questionnaire
IQ	intelligence quotient
IQR	interquartile range
LDL	low-density lipoprotein
L-DOPA	levodopa
LFD	low fat diet
LFS	low fat and sugar
M	mean
Max	maximal value
MET	metabolic equivalent of task
Met	methionine
Mg^{2+}	Magnesium cation
Min	minimal value
min	minute
mRNA	messenger ribonucleic acid
MSN	medium spiny neurons

n.s.	not significant
NAc	nucleus accumbens
NEO FFI	NEO Five Factor Inventory
NP tests	neuropsychological tests
OFC	orbitofrontal cortex
p	p-value
PA	physical activity
PBL	peripheral blood lymphocytes
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PET	positron emission tomography
PFC	prefrontal cortex
PKA	protein kinase A
PP1	protein phosphatase 1
qPCR	qualitative polymerase chain reaction
r	effect size/correlation coefficient
RNA	ribonucleic acid
ROS	reactive oxygen species
RPLP0	ribosomal protein, large, P0
rpm	rounds per minute
SD	standard deviation
s	second
SNP	single nucleotide polymorphism
SNr	substantia nigra pars reticulata
SOX 17	SRY-Box Transcription Factor 17
SPECT	single-photon emission computed tomography
T	thymine
TBP	TATA box binding protein
TH	tyrosine hydroxylase
TMT	Trail making test
TNF-α	tumor necrosis factor α transferase 1
tRNA	transfer RNA
UCPs	uncoupling proteins
UPPS	Urgency, Premeditation, Perseverance, Sensation Seeking Impulsive Behavior Scale
Val	valine
vlPFC	ventrolateral prefrontal cortex
VMAT	vesicular monoamine transporter
VNTR	variable number tandem repeat
vs	versus
WHR	waist-to-hip-ratio
WMT	Wiener Matrizen-Test 2 (Viennese Matrices Test 2)
YFAS	Yale Food Addiction Scale

2. Introduction

2.1 Obesity: a serious health issue

Obesity is a public as well as individual health issue of increasing importance. According to the World Health Organization, in 2016 39% of all adults worldwide were overweight (which corresponds to almost 2 billion people) of which 650 million were obese – with upward tendency, as the number of obese individuals almost tripled since 1975 (Obesity and overweight, 2020). In Germany, the situation is even more dramatic. The DEGS1 study including data from 2008 to 2011 found that more than half of the population is overweight (67.1 % of men and 53.0 % of women), and around 23 % are obese, with a rising trend especially for young adults (Mensink et al., 2013).

The severity of obesity-associated comorbidities, health risks and social disadvantages are commonly known. A 10-year follow-up study by Field et al. showed that obesity is highly associated with high blood pressure, cardiovascular diseases and metabolic dysfunctions like diabetes mellitus type II and hypercholesterolemia (Field et al., 2001). The risk of developing these comorbidities increases with the severity of obesity and a high BMI additionally often leads to acquiring multiple chronic conditions. As summarized by Flegal et al. obesity is also a major risk factor for rheumatoid arthritis, cancer or psychiatric disorders like major depression (Flegal et al., 2013) and even has consequences on a social level, as obese children have a higher number of absence days from school and are more often the target of bullying by peers (Apovian, 2016). Furthermore, severe obesity is associated with a higher general mortality (Flegal et al., 2013) qualifying obesity as a serious health issue and impairment of life quality.

2.2 Obesity impairs behavioral control and decision-making

People with obesity are often aware of the negative consequences their weight condition could have and feel the wish to lose weight by dieting or doing sports. However, many fail to incorporate their intent into their everyday life or continue it over a longer period of time to achieve a permanent weight loss. The question immediately arising is why these discrepancies occur between planned and actual behavior and what drives the deviation from the original goal?

One explanation is the modern environment that promotes acquiring excess weight. Energy-dense, high-caloric, and very palatable food is constantly and easily available and often even cheaper than homemade meals prepared with healthy ingredients, not mentioning the time-consuming procedure of choosing, buying, and cooking healthy groceries. Additionally, physical exercise is limited to a minimum due to sitting jobs and inactively spent free time (Hill & Peters, 1998; Jeffery & Utter, 2003; Leung et al., 2011). From an evolutionary point of view, reward-driven or hedonic feeding, which means consuming food above the extent of homeostatic needs, was necessary and reasonable to gain fat depots in times of abundant nutrition for times of scarcity. In modern food environments this well-adapted feeding behavior lost its purpose and can even be considered maladaptive (Pinel, Assanand, & Lehman, 2000). On the other hand, inhibition of hedonic feeding in support of the social group was also an important ability in the evolutionary context. Therefore, successful dieting may depend on a "hedonic inhibition model", when behavioral inhibition dominates over hedonic food craving (Appelhans, 2009).

Strikingly, there is extensive literature demonstrating that individuals with obesity lack in inhibitory control and reward-based decision-making abilities and seem to prefer immediate rewards despite future negative consequences or the loss of a delayed bigger reward (Brogan, Hevey, O'Callaghan, Yoder, & O'Shea, 2011; Pignatti et al., 2006). In line, Horstmann et al. found that obese

women, even in the face of unpleasant consequences, chose immediate salient monetary rewards more often than lean controls (Horstmann et al., 2011). Also, learning from negative outcomes seems to be impaired in obesity in the context of food (Horstmann et al., 2017) and in non-food context (Kube et al., 2018; Mathar, Neumann, Villringer, & Horstmann, 2017). The latter two studies suggested that sensitivity to changes in the motivational value of a reward is less pronounced in people with obesity. Further, obese individuals showed working memory deficits and failed to avoid negative outcomes in a reward learning task (Coppin, Nolan-Poupart, Jones-Gotman, & Small, 2014). Contrarily, Meemken et al. found that obesity is not associated with an impairments in reinforcement learning but with enhanced flexibility in reward-approaching. This increased flexibility could promote obesity-prone behavior (Meemken, Kube, Wickner, & Horstmann, 2018). Moreover, obese individuals are generally more susceptible to disturbances by internal or external cues. Obesity is associated with a higher degree of disinhibition, especially in the presence of tempting food cues, leading to opportunistic overeating and unhealthy food and lifestyle choices (Bryant, King, & Blundell, 2008). Additionally to these ambient cues, emotional states such as depression played a relevant role in the context of weight gain as examined in women over a 20 years period (Hays & Roberts, 2008) and a stressful work environment can additionally add to increased caloric intake (Scott & Johnstone, 2012).

In general, obese individuals exhibit a higher responsiveness of reward regions in the brain towards food (García-García et al., 2014) even after devaluating individual food motivation (Horstmann et al., 2015). These characteristics of decision-making in obesity may take effect especially in cost/benefit decisions and could lead to neglect of long-term goals in the face of rewarding short-termed pleasures.

2.3 Executive functions: the underlying cognitive mechanisms for goal-directed behavior

Decision making and behavioral control are higher order cognitive processes that depend on top-down executive functions including inhibition and interference control (selective attention), working memory, and cognitive flexibility (Diamond, 2013). Literature confirmed that executive functions play an essential role in eating and exercising behavior, weight loss success and snack food intake (Dohle, Diel, & Hofmann, 2018; Riggs, Spruijt-Metz, Sakuma, Chou, & Pentz, 2010; Will Crescioni et al., 2011). Executive function deficits are associated with impulsive/compulsive behavior, disinhibition and inflexibility of thinking, which are cognitive traits observed in addiction-like behaviors including hedonic overeating (Lee et al., 2013; Lokken, Boeka, Austin, Gunstad, & Harmon, 2009; Zhao et al., 2017) and therefore play a relevant role in the context of obesity acquisition.

Individuals with obesity show specific impairments and behaviorally relevant changes in executive functioning. Inhibitory control was restricted in individuals with a higher BMI during a Stop-Signal-Task with food cues but not with general cues, indicating impaired impulse inhibition selectively towards palatable food (Houben, Nederkoorn, & Jansen, 2014). Additionally, a higher BMI was associated with an attentional bias towards pictures depicting food items along with increased activation of attention- and reward-related brain regions, e.g. orbitofrontal cortex (OFC) and ventrolateral prefrontal cortex (vlPFC), insula and operculum (Yokum, Ng, & Stice, 2011). Deficits in the working memory domain were also found in obese individuals in several studies (Coppin et al., 2014; Gunstad, Paul, Cohen, Tate, & Gordon, 2006). A current meta-analysis indicated that obese individuals actually show impairments in all relevant basic domains of executive functioning, i.e. in inhibition, cognitive flexibility, working memory, decision-making, verbal fluency, and planning, with inhibition and working memory being impaired already in overweight individuals (Yang, Shields, Guo, & Liu, 2018). For the assessment of executive functioning a vast variety of different tasks is available that are more or less specific and sensitive for detecting impairments. Basic neuropsychological tests like the

Trail making test, the Digit symbol substitution test and the Digit span test are straightforward and easy to administer and were still sufficient to show executive impairments in association with obesity (Cohen, Yates, Duong, & Convit, 2011; Gunstad et al., 2007; Maayan, Hoogendoorn, Sweat, & Convit, 2011; Verdejo-García et al., 2010; Yau, Kang, Javier, & Convit, 2014) suggesting that these tests might provide a feasible method for a basic assessment of executive functions.

2.4 Executive functions: brain regions, pathways, and neurotransmitters

Executive functions are based on distinct neuronal loops called fronto-striatal circuits and depend on several neurotransmitter systems. The circuits are organized in segregated but parallel anatomical loops originating in the frontal cortex that project via the basal ganglia and the thalamus back to the frontal cortex (Alexander, DeLong, & Strick, 1986). Each loop is enrolled in different cognitive functions relevant for controlled and adaptive behavior (summarized by (Alvarez & Emory, 2006; Mega & Cummings, 1994; Tekin & Cummings, 2002). Fig. 1 shows an overview of involved neuroanatomical regions, pathways, and neurotransmitters.

The dorsolateral prefrontal circuit is commonly associated with general executive functions like working memory, response inhibition, set-shifting, reasoning, problem-solving, organizing or planning (Diamond, 2013; Nee, Wager, & Jonides, 2007). The orbitofrontal circuit is relevant for behavioral inhibition and control (Bryden & Roesch, 2015) and thus lesions in this circuit can lead to disinhibition and impulsivity up to antisocial behavior. The anterior cingulate circuit is part of the motivational system and associated with motivation-dependent selective attention as lesions of this circuit cause decreased social interaction and apathy (Appelhans, 2009).

The circuits are named after their frontal origin, the dorsolateral prefrontal cortex (dlPFC), the lateral orbitofrontal cortex (OFC) and the anterior cingulate cortex (ACC), respectively. From there, projections reach the striatum comprising mainly caudate nucleus, putamen, and the ventral striatum with the nucleus accumbens (NAc) and the olfactory tubercle. The dlPFC projects to the dorsolateral caudate nucleus, the OFC to the ventromedial caudate nucleus and the ACC to the ventral striatum. All parts of the striatum are then connected with the globus pallidus internus (GPi) and substantia nigra pars reticulata (SNr), each circuit with a slightly different part of these regions. There is a direct connection from the striatum to the GPi/SNr and an indirect connection reaching GPi and SNr via the globus pallidus externus and the subthalamic nucleus (Gerfen & Surmeier, 2011). The GPi and SNr send projections to the ventral-anterior or mediodorsal thalamus, depending on the circuit. Finally, the loops are closed by thalamic projections to the frontal origins of each loop. Additionally, there are “open loop” connections to and from other cortical and subcortical structures sending e.g. sensory or emotional input. The anatomy of the fronto-striatal loops as described here was summarized in several comprehensive reviews (Mega & Cummings, 1994; Tekin & Cummings, 2002).

Several neurotransmitters are relevant for signal transmission in these circuits. The frontal lobes project to the striatum with glutamate as excitatory transmitter. Striatal neuronal projections to the GPi and SNr, the so-called medium spiny neurons (MSN), use the inhibitory neurotransmitter GABA (gamma-aminobutyric acid), but the indirect pathway also includes a glutamatergic connection between subthalamic nuclei and the thalamus. The GPi and SNr project via GABA to the thalamus and the thalamus in turn sends excitatory glutamatergic signals back to the frontal cortex (Mega & Cummings, 1994). The neurotransmitter dopamine acts as a neuromodulator in all circuits through several inputs to the system (Floresco & Magyar, 2006; Tekin & Cummings, 2002). Via the mesocortical projections from the ventral tegmental area to the dlPFC, dopamine modulates frontal lobe activity related to general executive functions (Floresco & Magyar, 2006; Logue & Gould, 2014; Tekin & Cummings, 2002). The mesolimbic projection from the ventral tegmental area (VTA) to the nucleus

accumbens is an important connection for integrating positive and negative rewards, motivational and aversive stimuli and contributes to manifestation of personality traits like novelty seeking, extraversion and impulsivity (Alcaro, Huber, & Panksepp, 2007). The dopaminergic input to MSN in the striatum via the nigrostriatal pathway can modulate activity in all circuits. These projections from the substantia nigra pars compacta to striatal regions can either be inhibitory or excitatory, depending on the predominant receptor subtype. Activation of D1-like dopamine (DA) receptors on MSN has an excitatory effect on the activity of the direct pathway, whereas activation of D2-like receptors expressed on MSN can inhibit the indirect pathway (Keeler, Pretsell, & Robbins, 2014; Kravitz, O'Neal, & Friend, 2016). The direct pathway is for example involved in behavioral optimization in response to positive outcomes, whereas the indirect pathway subserves behavioral adaptation to negative feedback (Frank & Fossella, 2011; Sevgi et al., 2015).

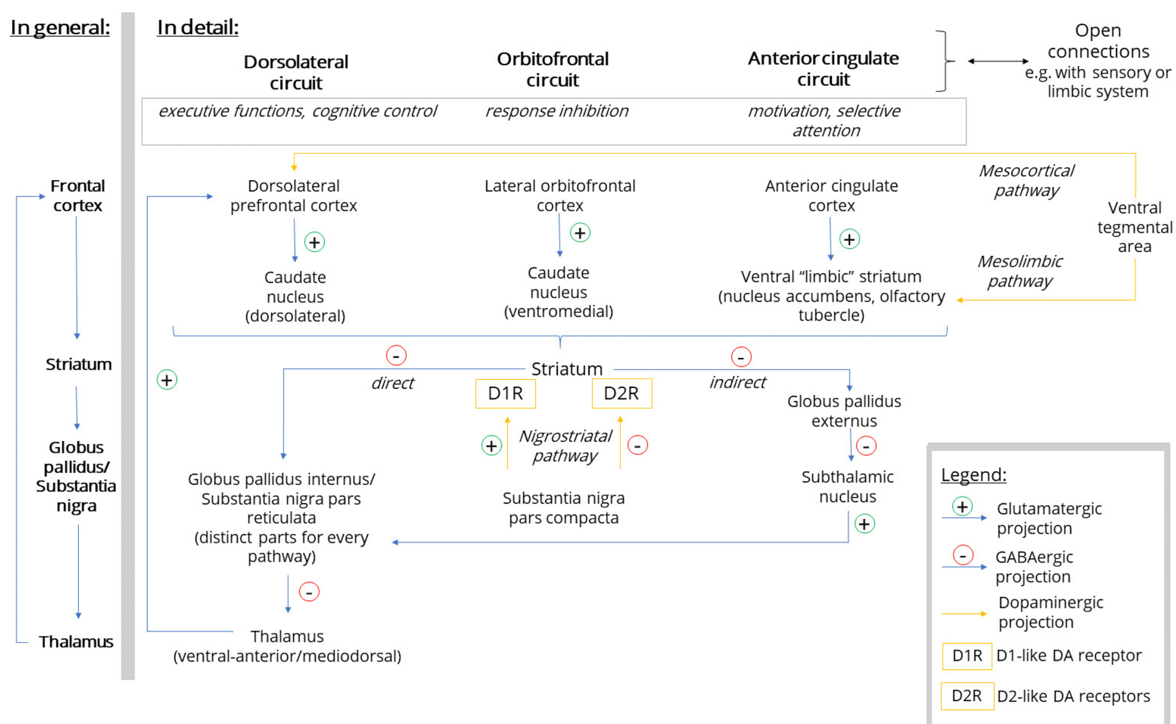


Figure 1: Fronto-striatal circuits. In general: all circuits originate in the frontal cortex and project via the striatum and globus pallidus/substantia nigra to the thalamus and back to the frontal lobes. Dorsolateral circuit: from the origin in the dorsolateral prefrontal cortex projections via glutamate reach the dorsolateral caudate nucleus. Orbitofrontal circuit: from the origin in the lateral orbitofrontal cortex projections via glutamate reach the ventromedial caudate nucleus. Anterior cingulate circuit: from the origin in the anterior cingulate cortex projections via glutamate reach the ventral striatum (nucleus accumbens and olfactory tubercle). Joint striato-thalamo-cortical projections: striatal neurons project via a direct inhibitory (GABAergic) connection or via an indirect connection to the globus pallidus internus (GPi) and substantia nigra pars reticulata (SNr). The indirect projection passes the globus pallidus externus (GABAergic) and the subthalamic nucleus (GABAergic) and reaches the GPi and SNr via an excitatory connection. GPi and SNr send inhibitory projections to the ventral-anterior and mediodorsal thalamus. The loops are closed by thalamic excitatory projections to the frontal origins of each loop. Dopaminergic input: the mesocortical pathway projects from the ventral tegmental area to the dlPFC, the mesolimbic pathway projects from the ventral tegmental area to the nucleus accumbens and the nigrostriatal pathway projects from the substantia nigra pars compacta to the striatum. Nigrostriatal signals activating D1-like receptors have an excitatory effect on the direct pathway and activation of D2-like receptors inhibits the indirect pathway. Open connections: connections to and from other cortical and subcortical structures send e.g. sensory or emotional signals.

The process of eating is represented in various brain regions that are part of this system (Berthoud, 2007). The frontal cortex (dlPFC, OFC and ACC) is involved in the mental representation of experiences with food, e.g. the palatability of a food item. Striatal regions like the nucleus accumbens and the ventral part of the globus pallidus mediate “liking”, i.e. the positive affect experienced when eating palatable food, via opioid receptors. The dopaminergic system is involved in the mediation of “wanting” of food or food craving via the mesolimbic projection to the nucleus accumbens. This “wanting” induces food-seeking-behavior, e.g. for sweet and palatable snacks, that can even have addiction-like traits (Avena, Rada, & Hoebel, 2008; García-García et al., 2014). Guo et al. were able to establish a connection between obesity/opportunistic eating and changes of dopamine receptor availabilty in different striatal regions measured with positron emission tomography (PET) (Guo, Simmons, Herscovitch, Martin, & Hall, 2014). This indicates differences in dopaminergic transmission associated with obesity that could increase susceptibility for overeating. Several reviews gave an apt description of the interaction between the appetitive and the inhibitory cognitive system regarding eating behavior (Appelhans et al., 2012; Horstmann, 2017). The appetitive system, here the mesolimbic pathway, mediates the hedonic motivation for rewarding and pleasurable behaviors like eating, gambling or addictive drug use. Opposing inhibitory signals coming from the executive system allow individuals to consider the consequences of hedonic behaviors and potentially delayed larger rewards. Appelhans et al. mention several examples of studies that show that the limbic system could be the main driver of hedonic overconsumption of palatable food without a metabolic need, while the dlPFC is involved in inhibiting such behavior (Appelhans et al., 2012; Hall, Vincent, & Burhan, 2018). The balance between both systems contributes to long-term successful weight maintenance whereas an imbalance could consequently promote obesity development (see also (Le et al., 2007).

2.5 The dopaminergic signal transmission system

2.5.1 The dopaminergic synapse

The neurotransmitter and neuromodulator dopamine is synthesized predominantly in the VTA and SNr in presynaptic dopaminergic neurons (Dahlstroem & Fuxe, 1964). Fig. 2 depicts the main processes of DA synthesis, signal transmission and signal termination graphically. DA is a monoamine, or more specifically a catecholamine, derived from the amino acids L-tyrosine or phenylalanine, which can be transformed into tyrosine. Tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis, transforms L-tyrosine into the intermediate product levodopa (L-DOPA), which is then processed to DA by the DOPA-decarboxylase (DDC) (Christenson, Dairman, & Udenfriend, 1970; Klein et al., 2019). Subsequently, DA is transferred into vesicles by the vesicular monoamine transporter 2 (VMAT2) and stored until its exocytotic release into the synaptic cleft upon an excitatory signal reaching the presynapse (Eiden, Schäfer, Weihe, & Schütz, 2004).

There are two mechanisms of DA release, phasic firing, and tonic transmission. Phasic DA firing is triggered by excitatory action potentials reaching the presynaptic neurons and lead to a fast but short (in the range of milliseconds) spike of DA concentration in the synaptic cleft. Tonic DA transmission describes a constant release of smaller DA concentrations over several seconds to minutes which is regulated independently of action potentials by the activity of surrounding neurons and negative dopaminergic feedback (Floresco, West, Ash, Moore, & Grace, 2003). For optimal functionality of the DA system certain levels of phasic and tonic DA are required for instance in the context of memory functioning and cognitive control (Cools & D'Esposito, 2011; Mattay et al., 2003). These optimal levels could be disturbed by a non-linear effect of weight gain on DA transmission such

that being overweight might be accompanied by a reduction in dopamine tone and associated exaggerated phasic DA responses in the striatum, whereas severely obese individuals might show an increased dopamine tone, but blunted phasic firing in the striatum (Horstmann, Fenske, & Hankir, 2015). Phasic DA release is a rapid signal for incoming new rewarding stimuli, whereas constant tonic DA release indicates the engagement of an individual with its environment during positive and negative emotional arousal. The constant presence of DA in tonic signaling activates the autoinhibitory D2-type receptors pre- and postsynaptically and therefore reduces the excitatory effect of glutamatergic cortical projections so that top-down control of behavior from the cortex may be attenuated by high tonic DA. Additionally, high levels of tonic DA inhibit phasic DA release by reducing the likelihood of a DA burst via the inhibitory presynaptic D2-like receptors but simultaneously increase the total level of released DA when a phasic burst occurs (Alcaro et al., 2007). In conclusion, overweight might be associated with a higher susceptibility for appetitive reward (more phasic firing), but individuals with severe obesity might have a less excitable reward system which at the same time can signal reward even more powerfully when the level of reward is high enough.

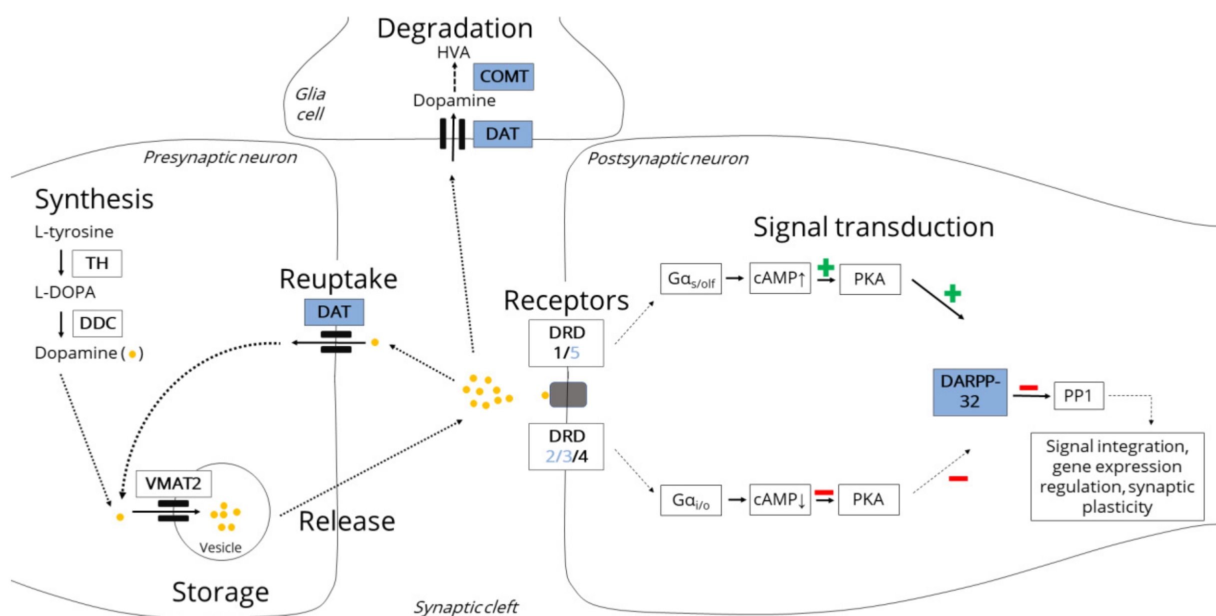


Figure 2: Signal transmission in a dopaminergic synapse. Presynaptic neuron (left): DA is synthesized from L-tyrosine. Tyrosine hydroxylase (TH) converts tyrosine to L-DOPA, which is transformed into DA by the DOPA-decarboxylase (DDC). DA is then transported into vesicles via the vesicular monoamine transporter 2 (VMAT 2), where it is stored until its release. Postsynaptic neuron (right): DA-receptors (DRDs) are either coupled to $G_{\alpha s/olf}$ (D1-like family: DRD1/5) or to $G_{\alpha i/o}$ (D2-like family: DRD2/3/4). $G_{\alpha s/olf}$ increases the cytosolic cAMP (3'-5'-cyclic adenosine monophosphate) concentration, which activates protein kinase A (PKA). $G_{\alpha i/o}$ decreases cAMP concentration, which inhibits PKA activity. PKA phosphorylates dopamine and cAMP-regulated phosphoprotein 32 (DARPP-32) and by that converts it into an active protein, which is able to inhibit protein phosphatase 1 (PP1). DARPP-32 is involved in a complex network of signaling pathways, which finally affects e.g. signal integration from other pathways or gene expression regulation causing synaptic plasticity. Synaptic cleft/glia cell: the DA signal is terminated mainly by reuptake of DA via DAT into the presynaptic cell, where it can be recycled and stored again in vesicles for a second release. DAT can also transfer DA into glia cells for degradation by catechol-O-methyltransferase (COMT). The end product of degradation is homovanillic acid (HVA). Blue marks: genes of interest for the mRNA expression analyses (see section 4.4.1). Source: the idea for this figure is conceptionally derived from a paper by Ramos-Lopez et al. (Ramos-Lopez, Riezu-Boj, Milagro, Martinez, & MENA Project, 2018a).

Released DA in the synaptic cleft binds to DA receptors (DRDs) inducing a postsynaptic signal. However, DA cannot clearly be classified as excitatory or inhibitory neurotransmitter, as DRDs are coupled to

different G-proteins, which initiate different signal cascades. The family of D1-like receptors – DRD1 and DRD5 - are coupled with a $G\alpha_{s/olf}$ protein. Their activation leads to increased productivity of the protein adenylyl cyclase resulting in higher intracellular 3′-5′-cyclic adenosine monophosphate (cAMP) levels, which stimulates the activity of the protein kinase A (PKA). On the contrary, the D2-like receptor family – DRD2, DRD3 and DRD4 - are connected with a $G\alpha_{i/o}$ protein, which inhibits the adenylyl cyclase and therefore, inversely to D1-like receptors, decreases intracellular concentration of cAMP thus attenuating the activity of PKA (Klein et al., 2019; Missale, Nash, Robinson, Jaber, & Caron, 1998).

The PKA has several targets like ion channels (e.g., calcium and potassium), receptors (e.g. glutamate and GABA receptors), and proteins such as the cAMP response element-binding protein (CREB) and dopamine and cAMP-regulated phosphoprotein 32 (DARPP-32). DARPP-32 is one of the most interesting molecules within the dopaminergic downstream signaling pathway. The connection of PKA with DARPP-32, which is expressed in medium spiny neurons of the striatum, modulates the downstream PKA signal. The PKA phosphorylates DARPP-32 and by that converts DARPP-32 into an active protein, which is able to inhibit protein phosphatase 1 (PP1) (Greengard, 2001). Further effects of phosphorylation and dephosphorylation within the DARPP-32/PP1 cascade are manifold. For instance, DARPP-32 mediates the interaction between dopaminergic and glutamatergic pathways which is necessary to integrate glutamatergic signals reaching the striatum from the cortex. Relevant in this context is for example that on the one hand the DARPP-32/PP1 cascade regulates sensitivity of glutamate receptors (Greengard, 2001) and on the other hand glutamate counteracts the DRD1 signal by dephosphorylating DARPP-32 (Nishi et al., 2017). Additionally, PP1 is involved in gene expression regulation by histone dephosphorylation. Thus, DARPP-32 activation in response to phosphorylation by PKA inhibits PP1 and reduces its dephosphorylation activity. This enhances gene expression and contributes to synaptic plasticity (Klein et al., 2019). A study in mice, where *DARPP-32* was inactivated, showed that responses to DA agonists and antagonists were reduced or even abolished. This indicates that DARPP-32 plays a central role in dopaminergic signal transmission (Fienberg et al., 1998).

The complex downstream effects of DA receptor activation not only depend on induced second messenger responses or the activation of other receptors and ion channels as described above, but also on the receptor subtypes expressed on the target cell and their exact localization, as DRD distribution and abundance varies across different brain regions. D1-like receptors are mostly located in the striatum, SNr, amygdala and in the frontal cortex, D2-like receptors are also expressed in the striatum and amygdala and additionally in the globus pallidus, VTA, hypothalamus, hippocampus, pituitary and in cortical areas. The opposingly functioning receptors DRD1 and DRD2 are the most frequently expressed receptors and are commonly co-expressed. Furthermore, DRD2 and DRD3 can also be expressed not only postsynaptically but also on the presynaptic neuron and function as DA auto-receptors inhibiting DA release (Beaulieu & Gainetdinov, 2011). DRD4 is the least relevant receptor in the context of executive functioning, as it can mainly be found in the retina (Klein et al., 2019). Besides the differences in localization, DRDs also show different affinity for DA. D2-like receptors have an up to 100-fold greater binding affinity for DA than D1-like receptors, potentially due to different roles of the receptor subtypes in DA signaling. Hence, D1-like receptors could be preferentially activated by phasic DA release, when higher concentrations of DA reach the synaptic cleft, whereas D2-like receptors could be involved in tonic signaling, as they can detect lower DA levels (Beaulieu & Gainetdinov, 2011; Klein et al., 2019).

The dopaminergic signal transmission is generally terminated by DA reuptake via the dopamine transporter (DAT). After reuptake into the presynaptic neuron, DA can be recycled and restored in vesicles. Accumulating DA in the cytosol due to leakage from the vesicles can be degraded by the monoamine oxidase (MAO) or by the catechol-O-methyltransferase (COMT). Additionally, DA can be transported into glia cells, where it is degraded also by MAO or COMT (Meiser, Weindl, & Hiller, 2013).

Dopamine degradation via COMT is especially relevant for determining extracellular DA levels in the prefrontal cortex (PFC) as DAT is less abundant in prefrontal than in striatal regions (Lewis et al., 2001; Sesack, Hawrylak, Matus, Guido, & Levey, 1998; Slifstein et al., 2008) and therefore DA reuptake is less relevant for signal termination in the PFC than DA degradation by COMT. To date it is unclear whether COMT in the PFC is either membrane-bound and thus active extracellularly in the synaptic cleft (Chen et al., 2011) or if DA is transported into glia cells and neurons via a yet unknown DAT-independent reuptake mechanism (Schott et al., 2010).

2.5.2 Genetic variability affecting dopaminergic transmission

Functional Single Nucleotide Polymorphisms (SNPs) can for instance influence gene expression or have functionally relevant effects on physiological mechanisms, e.g. receptor binding affinity and protein stability. SNPs supposedly contribute to differences in baseline DA transmission and subsequently influence cognition and behavior. The following paragraph characterizes relevant SNPs within the dopaminergic system and their functional effects.

rs4680 in *COMT* (Val158Met polymorphism)

One well-studied SNP is rs4680 (Val158Met polymorphism) in *COMT*. The wildtype allele guanine (G) coding for the amino acid valine (Val) can be substituted by adenine (A), causing the incorporation of the amino acid methionine (Met) instead. The so-called Met allele gives rise to a thermolabile protein (Lotta et al., 1995), which causes a decrease of approximately 40% in COMT activity in the PFC (Chen et al., 2004). The observed effect increases with the number of Met alleles ("gene dose effect") as homozygous Val/Val carriers have highest COMT activity, heterozygous Val/Met carriers show reduced activity on an intermediate level and lowest COMT activity is observed in homozygous Met/Met carriers (Berryhill, Wiener, Stephens, Lohoff, & Coslett, 2013; Smolka et al., 2005). This decrease in COMT activity in carriers (homozygous and heterozygous) of the Met allele translates into higher levels of extracellular DA in the PFC and is related to cognitive abilities.

The effect of the Met allele on cognitive functions is illustrated by the commonly accepted Worrier/Warrior hypothesis (Stein, Newman, Savitz, & Ramesar, 2006). It implies that individuals carrying the Met allele are called "worriers" because they are advantaged in exploring and processing information and in tasks of executive functioning like memory (Aguilera et al., 2008; Bellander et al., 2015; Berryhill et al., 2013; Bruder et al., 2005), cognitive flexibility (Mitaki et al., 2013) and cognitive control (Cools & D'Esposito, 2011). Contrarily, Val/Val carriers, the "warriors", seem to be more efficient in processing of adverse stimuli, have higher resilience against stress and anxiety and lower perception of pain (Xu, Ernst, & Goldman, 2006), however at the expense of reduced executive functioning (Egan et al., 2001). Congruent effects of *COMT* genotype can be found on a functional level. Smolka et al. showed that Val carriers exhibit lower activation of limbic brain regions in response to unpleasant stimuli, indicating lower emotional engagement in averse emotional processing in Val carriers (Smolka et al., 2005). On the other hand, Val carriers show higher brain activity in PFC and ACC (Egan et al., 2001) and greater functional connectivity within the executive network (Tunbridge, Farrell, Harrison, & Mackay, 2013) while performing tasks of executive functioning. A similar association between the Val allele and prefrontal activation during executive processing was found meta-analytically by Mier et al. and they also showed a negative association between the presence of the Val allele and prefrontal activation during processing of emotional information, indicating an advantage for Val allele carriers (Mier, Kirsch, & Meyer-Lindenberg, 2010).

DRD2

rs1800497 ("Taq1A")

The polymorphism rs1800497, also described as the Taq1A polymorphism, is located over 10,000 bp downstream of the *DRD2* gene in exon 8 of the Ankyrin repeat and kinase domain containing 1 (*ANKK1*) gene. The base thymine (T) at this locus codes for the minor A1 allele, whereas cytosine (C) corresponds to the major A2 allele. Presence of the A1 allele is associated with lower striatal D2 receptor binding potential which could be due to lower receptor density (Gluskin & Mickey, 2016; Jönsson et al., 1999a). Most likely, the Taq1A SNP is in linkage disequilibrium with another SNP directly affecting *DRD2* gene expression (Zhang et al., 2007) and is therefore indirectly associated with DA transmission.

This difference in receptor availability could be the reason for observed effects of the A1 allele on executive functioning. A1 carriers for instance show impairments in working memory performance (Berryhill et al., 2013; Stelzel, Basten, Montag, Reuter, & Fiebach, 2009), which is in line with findings of Salami et al. who found that D2 receptor density in and outside of the striatum correlates with neural response in fronto-striatal circuits during a working memory task and affects working memory performance (Salami et al., 2019). Furthermore, the SNP might have an effect on cognitive flexibility. However, the direction of effect is unclear as one study reported less flexibility (Persson & Stenfors, 2018) but other studies indicated increased flexibility (Markett, Montag, Walter, Plieger, & Reuter, 2011; Stelzel, Basten, Montag, Reuter, & Fiebach, 2010). Additionally, being an A1 allele carrier is associated with greater impulsivity (White, Morris, Lawford, & Young, 2008) and impaired negative outcome learning (Klein et al., 2007; White et al., 2008).

rs6277 ("C957T")

Another SNP potentially affecting DRD2 density in the striatum is the C957T polymorphism within the *DRD2* gene. Even though the base exchange from cytosine to tyrosine is synonymous meaning that the base triplet is still coding for the same amino acid, *in vitro* experiments showed that the presence of the T allele decreased *DRD2* translation induced by changes in folding of the transcribed *DRD2* mRNA and accelerated mRNA decay and attenuated *DRD2* upregulation in response to a DA signal (Duan et al., 2003).

This might be translatable to the human brain, as Hirvonen et al. (Hirvonen, Lumme et al., 2009) showed in a PET study with healthy subjects that the number of C alleles in the C957T polymorphism predicted higher extrastriatal DRD2 availability (C/C > C/T > T/T). Contrarily, the opposite association was observed for striatal regions, where the number of T alleles was associated with increasing DRD2 availability (Hirvonen, Laakso et al., 2009; Smith et al., 2017). Hirvonen et al. however pointed out that this higher striatal availability might be driven not by T-allele-associated higher receptor expression in the striatum, but by SNP-related alterations in DRD2 affinity and striatal DA levels that might provide a possible hint for an explanation for these conflicting results (Hirvonen, Laakso et al., 2009).

The SNP also influences executive functioning. Homozygous C-carriers showed reduced spatial memory performance, spatial planning abilities and decreased sustained attention (Klaus et al., 2017). Further, a disadvantageous effect of the C allele was shown for working memory (Xu et al., 2007) and set-shifting (Rodriguez-Jimenez et al., 2006), whereas Li et al. reported both positive and negative effects of C957T on working memory performance, depending on the task used (Li, Bäckman, & Persson, 2019).

DAT rs28363170

A variable number tandem repeat (VNTR) in the 3' untranslated promotor region of *DAT* was first described by Vandenbergh et al. in 1992 (Vandenbergh et al., 1992). The repeat consists of 40 bp that are repeated between 3 and 11 times, most frequent are the alleles with 9 or 10 copies. Inconsistent

results were found for the effect of the VNTR on DAT availability. The 10 repeat allele was associated with higher DAT availability *in vitro* (VanNess, Owens, & Kilts, 2005) and *in vivo* (Heinz et al., 2000), whereas several studies found an opposite association with 9-repeat carriers having higher striatal DAT binding/availability than 10-repeat carriers (Jacobsen et al., 2000; van de Giessen et al., 2009; van Dyck et al., 2005).

Independent of the actual direction of effects on molecular level, the VNTR in *DAT* might affect cognitive functioning by influencing DAT availability and thus DA transmission. In line, homozygous carriers of the 9-repeat allele had better cognitive flexibility (Fagundo et al., 2014), whereas homozygous 10-repeat carriers were more focused during working memory performance indicated by lower activation in the cortical working memory network (Bertolino et al., 2006) but also improved less during working memory training (Brehmer et al., 2009). However, similar to the inconsistent results about the effect of the VNTR on DAT availability, a meta-analysis including several cognitive functions, e.g. working memory, inhibition and executive functions, was not able to show a significant effect of the VNTR on cognitive functioning (Ettinger, Merten, & Kambeitz, 2016).

rs907094 in *DARPP-32*

The SNP rs907094 within the *DARPP-32* gene was shown to be part of a haplotype that affects *DARPP-32* gene expression and striatal volume and connectivity (Meyer-Lindenberg et al., 2007). The T allele might increase *DARPP-32* mRNA expression in the striatum (Frank, Doll, Oas-Terpstra, & Moreno, 2009; Meyer-Lindenberg et al., 2007) and consequently facilitate striatal D1-related dopamine transmission (Gershman & Tzovaras, 2018) which regulates fronto-striatal circuit activity.

Homozygous T carriers are less exploratory, both in random (random exploration pattern in uncertain circumstances) and direct (selective exploration of uncertain options to gain information about them) exploration (Gershman & Tzovaras, 2018) and learn better from probabilistic positive reinforcements (Frank et al., 2009) and positive prediction errors (Frank, Moustafa, Haughey, Curran, & Hutchison, 2007). Doll et al. showed that the T allele is associated with more model-free learning, which means that choice decisions are based on previous actions and their outcomes and less on predicted outcomes derived from model rules (Doll, Bath, Daw, & Frank, 2016). Additionally, the SNP might play a role in the context of memory (Schuck et al., 2013) and for attention regulation, as homozygous T carriers were able to more flexibly adjust their auditory attention according to task requirements (Li et al., 2013).

Fat mass and obesity-associated protein (FTO)

rs9939609

The SNP rs9939609 in intron 1 of the *FTO* gene was first described in 2007 by Frayling and colleagues (Frayling et al., 2007). They indicated that the A allele in this common variant is a risk allele predisposing for higher BMI and diabetes mellitus type 2 (Frayling et al., 2007). In line, the A-allele compared to T/T was associated with food consumption and dietary preferences. A-allele carriers consumed more snacks and high-caloric food like meat and cheese and also juice consumption was higher for A-carriers within an overweight group. Additionally, A-allele carriers tend to underreport their energy intake more than homozygous T-carriers (Brunkwall et al., 2013), had a higher energy intake during a dietary lifestyle intervention (Haupt et al., 2009) and reported higher food cravings (Dang et al., 2018).
The SNP is especially relevant in the context of DA signaling, as literature indicates an interaction between the DA-related Taq1A polymorphism and this *FTO* SNP (reviewed here (Sun, Luquet, & Small, 2017). For instance, it was suggested that the risk allele A affects striatal dopaminergic signaling by increasing autoinhibition of DA-neurons in the midbrain, which in turn decreases signal transmission from the VTA and SNr to the striatum (Hess et al., 2013). The exact mechanism how *FTO* could affect the dopaminergic system is not yet clear but an association between variants in *FTO* SNP and

availability of DRD2 seems not to be present (Dang et al., 2018). Still, individuals carrying the *FTO* risk allele in combination with the Taq1A risk allele performed worse in negative but not positive outcome learning and showed reduced responsive activation in the VTA and SN during the applied learning task (Sevgi et al., 2015). This interaction is also applicable for obesity. The FTO risk allele was only able to affect body fat distribution and insulin sensitivity when the Taq1A risk allele was present as well (Heni et al., 2016). The SNP rs9939609 is thus included in the analyses of this study to explore potential relationships with diet, *DRD2* mRNA expression and executive functions.

rs8053740

The SNP rs8053740 lies in intron 3 of *FTO* and was among others shown to be associated with obesity in a Sorbian population – a self-contained population with Slavic origin living in Eastern Germany. This population is slightly less genetically heterogenous than the average European population and thus genetic signals could be more pronounced in this population, but it is still comparable to the European average. In this population, the minor C allele of the SNP was correlated with BMI and fat mass (Tönjes et al., 2010). Liu et al. were able to show *in silico* (by computational simulation) that this minor allele could be functionally relevant as it is related to a novel transcription binding site for SRY-Box Transcription Factor 17 (*SOX17*), which plays an important role in embryonic and cell development (Liu et al., 2015). This prediction and the association with BMI indicate the relevance of this SNP in obesity and thus interactions with the DA system and DA-dependent cognition should be target of exploration.

In general, due to their potential effects on both cognitive performance and mRNA expression, the SNPs described above need to be considered and controlled for as potential experimental cofactors in this study.

2.6 High fat/sugar diet: the driver of obesity-related changes in the dopaminergic system?

2.6.1 Gene expression differences in association with obesity

Mechanisms of gene expression regulation

Gene expression describes the cellular process of transforming genetic information contained in the deoxyribonucleic acid (DNA) into a functional protein, e.g. cell structure protein, enzyme, receptor, or messenger such as a hormone or transmitter. Fig. 3 depicts the process of gene expression schematically. The genetic information is encrypted within the specific base sequence of the DNA (genotype), which is either constantly or upon an induction signal transcribed into ribonucleic acid (RNA), specifically pre-messenger RNA, in the cell nucleus by enzymes called RNA polymerases. The pre-messenger RNA copy can then undergo various modifications before it becomes the finished messenger RNA (mRNA). These modifications include polyadenylation (attaching a multiple adenine sequence to the 3' end of the RNA to protect it from degradation), splicing (removal of non-coding intron sequences) or alternative splicing (variable removal of introns and exons). With alternative splicing it is possible to obtain several different mRNAs from identical pre-messenger RNAs which increases the heterogeneity of produced proteins. The mRNA is then transported into the cytosol and to the endoplasmatic reticulum for translation into proteins by ribosomes. During the process of translation nucleotide triplets ("codons") in the protein-coding region of the mRNA are matched to corresponding triplets ("anticodons") of transfer RNAs (tRNA) which carry amino acids. The amino acid belonging to one particular codon is always identical (genetic code). The amino acid sequence is then connected by the ribosome to form an amino acid polypeptide. For correct functionality of the protein it needs to be folded into a characteristic three-dimensional structure. This structure is encoded in the amino acid sequence and folding happens automatically, assisted be chaperones. Sometimes the

protein is modified post-translationally, e.g. by changing or cleaving chemical bonds or covalently adding functional groups, to achieve or adapt its final structure and function. The finished protein can then be translocated to its predetermined site of action.

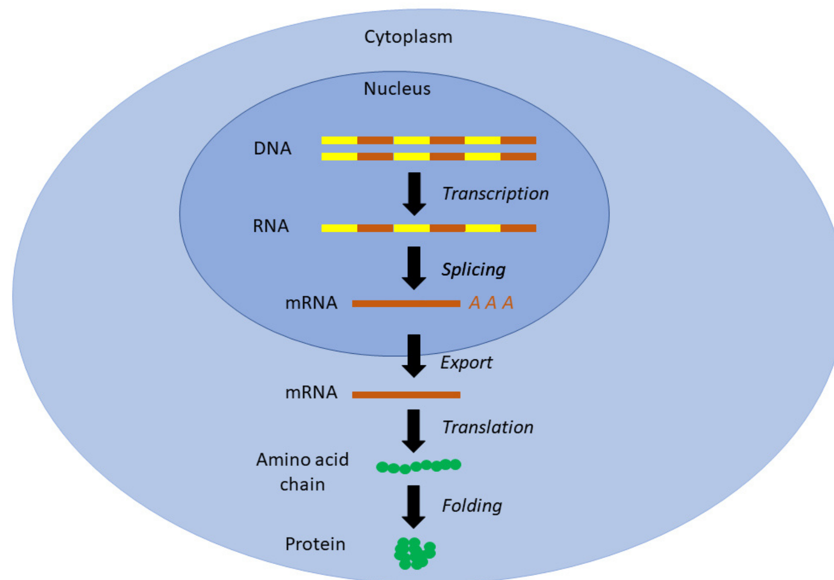


Figure 3: Gene expression. The DNA, located in the cell nucleus, is a double-helical structure (not shown) consisting of protein-coding (brown) and non-coding (yellow) areas. The mechanism of transforming genetic information to functional gene products (proteins) is called gene expression and consists of several steps. Transcription: a raw RNA copy complementary to a DNA single strand is synthesized by RNA polymerases in the cell nucleus. Splicing: the primary RNA copy undergoes various modification steps, including splicing (removal of non-coding intron sequences) and alternative splicing (variable removal of introns and exons). Export: the modified mRNA leaves the nucleus and enters the cytoplasm. Translation: nucleotide triplets (codons) in the protein-coding region are matched to their corresponding amino acid. The amino acid sequence is then connected by the ribosome to form an amino acid polypeptide (Amino acid chain). Folding: folding of the peptide chain into its characteristic three-dimensional structure completes the process of protein production**.**

Gene expression is highly regulated to adapt the amount of gene product and the timing of its appearance to current needs, e.g. due to a changing environment, cell damage or other external stimuli, and to enable cell differentiation and functionality. Some genes are constantly active (e.g. genes necessary for basic cell functions and structure), and some are facultatively active or inducible when needed.

Regulation of gene expression can happen on all mechanistic levels. On transcriptional level, direct binding of transcription factors to the DNA strand regulates the accessibility of the DNA for RNA polymerases, which can cause gene expression enhancing (more expression) or silencing (less or no expression). The activity of transcription factors can also be regulated by modifications such as phosphorylation of the factor, which often happens within downstream signaling pathways of receptors on the cell surface. Other important regulatory mechanisms are covalent modifications in and around the DNA molecule. Important examples, among many, are methylations of the DNA base cytosine or acetylation/phosphorylation of DNA-packaging histone molecules. DNA is often methylated at CpG cites (where a cytosine is followed by a guanine nucleotide) and methylated DNA sections tend to be downregulated and less transcribed (with some exceptions), while histone acetylation reduces the electrostatic connection between the DNA molecule and the histones, loosens chromatin structure and therefore upregulates gene expression by facilitating transcription. These processes are generally referred to as "epigenetics", as phenotypical differences are not caused by alterations of the DNA base

sequence itself but happen “in addition to” genetically coded features (Felsenfeld, 2014). Gene expression regulation on a translational level can be achieved by controlling the active transport of the mRNA molecule from the nucleus to the cytoplasm, protect the mRNA molecule from degradation (e.g. by the poly-A sequence) or induce degradation, e.g. via other types of RNA like micro RNA or small interfering RNA (Carthew & Sontheimer, 2009).

Obesity-associated differences in dopaminergic gene expression

Obesity generally affects epigenetic mechanisms and was shown to be associated with epigenetic changes, such as methylation differences in several metabolically relevant candidate genes like leptin, POMC and hormonal transporters and receptors. These epigenetic changes seem to be at least partly reversible by lifestyle change, weight loss or bariatric surgery (van Dijk et al., 2015).

Also, within the DA system specific changes in gene expression were indicated in association with obesity. A post-mortem study with human brain samples of obese vs. normal-weight individuals found lower striatal DAT density (radioligand binding assay), lower *DAT* mRNA expression in the midbrain and lower mRNA expression of *TH* in the SN. Additionally, mRNA levels of *DAT* and *TH* were negatively correlated with BMI (Wu, Garamszegi, Xie, & Mash, 2017). Mansur et al. observed lower mRNA levels of *VMAT2, DRD2, DRD5* and *DAT* in post-mortem dlPFC samples of individuals with major psychiatric disorders (bipolar disorder, major depression and schizophrenia), which were moderated by a higher BMI (Mansur et al., 2018). On a peripheral level obesity-related changes in the DA system were found in blood cells. Leite et al. showed reduced expression of *DRD2, DRD4, DRD5* and *TH* in white blood cells of centrally obese individuals and they found that expression levels of *DRD2* and *DRD5* correlated negatively with weight, BMI and waist circumference as well as with lower plasma levels of leptin and inflammatory markers (Leite, Lima, Marino, Cosentino, & Ribeiro, 2016). On the basis of epigenetics, Ramos-Lopez et al. found that BMI correlated with methylation patterns of CpG sites that regulate genes within the DA pathway, e.g. *VMAT1, DDC, DRD5, DAT* and several downstream molecules. Additionally, CpG methylation patterns in *VMAT1* and *DAT* correlated with total energy intake and intake of carbohydrates (Ramos-Lopez et al., 2018a). However, the underlying mechanisms of how obesity affects epigenetics or gene expression in the DA system are not yet fully understood and need further exploration.

2.6.2 Diet-associated alterations in the dopaminergic system

Accumulating evidence from rodent literature that allows the investigation of causal relationships between obesity development and the DA system indicates that in fact a diet high in saturated fat and sugar leads to those changes observed in obesity. Several studies found diet-induced alterations regarding the expression of genes in the DA system of the hypothalamus and in regions associated with the reward circuitry (VTA, SN, NAc, PFC) that are explained in detail hereafter.

Ad libitum access to high fat and sugar (HFS) diet reduced mRNA expression of *DRD1* in the NAc specifically in obesity-prone compared to obesity-resistant rats, an effect that was present even after withdrawal from HFS diet (Alsiö et al., 2010). Similarly, unrestricted access to high fat diet (HFD) reduced expression of *TH* in the midbrain, but this effect was reversible by switching to a normal diet (Li et al., 2009). Strikingly, a downregulation of *DRD1, DRD2* and *TH* was also present, when animals had only calorie-restricted access to HFD (Alsiö et al., 2010; Li et al., 2009). In line, administration of HFD reduced DRD2 protein levels in the NAc of rats, which correlated with impulsive and maladaptive behavior in response to food reward (Adams et al., 2015). Reduced striatal DA clearance most likely due to lower DAT activity (Baladi, Horton, Owens, Daws, & France, 2015; Cone, Chartoff, Potter, Ebner, & Roitman, 2013) and reduced membrane-associated DAT expression in the striatum (Cone et al.,

2013) were also found after high fat feeding. Additionally, striatal responsiveness upon stimulation (Barry et al., 2018; Carlin, Hill-Smith, Lucki, & Reyes, 2013) as well as mesolimbic DA turnover and reward-responsiveness were attenuated after HFD intake (Davis et al., 2008) and decreased striatal DRD2 availability was observed (Barry et al., 2018). Furthermore, broad effects on gene expression regulation were reported in response to HFD feeding with altered mRNA levels of *COMT, DAT, TH, DARPP-32, DRD1* and *DRD2* in different brain regions, i.e. VTA, PFC and NAc (Carlin et al., 2013). Similarly, alterations in mRNA expression of *TH, DAT, DRD1, DRD2* and *DARPP-32* in the hypothalamus and reward circuitry were shown in a mouse model of diet-induced obesity after chronic high fat feeding (Vucetic, Carlin, Totoki, & Reyes, 2012). Isocaloric feeding with saturated fat (palm oil) but not monounsaturated fat (olive oil) reduced reward sensitivity and altered DA signaling in the striatum, indicating a selectivity of effects for unhealthy saturated fatty acids (Hryhorczuk et al., 2016), while continuous *ad libitum* access to a low dose of sugar in drinking water (1%) mimicking the constant consumption of hidden sugars in beverages and refined food in western-style diets also changed *DRD1* and *DRD2* expression in the striatum (Hakim & Keay, 2019).

Dietary effects on DA-dependent executive functioning, potentially demonstrating the functional relevance of described alterations on gene expression level, were found both in humans and in rodents. A study by Limbers et al. found that higher consumption of saturated fat was associated with lower levels of inhibition assessed with a test battery of executive functioning in a sample of healthy young adults (Limbers & Young, 2015). A review about cognitive impairments associated with intake of HFS “Western” diet found hints in humans and more direct causal links in animal models that consumption of simple carbohydrates and saturated fatty acids is associated with impairments especially in hippocampus-based memory and learning, i.e. learning of response inhibition to not reinforced actions. The authors indicate that these impairments appear before the onset of obesity and might elicit obesity-promoting behavior (Kanoski & Davidson, 2011).

In conclusion, animal literature accumulates that shows diet-induced changes in dopaminergic transmission in relation to cognitive alterations and it is suggested that these alterations might also be present in humans, but evidence is still not extensive enough to draw causal conclusions or even proof a direct connection. This study aims to contribute to filling this gap in literature.

2.7 Potential mechanistic links between diet and the dopaminergic system

There are several potential pathways by which diet could have an impact on the DA system. The following paragraph provides an introductory overview that is not meant to be comprehensive.

2.7.1 The gut-brain axis: direct signaling of metabolic state via hormones, nutrients and vagal afferences

Food intake is predominantly regulated by the hypothalamus where several central orexigenic (appetite-stimulating) and anorexigenic (appetite-suppressing) mediators adapt feelings of hunger to current homeostatic needs. Metabolic hormones like insulin and leptin are essential for forwarding these homeostatic signals from the periphery to the brain. Insulin is released from pancreatic ß-cells after food intake in response to elevated blood glucose levels, provokes glucose internalization and metabolism in cells and is a signal for central suppression of appetite (Woods, Lotter, McKay, & Porte, 1979). Another suppressor of appetite is leptin, which is mainly synthesized by white adipocytes. Its release correlates with adipocyte size and fat mass and conveys information about energy content stored in adipocytes to the hypothalamus (Brennan & Mantzoros, 2006). Levels of both hormones are

often increased in obese individuals (Dunn et al., 2012), while insulin and leptin sensitivity is attenuated both peripherally and centrally (Ramos-Lobo & Donato, 2017).

For the role of insulin and leptin in central processes it is crucial that they can cross the blood-brain-barrier and bind to specific receptors to take their effect. At a specific hypothalamic site called the median eminence the blood-brain barriers is incomplete and allows circulating metabolic messengers to directly access the central nervous system (CNS) where they act on hypothalamic nuclei to signal energy intake (Suzuki, Simpson, Minnion, Shillito, & Bloom, 2010). However, hedonic regulation of food intake via DA is also affected by metabolic hormones. Insulin and leptin can, in addition to gaps in the blood-brain barrier, cross it via active transport. As these transporting mechanisms are saturable, short-termed insulin and leptin signals induced by food intake may not be equally well translated to the CNS in the chronic state of elevated insulin and leptin levels associated with obesity (Figlewicz & Benoit, 2009). Expression of insulin and leptin receptors in the DA system was shown for the VTA and SNr enabling both hormones to directly affect DA signaling (Figlewicz, Evans, Murphy, Hoen, & Baskin, 2003). For instance, insulin binding in the VTA causes reduced DA release in the NAc, which may underlie the physiological process of insulin-mediated inhibition of food-seeking behavior (Naef, Seabrook, Hsiao, Li, & Borgland, 2019). Leptin administration into the VTA had a similar effect, i.e. decrease of food intake (Morton, Blevins, Kim, Matsen, & Figlewicz, 2009). In an animal study, the expression of the DA synthesis enzyme TH was shown to be decreased after high-fat feeding in the VTA, SN and also in the ventromedial hypothalamus and the decrease correlated with an increase in leptin levels (Li et al., 2009). In addition to this direct impact on the VTA, leptin was shown to influence reward-guided behavior via hypothalamic projections to the VTA (Liu, Bello, & Pang, 2017). Striatal DA signaling can also be affected by metabolic hormone action. For example, insulin sensitivity was negatively associated with DRD2 availability in ventral striatum, while leptin levels were positively associated with DRD2 in the dorsal striatum (Dunn et al., 2012). Kleinridders & Pothos recently reviewed evidence for the expression of insulin receptors in striatal dopaminergic regions and summarized that insulin can affect DA signaling in at least three ways on a molecular level. By inducing *DAT* expression it increases DA reuptake, by affecting protein expression of the degrading enzyme MAO it alters the duration of DA action on the postsynapse and finally insulin might affect signaling frequency of striatal dopaminergic neurons (Kleinridders & Pothos, 2019), as e.g. Cai et al. showed in mice that the loss of astrocytic insulin signaling decreases DA release from the observed brain slices (Cai et al., 2018). These mechanisms might explain how insulin physiologically decreases the rewarding property of food when the body is not in metabolic need of food intake. This effect might thus be diminished in insulin resistant individuals potentially leading to hedonic overeating in obesity. Concerning leptin, it was shown that it affects the mesolimbic DA system by acting on leptin receptors of neurons in the lateral hypothalamus, which plays an important role in the control of eating behavior and energy balance (Leinninger et al., 2011).

Apart from indirect effects of nutrients on the CNS mediated by metabolic hormones, additional direct “nutrient sensing” properties are assumed, meaning direct effects of food components on neuronal processes. Nutrient sensing is suggested for most sugars, fats and carbohydrates which induce striatal DA release and thus lead to operant conditioning for these ingredients (McCutcheon, 2015). To confirm direct sensing effects for glucose independent of a hormonal response, it was demonstrated in animal models that glucose sensing happens already before intestinal absorption (McCutcheon, 2015). Similar indications exist for the lipid metabolism, as centrally available triglycerides reduce striatal D2-dependent signaling via a mechanism that involve lipoprotein lipase activation (Berland et al., 2020) and cholesterol affects vulnerability of dopaminergic neurons in the SN leading to neuronal loss, which was shown to be in causal relationship with

Parkinson`s disease (Paul et al., 2017). Nevertheless, evidence for direct nutrient sensing is still inconclusive.

Another pathway connecting food intake and brain responses is the vagal nerve, the main parasympathetic connection between the intestinal tract and the CNS. Vagal afferences from the gut convey information such as gastric distension as well as levels of nutrients and gut hormones to the hypothalamus and to the brain stem (Suzuki et al., 2010). Moreover, Han et al. were able to establish the anatomical link between the vagal sensory ganglion and the substantia nigra enabling signals from the gut to directly interact with the reward system (Han et al., 2018).

2.7.2 Chronic inflammation

Obesity has been causally linked to chronic low-grade inflammation which contributes to its negative consequences such as the development of type 2 diabetes. Adipocytes were shown to be actively involved in initiation of this chronic inflammatory state, as they have properties of immune cells, i.e. activation of the complement system and production of inflammatory cytokines like tumor necrosis factor α (TNF-α) or interleukin-6 (IL-6) (Hotamisligil, 2006). Leptin as a fat-related metabolic factor is also considered to act as a proinflammatory cytokine by inducing release of TNF-α, IL-6 and IL-12 from macrophages (Lord, 2006). In turn, TNF-α and IL-6 are, besides their proinflammatory effects, interconnected with metabolic regulation. For instance, TNF-α affects signaling of the insulin receptor and might therefore causally contribute to the development of insulin resistance, while IL-6 is part of the regulation of glucose and lipid metabolism (Coelho, Oliveira, & Fernandes, 2013).

An inflammatory state in the body generally causes fatigue, psychomotor slowing and cognitive impairment that is at least partially attributed to disruptions of basal ganglia DA signaling (Felger & Miller, 2012). In line, motivation and reward perception were shown to be strongly affected by proinflammatory cytokines (Shields, Moons, & Slavich, 2017), as loss of motivation (Felger et al., 2013) and reduced reward sensitivity, indicated by decreased signaling in the NAc during reinforcement learning (Treadway et al., 2017), are among the first behavioral changes observed in association with inflammation. Regarding executive cognition, blood levels of the proinflammatory highly-sensitivity C-reactive protein (hsCRP) were associated with reduced attentional set shifting (Lasselin et al., 2016). However, a review by Shields et al. aimed to summarize influences of chronic inflammation on self-regulation, including executive functioning, and found that evidence is yet unclear if and how inflammation might affect distinct executive functions in humans but they indicated that self-control is in general affected by inflammation (Shields et al., 2017).

To assume a direct effect of peripheral inflammation on neuronal processes it is necessary that inflammatory signals can enter the CNS. Several mechanisms of passage, that are partly similar to those for nutrients and metabolic hormones were summarized by Felger and Miller. They propose that inflammatory cytokines can enter through leaks in the blood-brain-barrier or via active carrier-mediated transport. Additionally, cytokines can activate endothelial cells and macrophages located in brain vessels and central immune cells like microglia and astrocytes that transfer the proinflammatory signal by further producing cytokines upon their activation. And finally, cytokines can locally activate vagal nerve afferents to transfer the inflammatory signal to various brain regions like the hypothalamus (Felger & Miller, 2012). On the other hand, a recent review indicated that especially saturated fatty acids might directly cause neuroinflammation e.g. by activating microglia cells (Guillemot-Legris & Muccioli, 2017). This suggests a potential impact of diet on the CNS not only indirectly via peripheral inflammatory cytokines but also directly without peripheral inflammation.

Decreased levels of the DA precursor tyrosine (Capuron et al., 2011) as well as decreased turnover of phenylalanine to tyrosine and tyrosine to DA can be observed in response to

proinflammatory cytokines (Neurauter et al., 2008), leading to decreased DA synthesis. Neurauter et al. attributed decreased DA turnover to an inhibition of the main enzyme TH caused by lower availability of its essential cofactor tetrahydrobiopterin (BH4) in association with inflammation (Felger & Treadway, 2017). DA stability, release capacity and reuptake may also be affected by inflammation. The inflammatory cytokines IL-1β and TNF-α were shown to decrease expression of VMAT2, which could make DA more vulnerable to cytosolic degradation (Kazumori et al., 2004). However, this was only studied in cell culture of gastric cells and is therefore to date a theoretical consideration. Furthermore, higher expression of DAT in inflammation leading to decreased dopaminergic signaling has been proposed to be involved (Felger & Treadway, 2017), though evidence is also tenuous here. In addition to potential inflammation-associated reduction in DA synthesis and extracellular activity, lower striatal DRD expression may further contribute to decreased DA signaling. Lower striatal DRD2 binding in PET was observed both in non-human primates after cytokine application (Felger et al., 2013) and in obese patients (Felger & Treadway, 2017). Finally, chronic activation of microglia by proinflammatory stimuli can cause neurotoxicity and lead to DA cell loss (Block & Hong, 2007), an effect which could even be exacerbated by HFD intake (Bousquet et al., 2012). This is a key feature in Parkinson's disease, but might also be relevant in the context of obesity.

Taken together, it is possible that chronic (neuro)inflammation related to diet can impact several aspects of DA signaling, e.g. DA synthesis, storage, and transmission and might thus contribute to cognitive patterns observed in obesity.

2.7.3 Epigenetic and enzymatic modifications directly through dietary ingredients

Several factors that are associated with obesity, e.g. inflammation, hypoxia and oxidative stress, might directly or indirectly affect epigenetic processes like DNA methylation (Campión, Milagro, & Martínez, 2009). Moreover, also dietary properties like protein content, vegetarian diet and intake of folic acid, vitamins and fatty acids were associated with changes in methylation patterns (Campión et al., 2009). Cuyàs et al recently provided theoretical computational evidence that oleacein from extra virgin olive oil inhibits the activity of the DA-degradation enzyme COMT (Cuyàs et al., 2019). These results are mainly based on animal literature, cell culture experiments and computation and are therefore very limited in their informative value about general or even dopamine related processes. However, this indicates that there are putative ways of how diet might directly affect epigenetics and enzyme functioning also in the context of DA transmission.

In summary, there are several hints that metabolic hormones and nutrients, low-grade chronic inflammation and direct epigenetic and enzymatic modifications might be mediators between dietary style and observed effects on the DA system, but conclusive evidence for these connections in humans is still very limited.

2.8 Further external influences and interactions

2.8.1 Physical activity: bidirectional interaction with the dopaminergic system

Physical activity (PA) needs to be considered as a possible confounder for cognitive performance. Several intervention studies found improvements in cognitive performance after physical training. For instance, a physical intervention with intense aerobic exercise in a sample of healthy young but otherwise sedentary man found improved hippocampal learning performance in a face-matching task after 5 weeks of training (Griffin et al., 2011). Another study reported that already an intense short-term exercise (2x 3 minutes sprint) improved learning (Winter et al., 2007) and better performance in

certain subcategories of the Stroop color word test assessing executive functions were found after intense exercise on a cycle ergometer (Ferris, Williams, & Shen, 2007). Performance in executive-related tests such as the Digit span test, Trail making test, and Stroop test as well as in a Logical Memory task increased with each time point of a three-months exercise program for elderly women (Vedovelli et al., 2017). Many studies suggested that peripheral biomarkers could either directly or indirectly be mechanistically involved in mediating effects of PA on cognitive performance. Frequently mentioned biomarkers are the brain-derived neurotrophic factor (BDNF), the insulin-like growth factor (IGF-1) and the catecholamines epinephrine, norepinephrine and dopamine, that were consistently increased in serum after physical activity interventions (Ferris et al., 2007; Griffin et al., 2011; Hung, Tseng, Chao, Hung, & Wang, 2018; Skriver et al., 2014; Vedovelli et al., 2017; Winter et al., 2007). The most appealing biomarker in this context might be BDNF, as it is a vital mediator of neuronal protection, synaptic plasticity, and neurogenesis.

The reverse direction of effects, i.e dopaminergic effects on PA and the motivation to move, also requires attention. Several studies indicated that altered DA signaling, especially in the striatum, contributes to development and maintenance of obesity by affecting PA. In a mouse model of impaired striatal DA transmission due to DRD2 knockout the obesity-prone knockout mice did not show increased appetitive motivation for food but in fact lower motivation to move in a free exercising environment (Beeler, Faust, Turkson, Ye, & Zhuang, 2016). In line, a similar functional effect was proposed by Friend et al., who found reduced striatal D2-receptor binding in obese mice after HFD intake. To establish causality, they removed DRD2 from medium spiny neurons in the striatum of lean mice and observed reduced moving activity but no increased susceptibility for obesity in these mice while restoring DRD2 signaling in obese mice increased activity. They propose that reduced D2R signaling in the striatum might thus contribute to inactivity in obesity but they could not find evidence that this is causal to obesity-development (Friend et al., 2017).

Therefore, PA needs to be balanced between diet groups to exclude a potential bias when analyzing DA-related cognitive functions. Further, exploring effects of SNPs that potentially determine baseline DA signaling might be interesting in this context.

2.8.2 Thyroid dysfunction: a potential confounder

DA has been recognized as an inhibitor of the thyroid hormonal axis and decreases the release of thyroid-stimulating hormone (TSH) (Besses, Burrow, Spaulding, & Donabedian, 1975; Kaptein, Kletzky, Spencer, & Nicoloff, 1980; Scanlon et al., 1979). However, evidence from animal literature indicates that thyroid dysfunction in turn also affects DA signaling. Associations between thyroid dysfunction and decreased dopaminergic transmission were observed in animal models of pharmacologically induced hypothyroidism with reduced activity of TH and lower DA levels in the striatum, increased COMT and MAO activity in striatum and midbrain (Rastogi & Singhal, 1979) and lower striatal DRD2 binding potential (Kalaria & Prince, 1986; Vaccari & Timiras, 1981). On a behavioral level, chronic hypothyroidism increased exploration of a new environment, a behavior that is linked to mesolimbic DRD1 activation, whereas hyperthyroidism increased movement activity but did not affect exploration (Sala-Roca, Martí-Carbonell, Garau, Darbra, & Balada, 2002). Consequently, severe thyroid dysfunctions indicated by strongly increased or decreased serum TSH levels should be excluded when studying the effect of diet on the DA system.

2.9 Validity of leukocytes as a peripheral proxy for the central dopaminergic system

Accessing central DA transmission in humans is obviously challenging, as the acquisition of human brain samples is restricted to extraction after death, which naturally biases physiological processes (Bakulski, Halladay, Hu, Mill, & Fallin, 2016). Imaging methods like functional magnetic resonance imaging (fMRI), positron emission tomography (PET) or single-photon emission computed tomography (SPECT) are a frequently used workaround. Even specific markers for several parts of the DA system are available, e.g. 6-[18F]Fluoro-L-dopa (FDOPA) or radiofluorinated L-m-tyrosines as a markers for DA synthesis, storage and turnover, radio-labelled tropanes for DAT imaging, [11C]raclopride for labelling striatal DRD2/3 and [18F]fallypride for extrastriatal DRD2/DRD3 imaging in PFC or ACC. However, these imaging procedures are technically demanding and still provide only indirect estimations of actual physiological features (Peng, Doudet, Dhawan, & Ma, 2013) and also the exposure to radiation needs to be considered. Thus, studying easily accessible peripheral biomarkers as a potential proxy for central DA transmission represents another workaround for approaching the topic. In peripheral blood leukocytes the main players of DA transmission were found to be present, i.e. TH, DAT, DRD2/3/4/5, and VMAT2, but not DRD1 (Buttarelli, Fanciulli, Pellicano, & Pontieri, 2011; Gladkevich, Kauffman, & Korf, 2004; Kirillova et al., 2008) and it has been hypothesized that their peripheral expression could be indicative of the expression in the CNS. Several studies aimed to verify this hypothesis from the viewpoint of different backgrounds. For a complete overview of relevant studies and their main findings see Table S14 (section 9.1.1)

First evidence comes from studies trying to find diagnostic or prognostic markers for neuropsychiatric diseases that are known to be causally related to disruptions in the DA system, i.e. Parkinson's disease, migraine, schizophrenia/psychosis, addiction, and eating disorders such as anorexia nervosa and bulimia (see Table S14, evidence category 1). Alterations in peripheral gene expression in the DA system found in patients with these diseases and correlations with symptom manifestation provide a strong hint that peripheral observations could thus have a central correlate (Barbanti et al., 1999; Barbanti et al., 2000; Caronti et al., 1999; Cui, Prabhu, Nguyen, Yadav, & Chung, 2015; Czermak, Lehofer, Wagner et al., 2004; Frieling et al., 2010; Goodarzi, Vousooghi, Sedaghati, Mokri, & Zarrindast, 2009; Ilani et al., 2001; Kwak, Koo, Choi, & Sunwoo, 2001; Liu et al., 2013; Nishihara et al., 2011; Pellicano et al., 2007; Urhan-Kucuk, Erdal, Ozen, Kul, & Herken, 2011; Vousooghi, Zarei, Sadat-Shirazi, Eghbali, & Zarrindast, 2015). Additionally, three reviews summarized evidence for and against the use of peripheral markers in neuropsychiatric research and argued in favor of its advantages despite several limitations (Bakulski et al., 2016; Buttarelli et al., 2011; Gladkevich et al., 2004).

To further validate this argumentation, several studies performed post-mortem comparisons between blood cells and brain tissue (see Table S14, evidence category 2) and found that gene expression and epigenetic marks are at least to some extent comparable (Davies et al., 2012; Kirillova et al., 2008; Masliah, Dumaop, Galasko, & Desplats, 2013). Another study correlated peripheral gene expression with monoamine metabolites in cerebrospinal fluid and found a significant correlation for the category of serotoninergic metabolites and at least trends for the DA metabolite HVA and *COMT* (Luykx et al., 2016).

Finally, there are already several studies using this method in the research domains of obesity and DA (see Table S14, evidence category 3) and therefore provide further justification for the use of peripheral markers in the context of challenging human research. For instance, Czermak et al. correlated gene expression in the DA system with a score for the personality trait of persistence (Czermak, Lehofer, Renger et al., 2004), Leite et al. with markers of obesity (Leite et al., 2016) and Ramos-Lopez et al. reported correlations between epigenetic methylation in the DA system and obesity (Ramos-Lopez, Riezu-Boj, Milagro, Martinez, & MENA Project, 2018b). All three studies used blood as surrogate or best accessible tissue for their analyses.

In summary, there is evidence pointing towards the validity of using blood leukocytes as a surrogate tissue for the brain when studying the human DA system. Thus, in this study leukocytes instead of brain tissue were used as target tissue for a first approach despite considerable limitations for generalizability and transferability of the obtained results (discussed in section 6.1).

2.10 Exploring uncoupling proteins and their connection with HFS diet

Uncoupling proteins (UCPs) are proton carriers located on the inner membrane of cell mitochondria. Their function is to dissipate the proton gradient that is built up across the inner mitochondria membrane for oxidative phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). ATP provides the cell's energy reservoir and thus by disrupting the gradient, this energy is instead released as heat (Chechi, Nedergaard, & Richard, 2014), a process called thermogenesis. Though similar in their basic structure and function, there are several different UCPs that are expressed in various tissues and have tissue-specific functions (Gaudry & Jastroch, 2019).

UCP-1, UCP-4 and UCP-5 might be relevant in the dietary context but are not expressed in blood cells

UCPs gained attention as a potential target for obesity treatment due to their ability to modify energy expenditure (Tutunchi et al., 2020). In this context, UCP-1 seems to be the most relevant UCP homologue. UCP-1 can almost exclusively be found in brown adipose tissue (BAT) (Chechi et al., 2014; Chondronikola & Sidossis, 2019) and it is responsible for non-shivering heat production in e.g. rodents and hibernators (Nicholls, Bernson, & Heaton, 1978). Decreased energy expenditure due to lower BAT activity and UCP-1 expression might therefore be mechanistically involved in susceptibility for diet-induced obesity (Labouesse et al., 2018). The physiological role of BAT in humans, however, is still under debate and evidence for the relevance of UCP-1 in human energy expenditure is not yet conclusive. Several studies and reviews indicated that humans have metabolically active BAT with UCP-1 positive adipocytes in neck and upper body regions and these depots could be at least partially inducible through a process called "browning" of white adipocytes by sympathetic stimulation (Chechi et al., 2014; Chondronikola & Sidossis, 2019; Frontini & Cinti, 2010; Nedergaard, Bengtsson, & Cannon, 2007; Porter et al., 2016) or by direct activation through leptin after food intake (Chechi et al., 2014). In cases of diet-induced abundance of energy, BAT recruitment might therefore contribute to maintenance of body weight as there is evidence in humans that higher BAT availability and activity might protect against obesity (Chondronikola & Sidossis, 2019). Nevertheless, increased UCP-1 expression in obese humans reflecting a compensatory upregulation of BAT activity was not sufficient for weight maintenance (Chechi et al., 2014). Thus, UCP-1 as well as UCP-4 and UCP-5 which are expressed almost exclusively in the brain and are discussed to protect neurons from oxidative stress (Ramsden et al., 2012) might provide valuable targets for this study, but are undetectable in blood tissue (Gene page - UCP, GTEx Portal, 2016) and can therefore not be explored in relation to HFS diet with the methods of this study.

Exploratory analysis of *UCP-2/3* mRNA expression in the context of HFD

UCP-2 and *UCP-3* mRNA is detectable in peripheral blood cells (Gene page - UCP, GTEx Portal, 2016) and they are thus suitable for exploratory analyses in the framework of this study. One main function of both UCP-2 and UCP-3 is the "mild uncoupling" providing a negative feedback loop in the regulation of oxidative stress. They are active only in the presence of specific activators like reactive oxygen species (ROS), reactive metabolic byproducts and fatty acids, especially polyunsaturated fatty acids

(Toda & Diano, 2014) which cause oxidative stress in the cell when their levels exceed the capacity of compensatory mechanisms and potentially lead to cell damage. By reducing the proton gradient, which is one main source of reactive species, UCP-2/3 protect cells against oxidative stress (Esteves & Brand, 2005; Gaudry & Jastroch, 2019; Mailloux & Harper, 2011).

UCP-2 is widely expressed in various tissues across the body, including spleen, pancreas, thyroid, lung, skeletal muscle, blood, adipose tissue and even the brain (Alán, Smolková, Kronusová, Santorová, & Jezek, 2009; Gene page - UCP, GTEx Portal, 2016), however mRNA expression seems to be much more widespread than actual protein expression (Pecqueur et al., 2001). As accumulating fat mass in diet-induced obesity increases ROS in fat tissue and in the blood (Furukawa et al., 2004) and even HFD induces oxidative stress (Alzoubi et al., 2018), UCP-2 might be relevant to counteract oxidative stress in high fat dieters. Additionally, UCP-2 is involved in hypothalamic regulation of food intake and glucose and fat metabolism. More specifically, higher UCP-2 expression is associated with higher fasting- as well as ghrelin-induced food intake mediated by increased activation of appetite-stimulating neurons in the hypothalamus signaled by ROS reduction (Toda & Diano, 2014). UCP-3 can be found predominantly in skeletal muscle and to a smaller extent also in the brain (Alán et al., 2009; Gene page - UCP, GTEx Portal, 2016). It e.g. exports fatty acids out of the mitochondria to allow higher rates of fatty-acid oxidation for energy production, as high levels of fatty acids in the mitochondria would limit further oxidation and additionally UCP-3 protects the cell from reactive fatty acid oxidants that would be formed inside of the mitochondria upon fatty acid accumulation (Brand & Esteves, 2005).

UCP-2 and UCP-3 might further be involved in modulation and protection of dopaminergic neurons. For instance, in a mouse model of Parkinson's disease, *UCP-2* overexpression was shown to protect dopaminergic neurons from oxidative stress and preserve locomotor function (Conti et al., 2005). Further, by creating temperature gradients through thermogenesis within CNS tissue, UCP-2 and UCP-3 might enhance extracellular neurotransmitter diffusion and convection (Fuxe et al., 2005; Rivera et al., 2006). This neuromodulatory function might be relevant in dopaminergic brain regions like the NAc, caudate putamen, amygdala, cingulate and frontal cortex SN, VTA, and also in the hypothalamus, where it might be part of the neuroendocrine regulation of food intake (Fuxe et al., 2005; Rivera et al., 2006). Additionally, UCPs could potentially affect neurotransmission directly by influencing presynaptic concentrations of calcium, which is a vital molecule in the regulation of neurotransmitter transport and metabolism (Andrews, Diano, & Horvath, 2005; Gaudry & Jastroch, 2019).

Taken together, even though the expression of *UCP-2* and *UCP-3* in peripheral blood cells is potentially not directly indicative for expression status and function in other tissues due to tissue-specific expression patterns (Pecqueur et al., 2001), the influence of HFD on *UCP-2* and *UCP-3* expression in blood cells might provide relevant hints towards a relationship between UCPs and HFS diet with a potential applicability for human obesity and thus their mRNA expression will be analyzed exploratorily in this study.

3. Research aims and hypotheses

This study aims to establish evidence for a potential association between a dietary preference for saturated fat and sugar and gene expression and cognition in the dopaminergic system. Obesity seems to have an impact on the DA system, but a potential link between HFS diet and DA is not yet well studied in humans independent of obesity. Therefore, analyzing diet-associated expression differences of genes within the DA signaling pathway in leukocytes combined with a potential cognitive phenotype related to executive functions within a sample with broad BMI range will allow to add to this new field of research.

Main hypotheses:

1. Individuals self-reporting to follow a high fat and sugar (HFS) diet have other mRNA expression levels of genes in the peripheral dopaminergic system than those reporting to follow a low fat and sugar (LFS) diet.
2. HFS compared to LFS diet consumption is associated with differences in neuropsychological tests performance.
3. The mRNA expression of DA-related genes in leukocytes mediates potential dietary effects on cognitive performance.

The third hypothesis slightly differs from the preregistered hypothesis ("mediates" instead of "is associated with", see https://osf.io/e3w8k) to more precisely illustrate that a mediation model was planned to test this hypothesis. This is in accordance with the preregistered analysis plan (see https://osf.io/e3w8k, part "Analysis plan").

4. Materials & Methods

4.1 Sample

4.1.1 Inclusion and exclusion criteria

For recruitment, the participant database of the Max Planck Institute for Human Cognitive and Brain Sciences and advertisements placed in public locations such as university facilities and online were used. In total, 109 volunteers were screened for the study, of which 75 participants were suitable and could be included.

The inclusion criteria were:

- male (due to the effect of the menstrual cycle on the dopaminergic system and executive functions, see e.g. Hidalgo-Lopez & Pletzer, 2017)
- healthy (for details see exclusion criteria)
- right-handed
- age between 18 and 40 years
- fluent German speaker
- DFS-Q score (for details see section 4.1.2) ≥ 62 (HFS) or ≤ 52 (LFS)

The exclusion criteria were:

- BMI below 18.5 or above 45 kg/m²
- personal or first-degree-relative history of psychiatric or neurological disease
- history of significant head injury (i.e. loss of consciousness)
- metabolic diseases, i.e. diabetes mellitus type I/II or thyroid dysfunction
- regular intake of psychotropic medication, illicit drug use, excessive alcohol use (on average no more than 20 g alcohol/day ≙ 1-2 beers with 0,5 l/day), smoking (no regular smoking within the last year, no cigarettes within the last 3 months)
- excessive self-reported physical exercise (> 6 h of vigorous activity per week) because of its effect on striatal DA (for details see section 2.8.1)
- contraindications for fMRI (as this project is part of a bigger study including fMRI imaging)
- score above 19 in the Beck Depression Inventory (BDI; Beck, Ward, Mendelson, Mock, & Erbough, 1961)
- mean score above 3.88 in the Eating Disorder Examination Questionnaire (EDE-Q; Fairburn & Beglin, 1994)
- IQ score < 85 in the Viennese Matrices Test 2 (WMT-2; Formann, Waldherr, & Piswanger, 2011)

The study was carried out in accordance with the Declaration of Helsinki and was approved by the Medical Faculty Ethics Committee of the University of Leipzig (ethics number: 400/18-ek). All participants gave written informed consent before taking part in the study.

4.1.2 Grouping based on the DFS questionnaire

According to their total score in the Dietary Fat and free Sugar – Short Questionnaire (DFS) (Francis & Stevenson, 2013) participants were assigned to either the group of high or low consumers of saturated fat and refined sugar (HFS vs. LFS). The DFS contains 24 questions asking for the average consumption frequency of certain food items over the last twelve months. There are five answer options ranging from 1x or less per month to 5x or more per week. Two additional questions ask for how often participants consumed fast food at a restaurant or via delivery service averaged over the last twelve

months and the number of teaspoons of sugar that were added to food and beverages in the last week. The lowest possible score is 26 (low intake of HFS diet) and the maximum score is 130 (high intake of HFS diet). A previous study showed that the DFS is a valid tool to assess intake of dietary fat and sugar (Fromm & Horstmann, 2019). Based on this study the cutoffs were defined as a total score ≤ 52 for the LFS group and a total score ≥ 62 for the HFS group. Participants scoring between these cutoffs (total score of 53 to 61) were excluded from the study. At the beginning of recruitment, the cutoff for the LFS group was 48 but had to be raised to 52 after one year of testing due to substantial problems in recruiting LFS participants (see updated preregistration: https://osf.io/w9e5y).

4.2 Study design

Fig. 4 provides an overview of the study design of this project. Participants were examined via online survey or phone call if they fulfilled all inclusion criteria (male, healthy, right-handed, age between 18 and 40 years, fluent German speakers, DFS-Q score ≥ 62 or ≤ 52) and no exclusion criteria (BMI ≤ 18.5 or ≥ 45 kg/m², personal or first-degree-relative history of psychiatric or neurological disease, history of significant head injury, metabolic diseases, regular intake of psychotropic medication, illicit drug use or excessive alcohol use, smoking, excessive physical exercise, contraindications for fMRI, BDI score > 19, mean EDE-Q score > 3.88, IQ score < 85). If the criteria were met, participants were invited to the institute for a screening where they provided informed consent and filled out the DFS, BDI, EDE-Q, and the WMT-2 (see section 4.1). Additionally, the screening day comprised an overnight-fasted blood drawing for mRNA expression analyses, genotyping of SNPs and measurements of metabolic and inflammatory markers and hormonal levels (see section 4.4, 4.5, and 4.6, respectively). The screening day was followed by two separate test days (lasting 3 h and 3.5 h). This was necessary as the present research project is embedded in a bigger study aiming to create a broad picture about the interplay between diet and the dopaminergic system on a genetic and behavioral level using e.g. functional magnetic resonance imaging (fMRI). The overarching study is preregistered here: https://osf.io/8pxgd. One behavioral test day included three neuropsychological tests of executive functioning, i.e. the Trail making test (TMT), the Digit symbol substitution test (DSST), and the Digit span test (DS), that were administered as pen and paper tests (see section 4.7). Moreover, six questionnaires assessing personality, eating behavior and physical activity were administered online (see section 4.8). The other test day comprised a second sequence of questionnaires as well as a repetition of the DFS to test the reliability of screening day scores. The complete study further included behavioral testing with and without simultaneous fMRI but the behavioral and MRI data is not part of this research project and is thus not further addressed here. The sequence of test days (with and without fMRI) was randomized and counterbalanced between groups. Following the last test day, a pedometer was handed out and participants were instructed to carry it for seven consecutive days to measure their step count during everyday activities (see section 4.9).

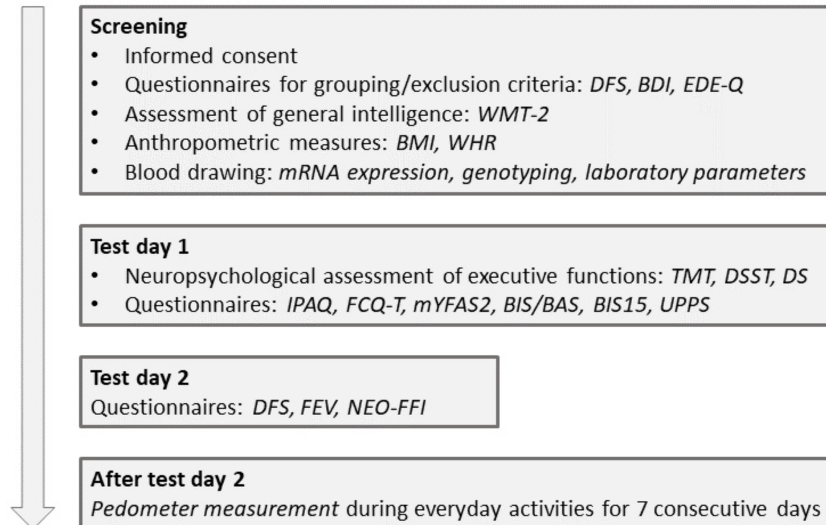


Figure 4: Overview of study design. Screening: participants gave informed consent and filled out the DFS for group assignment and the BDI, EDE-Q, and WMT-2 to approve suitability for inclusion. If inclusion criteria were met, BMI and WHR were obtained and overnight-fasted blood samples were drawn for mRNA expression analyses, genotyping, and laboratory assessments. Test day 1: neuropsychological assessment was performed using the Trail making test (TMT), the Digit symbol substitution test (DSST), and the Digit span test (DS) and questionnaires about physical activity, eating behavior and personality traits were administered. Test day 2: participants completed a second sequence of questionnaires about personality and eating behavior including a repeated measurement of the DFS score for confirming scoring reliability. After test day 2: participants carried a pedometer for 7 consecutive days after the last test day to measure everyday step count. Abbreviations: BDI: Beck Depression Inventory, BIS15: Barrat Impulsiveness Scale, BIS/BAS: Behavioral approach and avoidance system questionnaire, BMI: body mass index, DFS: Dietary Fat and free Sugar – Short Questionnaire, DSST: Digit symbol substitution test, DS: Digit span test, EDE-Q: Eating Disorder Examination Questionnaire, FCQ-T: Food Craving Questionnaire-Trait, FEV: Three-Factor Eating Questionnaire, IPAQ: International Physical Activity Questionnaire, mRNA: messenger ribonucleic acid, NEO-FFI: NEO-Five Factor Inventory, TMT: Trail making test, UPPS: Urgency, Premeditation, Perseverance, Sensation Seeking (UPPS) Impulsive Behavior Scale, WHR: waist-to-hip-ratio, WMT-2: Viennese Matrices Test 2, YFAS: Yale Food Addiction Scale.

4.3 Anthropometric measures

Weight and height were obtained on the screening day for all participants in overnight-fasted condition (at least 8 h). Weight was measured with an electronic body scale (accuracy of up to 0.1 kg) and height was measured using a fixed tape measure. Waist circumference (perimeter at the smallest point of the upper body usually slightly above the navel) and hip circumference (above hip bones) were obtained with a tape measure.

4.4 Gene expression analysis: mRNA quantification with quantitative polymerase chain reaction (qPCR)

4.4.1 Genes of interest and housekeeping genes

To create a broad picture of the mRNA expression profiles of genes within the DA pathway, mRNA levels of the following genes of interest were analyzed with quantitative polymerase chain reaction (qPCR) in blood leukocytes:

- *dopamine receptors D2/D3/D5* (*DRD 2/3/5*),
- *dopamine-and cAMP-regulated neuronal phosphoprotein 32* (*DARPP-32*),
- *dopamine transporter* (*DAT*),
- *catechol-O-methyltransferase* (*COMT*),
- *uncoupling proteins 2/3* (*UCP-2/3*) for exploratory analyses of potential associations between UCPs, diet and the DA system.

For standardization three housekeeping genes (HKG) were analyzed:

- *TATA box binding protein* (*TBP*)
- *hypoxanthine phosphoribosyl transferase 1* (*HPRT1*)
- *ribosomal protein, large, P0* (*RPLP0*)

4.4.2 Sample acquisition and RNA extraction

Peripheral blood samples with a volume of 3 ml were drawn with a Tempus™ Blood RNA Tube (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored at - 80 °C until further processing. Total mRNA was extracted from leukocytes with Norgen's Preserved Blood RNA Purification Kit I (Norgen, Biotec Corp., Thorold, ON, Canada). According to the manufacturer's protocol, the blood of each sample was diluted in Tempus™ Blood RNA Tube Diluent and centrifuged at 4000 rpm and 4 °C for 45 minutes (min) in an Eppendorf Centrifuge 5804 R (Eppendorf, Hamburg, DE). Additional 15 min were added to the suggested 30 min centrifugation time as the used centrifuge did not reach the recommended speed of 4500 rpm. After centrifugation, the RNA pellet was lysed in the supplied Lysis Solution and 95 % ethanol (JT Baker via Fisher Scientific GmbH, Schwerte, DE). The optional step for on-column DNA removal was performed by adding DNase I and Enzyme Incubation Buffer to the RNA samples, centrifuging the tubes for 1 min at 14000 rpm in a Heraeus Fresco 17 Centrifuge (Thermo Fisher Scientific Waltham, Massachusetts, USA) and pipetting the flow-through back on top of the column. After 15 min incubation at 28 °C in a Thermomixer compact (Eppendorf, Hamburg, Germany), each column was washed with Wash Solution for 3 times and the extracted RNA was diluted in 50 µl of Elution Solution and stored at -80 °C. RNA concentrations were measured with a Nanodrop 2000 Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and RNA quality was determined with gel electrophoresis on 2 % agarose gels (SeaKem® LE Agarose, Lonza, Rockland, ME, USA) with the RiboRuler High Range RNA Ladder, ready-to use (Thermo Scientific , Waltham, Massachusetts, USA) using a on a VWR® Imager2 (VWR International GmbH, Darmstadt, DE). Fig. 5 shows one representative agarose gel. The gel indicates good RNA quality with two bands for all RNA samples, one band slightly above 4000 bp (base pairs) for the 28S RNA (size = 4718 bp) and one band between 2000 and 1500 bp for the 18S RNA (size = 1874 bp). No additional bands or artefacts were visible.

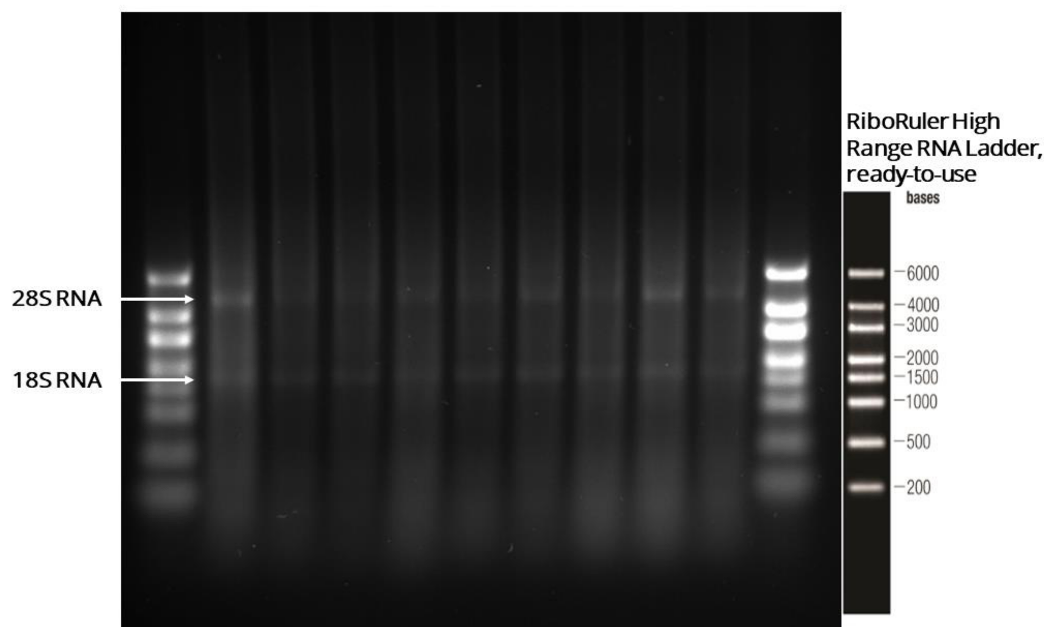


Figure 5: Agarose gel electrophoresis of RNA samples. RNA quality of all samples (nine representative samples are shown here) was determined on 2 % agarose gels with electrophoresis. The gel shows two bands for all RNA samples, one band slightly above 4000 bp for the 28S RNA (4718 bp) and one band between 2000 and 1500 bp for the 18S RNA (1874 bp). No additional bands or artefacts were visible, indicating good RNA quality.

4.4.3 Reverse transcription of RNA into complementary DNA

The gene expression measurement with qPCR is based on amplification of double-stranded material. As RNA is single-stranded, it is necessary to transform the RNA into double-stranded complementary DNA (cDNA). Thus, 500 ng of extracted RNA of each sample were reverse transcribed into cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany). Following the manufacturer's protocol, the template RNA dilution was incubated at 42 °C for 2 min on a ThermalCycler (Applied Biosystems, Foster, USA) with supplied gDNA Wipeout Buffer to remove traces of genomic DNA. Then the following reverse-transcription reaction components were added as a mastermix: Quantiscript Reverse Transcriptase (including RNase inhibitor), Quantiscript Reverse Transcription Buffer (including dNTPs and Mg^{2+}) and Reverse Transcription Primer Mix (mix of oligo-dT primers and random-primers) to reach a final reaction volume of 20 µl. Reverse-transcription was performed on a ThermalCycler (Applied Biosystems, Foster, USA) in two steps: incubation for 30 min followed by 3 min at 95 °C to inactivate the transcription enzyme. The transcribed cDNA was stored at -20 °C until further processing.

4.4.4 mRNA quantification with TaqMan® Gene Expression Assays

TaqMan® Gene Expression Assays (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were used to specifically quantify PCR products. As the number of original RNA copies correlates with the number of reverse transcribed cDNA molecules and with the number of amplified PCR copies, this molecular genetic method can be used to determine the relative amount of mRNA that was present in the original sample. Gene expression measurement with TaqMan Assays quantifies PCR products using fluorescence (Fig. 6).

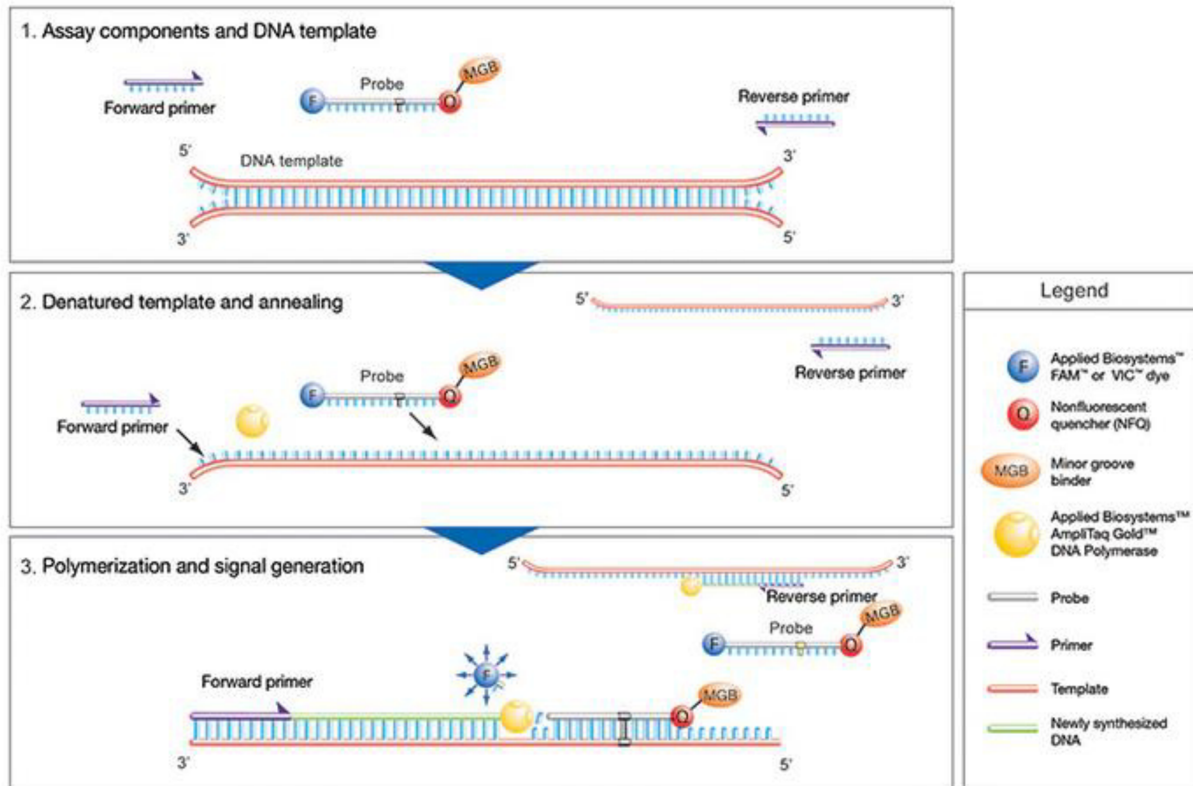


Figure 6: TaqMan Gene Expression Assay components and quantification procedure 1. Assay components and DNA template: components are a forward primer, a reverse primer, and a TaqMan probe. The probe is marked with the reporter dye FAM (F) at the 5'-end and a quencher (Q) at the 3'-end that suppresses the fluorescence signal, as long as the probe is intact. 2. Denaturation and annealing: after denaturation of the double-strand cDNA with heat, primers and the probe anneal to the single-strand cDNA at their specific predefined locus. 3. Polymerization and signal generation: during the following elongation period, the Taq-DNA-Polymerase synthesizes a DNA strand complementary to the single-strand cDNA template starting from the attached primer. If a probe is bound between forward and reverse primer it is segmented by the exonuclease activity of the polymerase, separating the quencher from the dye, which produces a luminescence signal proportional to the number of bound probes in the reaction sample. With each PCR cycle more reporter dye molecules are released. Source: Thermo Fisher Scientific website (How TaqMan Assays Work - Thermo Fisher Scientific – NL, 2020).

Each assay consists of two primers that are specific for a particular PCR product, one forward and one reverse primer, and a TaqMan probe. The probe is marked with the reporter dye FAM at the 5'-end and a quencher at the 3'-end that suppresses the fluorescence signal, as long as the probe is intact. After denaturation of the double-strand cDNA, the forward and reverse primers and the probe hybridize with a single-strand cDNA at their specific predefined locus. In the following elongation period, the DNA-polymerase synthesizes a DNA strand complementary to the single-strand cDNA template starting from the attached primer. If a probe is bound between forward and reverse primer it is segmented by the exonuclease activity of the polymerase, separating the quencher from the dye, which produces a fluorescence signal proportional to the number of bound probes in the reaction sample. With each PCR cycle more reporter dye molecules are released, and this fluorescence signal is measured after each amplification cycle ("real time") to realize quantification. The increase in intensity of the FAM fluorescence signal correlates with the number of specific mRNA copies in the original sample. The read-out of this procedure is the amplification cycle number (cT) at which the amplification fluorescence signal of the reporter dye FAM exceeds the background signal of an internal standard dye (ROX™ Passive Reference dye). The assay information is derived from the Thermo Fisher Scientific website (How TaqMan Assays Work - Thermo Fisher Scientific – NL, 2020).

4.4.5 Gene expression measurement

Transcribed cDNA was quantified using real-time polymerase chain reaction with TaqMan® Gene Expression Assays (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For details about the assays see Table S18 (section 9.1.2). According to the manufacturers qPCR protocol the PCR tubes for each sample were set up in 10 µl reactions with the Applied Biosystems™ TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and variable volume of cDNA template solution for each assay (volumes and corresponding cDNA amounts are summarized in Table S18). Required amounts of template cDNA for producing a sufficient qPCR detection rate were experimentally determined during the establishment of the experiment. This step was necessary due to unexpectedly low expression of the genes of interest in the obtained blood samples combined with a limited reservoir of extracted RNA. Separate 96-well plates (Sapphire Mikroplatte, 96 Well, Greiner Bio-One GmbH, Frickenhausen, Deutschland) were used to quantify cDNA transcripts of each gene for all 75 samples in single measurements. The qPCR was carried out on an Applied Biosystems 7500 Real-Time PCR System with the 7500 Real Time PCR Software (all supplied by Thermo Fisher Scientific, Waltham, Massachusetts, USA) using the PCR program described in Table 1.

Table 1: Real-time PCR protocol.

<table>
<tr><th>Temperature</th><th>Duration</th><th>Function</th></tr>
<tr><td>50 °C</td><td>2 min</td><td>Uracil-N glycosylase (UNG) incubation</td></tr>
<tr><td>95 °C</td><td>10 min</td><td>Denaturation</td></tr>
<tr><td>95 °C</td><td>15 sec</td><td rowspan="3">Amplification: 40 cycles</td></tr>
<tr><td>60 °C</td><td>1 min</td></tr>
<tr><td colspan="2">Fluorescence measurement</td></tr>
<tr><td>10 °C</td><td>∞</td><td>Hold</td></tr>
</table>

4.4.6 Standardization and output

Obtained cT values were corrected for qPCR efficiency by using a standard curve of commercially purchased Universal Human Reference RNA (Thermo Fischer Scientific, Waltham, Massachusetts, USA) transcribed into cDNA, which was diluted in series of 1:3. The efficiency value E is obtained from the slope of a linear model fitted to the log-transformed concentrations of this standard dilution series and their corresponding cT values with a theoretical maximal efficiency of 2 (each sample is replicated in each cycle) and a minimal efficiency of 1 (no replication). E is calculated as $10^{-1/slope}$ (Pfaffl, 2004). Expression values of the target genes *DRD2/3/5, DARPP-32, DAT, COMT, UCP-2,* and *UCP-3* were standardized with reference to a HKG. This minimizes the impact of external influencing factors such as low sample quality, inaccuracies of RNA quantification in the spectrometer measurement or incomplete transcription of RNA into cDNA. These influences are believed to impact gene expression levels of both the target gene and the HKG to the same extent and can therefore be accounted for by standardization. The most stably expressed HKG between and within diet groups was determined independently with two different excel-based softwares: Bestkeeper (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004) and Normfinder (Andersen, Jensen, & Ørntoft, 2004). For detailed information about these tools see the supplementary material in section 9.1.3. Both tools indicated *HPRT1* as the best HKG for standardization. *HPRT1* was therefore used as reference in the following calculation of arbitrary units (AU), which are the standardized and efficiency-corrected relative values of RNA expression used for further statistical calculations:

$$AU = \frac{Etar^{-cTtar}}{Eref^{-cTref}}$$

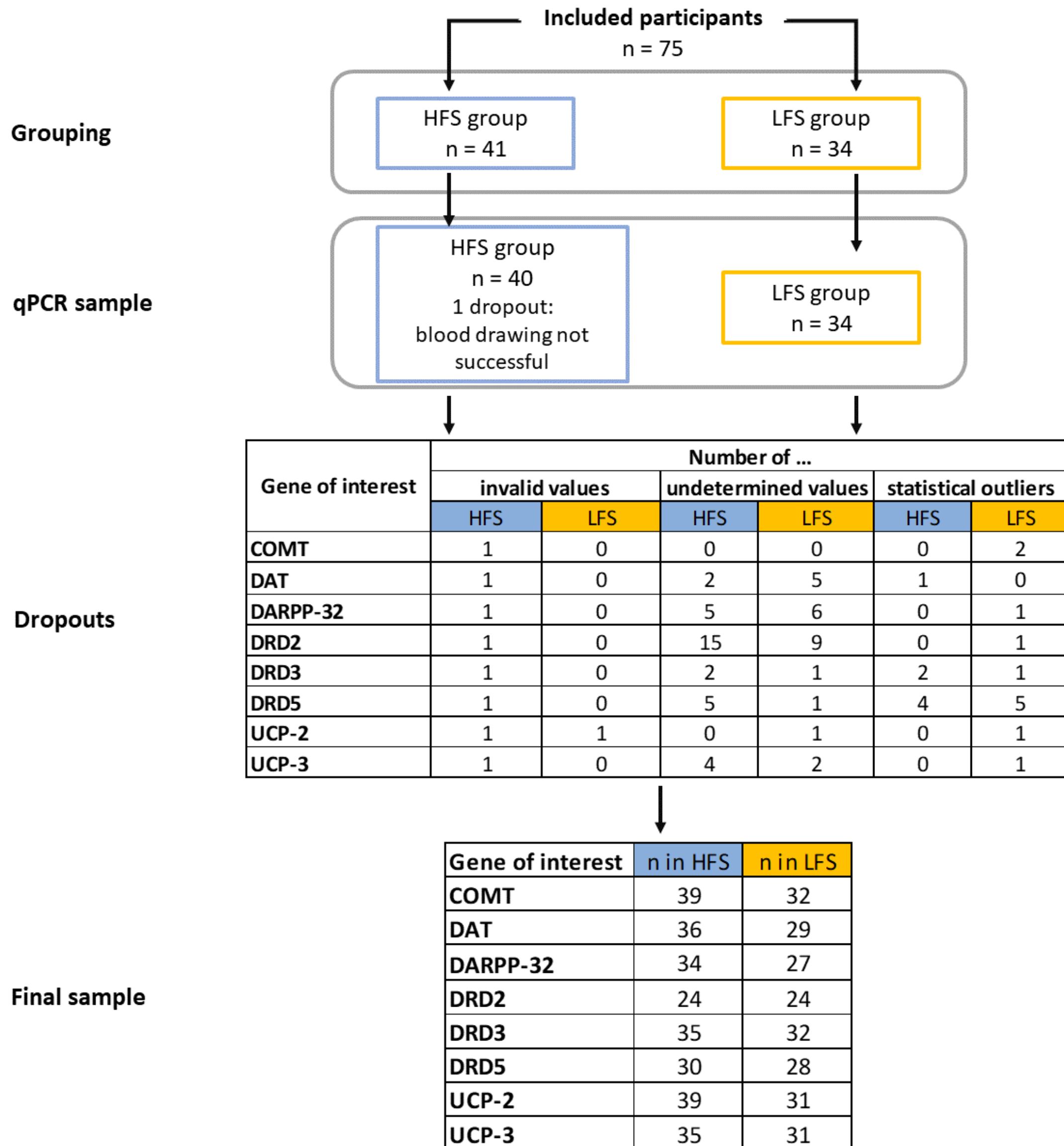
AU: arbitrary unit (describes RNA expression level)
E_{tar}*: efficiency of the qPCR of the respective target assay*
E_{ref}*: efficiency of the qPCR of the HPRT1 assay*
cT_{tar}*: threshold cycle (cT) of target assay for particular sample*
cT_{ref}*: threshold cycle (cT) of the HPRT1 assay for particular sample*

The equation is based on the commonly accepted formula $2^{-\Delta cT}$ that does not account for qPCR efficiencies and an equation that integrates efficiency corrections (Pfaffl, 2004) . As the equation proposed by Pfaffl is specialized for a within-subject design, the equation was adapted for the data of this study. A systematic derivation of the adapted formula from both the commonly accepted formula and the formula with efficiency correction can be found in supplementary material 9.1.4. The final value (AU) is a relative expression value for each gene of interest for each sample and describes the relative amount of mRNA for the respective gene that was present in the original blood sample. Comparing relative expression values between diet groups shows if mRNA levels of genes related to the DA system are elevated or decreased in leukocytes in one of the groups.

4.4.7 Final sample for mRNA expression analyses

In total, 75 participants were included in the study, of which 41 were in the HFS group and 34 in the LFS group. There were several dropouts during sample acquisition and processing that are summarized in Fig. 7. For one HFS participant it was not possible to draw blood samples and therefore there was no RNA available for analyses. One HFS sample had to be removed due to a pipetting mistake during sample preparation for qPCR. One sample from the LFS group presumably was of low quality, as gene expression values for this sample were two times not measurable, five times a statistical outlier and only one value for UCP-2 would have been valid for data analysis. It was agreed on not to include this sample into further analysis steps. Most likely due to the generally low expression of the genes of interest in leukocytes there were several cases where no cT value was determinable. The number of these dropouts is displayed in Fig. 7 in the table column “undetermined values”. Finally, a statistical outlier analysis was performed and values outside of the 2.2-times interquartile range were excluded. This results in different sample sizes for each gene of interest. Matching of groups in age and BMI was checked for all subsamples.

It was not possible to measure the expression of more potentially interesting DA-related genes, i.e. *tyrosine hydroxylase (TH)*, *DOPA-decarboxylase (DDC)*, *vesicular monoamine transporter 2 (VMAT2)* and *dopamine receptor D1 and D4 (DRD1/4)* . This was due to technical issues, i.e. the ordered *TH* assay did not deliver sufficient expression data already during establishment with reference RNA, where TH should certainly be expressed, expression of *DDC* and *DRD4* was not detectable, *DRD1* is generally not expressed in blood (Gladkevich et al., 2004; Kirillova et al., 2008) and an assay for*VMAT2* was commercially not available. All materials used for mRNA quantification are listed in Table S16 (section 9.1.2).



Gene of interest	Number of ...					
	invalid values		undetermined values		statistical outliers	
	HFS	LFS	HFS	LFS	HFS	LFS
COMT	1	0	0	0	0	2
DAT	1	0	2	5	1	0
DARPP-32	1	0	5	6	0	1
DRD2	1	0	15	9	0	1
DRD3	1	0	2	1	2	1
DRD5	1	0	5	1	4	5
UCP-2	1	1	0	1	0	1
UCP-3	1	0	4	2	0	1

Gene of interest	n in HFS	n in LFS
COMT	39	32
DAT	36	29
DARPP-32	34	27
DRD2	24	24
DRD3	35	32
DRD5	30	28
UCP-2	39	31
UCP-3	35	31

Figure 7: Dropouts during mRNA sample processing and final sample size for gene expression analyses. The figure shows the mRNA sample development. One HFS sample had to be removed due to a pipetting mistake during sample preparation for qPCR (“invalid values”). Further, the qPCR measurement was several times not successful (“undetermined values”) and values outside of the 2.2x interquartile range were removed from the analysis (“statistical outliers”). The sample size for each diet group and gene of interest for the actual statistical analyses is provided in the undermost table.

4.5 Genotyping

4.5.1 SNP description

Single nucleotide polymorphisms (SNP) and a various number tandem repeat (VNTR) were genotyped to enable controlling for potential confounding effects of genotype on mRNA expression and cognitive performance. The analyzed SNPs are specified in Table 2.

Table 2: SNPs of interest. The table provides details about the analyzed SNPs ("SNP specification"), labelling of alleles with VIC (1) and FAM (2) as defined by the supplier, details if and how alleles were grouped in risk allele carrier vs. non-carrier where appropriate ("allele grouping") and the coding of alleles or allele groups in category 0, 1 and 2 for further statistical analyses that was used in this manuscript**.**
* The SNP rs8050136 within the *FTO* gene is in complete linkage disequilibrium with rs9939609 (rs8050136 (SNP) - Linkage disequilibrium - Homo_sapiens - Ensembl genome browser 99, 2020). This means that the more frequent C allele in rs8050136 is always inherited with the more frequent T allele in rs99396009 and the less frequent A alleles on both SNPs are also jointly inherited. Therefore, rs8050136 can be used as a proxy for rs9939609 (Haupt et al., 2009) as directly genotyping rs9939609 is methodologically challenging.

SNP specification	Labelling (1: VIC, 2:FAM)	Allele grouping	Allele coding
COMT rs4680 NG_011526.1:g.27009G>A	1 = A 2 = G	No grouping (gene dose effect)	0 = AA 1 = AG 2 = GG
DRD2/ANKK1 rs1800497 (Taq1A polymorphism) NG_012976.1:g.17316G>A	1 = T = A1 allele 2 = C = A2 allele	Carriers of risk allele A1 (T/T + T/C) vs. A2/A2 (C/C)	1 = TT + TC 2 = CC
DRD2 rs6277 NG_008841.1:g.67543C>T	1 = T 2 = C	No	0 = TT 1 = TC 2 = CC
FTO rs9939609 NG_012969.1:g.87653T>A	Proxy: rs8050136 * 1 = A (proxy: A) 2 = T (proxy: C)	No	0 = AA 1 = AC 2 = CC
FTO rs8053740 NG_012969.1:g.142838G>A, C	1 = C 2 = G	No	0 = CC 1 = CG 2 = GG
DARPP-32 rs907094 NG_030330.1:g.12195G>A	1 = A/T 2 = G/C	Carriers of the C allele vs. T/T	1 = TT 2 = TC + CC
DAT (*SLC6A3*) VNTR rs28363170 NG_015885.1:g.56644_56683dup	1 = 9 repeats 2 = 10 repeats	Carriers of 9-repeat allele vs. 10/10 carriers	1 = 9/9 + 9/10 2 = 10/10

4.5.2 Genotyping with TaqMan® Genotyping Assays

Genotyping of SNPs is a molecular genetic analysis that is used to determine genetic variants of individuals within a population. Genotyping with TaqMan® Assays (Applied Biosystems, Foster, USA) allows the discrimination of alleles within SNPs by specifically quantifying PCR products using fluorescence signals. The assays again consist of two allele-specific primers, one forward and one reverse primer, but in contrast to gene expression assays (section 4.4.4) the genotyping assays contain two allele-specific probes that are marked with two different reporter dyes, either VIC or FAM, at the 5'-end. Also in the genotyping probes a quencher at the 3'-end suppresses the fluorescence signal as long as the probe is intact. For the PCR, assay components are added to a genomic DNA sample instead of the cDNA sample used for gene expression measurements. The following amplification sequence of repeated denaturation, hybridization (annealing) and elongation (DNA polymerization) steps follows the principle described in section 4.4.4 with the difference of using AmpliTaq Gold® DNA-Polymerase instead of Taq-DNA-Polymerase. Fig. 8 shows the assay components and the PCR procedure.

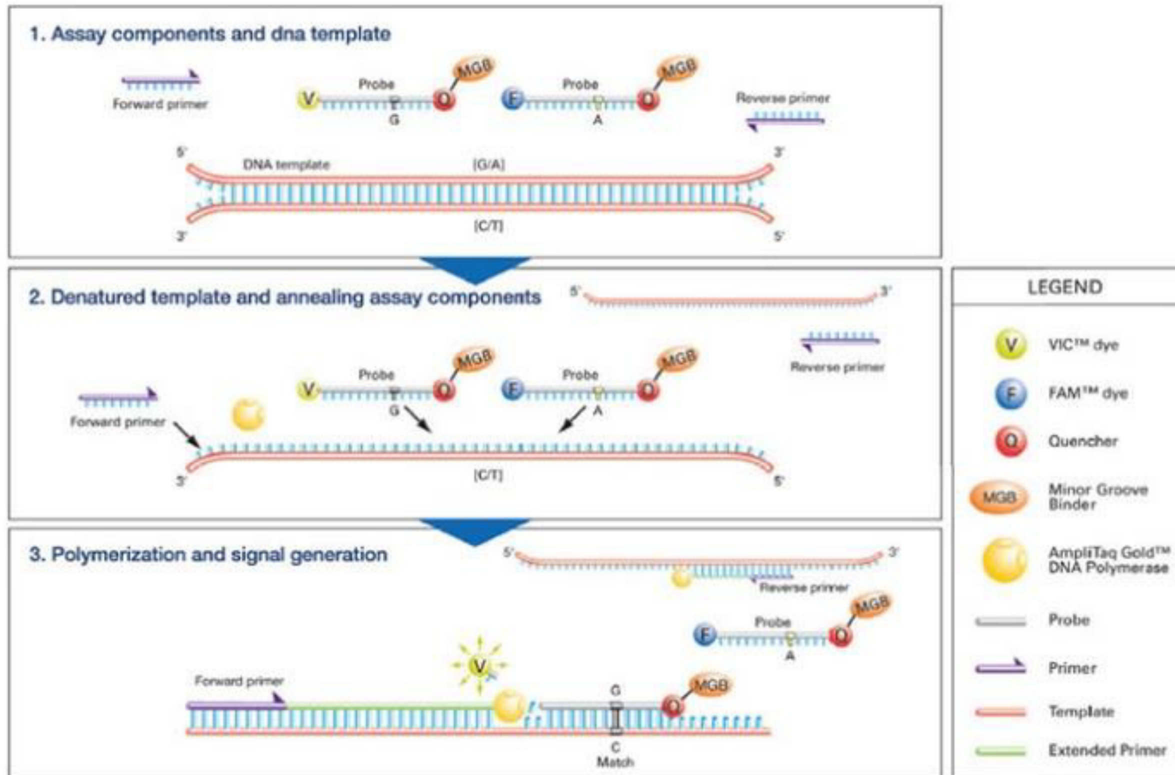


Figure 8: TaqMan genotyping assay components and allele detection with PCR 1. Assay components and DNA template: the assays contain a forward and a reverse primer and two allele-specific probes. The probe for allele 1 is marked with VIC (V) dye and the probe for allele 2 with FAM dye (F). 2. Denaturated template and annealing assay components: after denaturation primers and probes can bind to the single-stand DNA at their predefined specific locus ("annealing"). 3. Polymerization and signal generation: during elongation the AmpliTaq Gold® DNA-Polymerase synthesizes a DNA strand complementary to the single-strand DNA template starting from the attached primers. The bound probe is segmented by the exonuclease activity of the polymerase, separating the quencher from the dye, which produces a luminescence signal of either VIC or FAM depending on the allele present in the sample. Source: Thermo Fisher Scientific website (How TaqMan Assays Work - Thermo Fisher Scientific – NL, 2020).

Similar to the process described in 4.4.4, the polymerase segments the probes and thus separates the quenchers from the dyes. This produces luminescence signals of either VIC or FAM depending on the allele present in the sample. The increase of VIC reporter dye fluorescence signal indicates the presence of allele 1 and an increase in FAM fluorescence signal indicates allele 2 (How TaqMan Assays Work - Thermo Fisher Scientific – NL, 2020). Detection of the fluorescence signal allows to assign if the DNA sample shows either homozygous presence of allele 1 (only VIC signal), homozygous presence of allele 2 (only FAM signal) or heterozygous presence of both alleles (VIC and FAM signal equally present) in the SNP locus. An exemplary signal detection output is shown in Fig. 9.

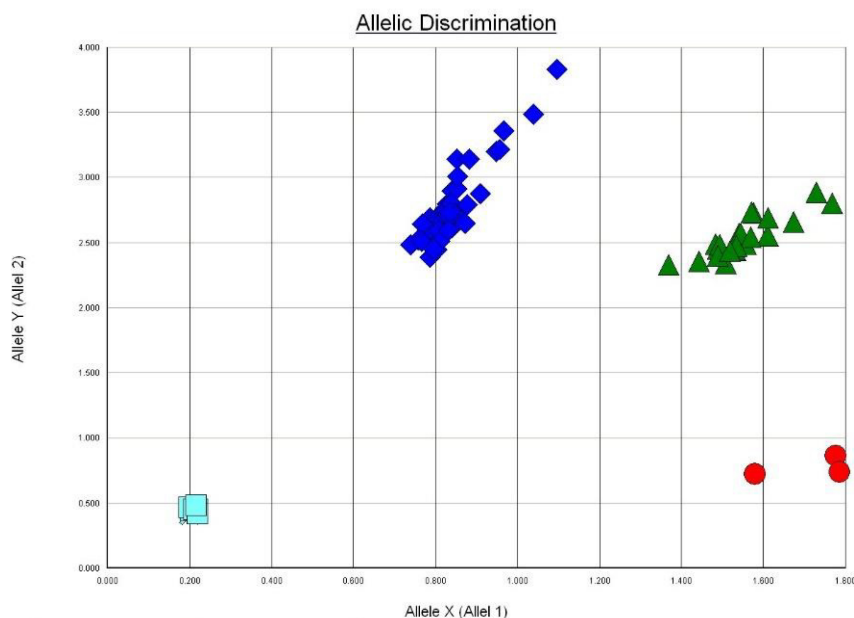


Figure 9: Exemplary output for the Taq1A polymorphism in *DRD2/ANKK1*. The x-axis shows the fluorescence signal of VIC (allele 1) and the y-axis the fluorescence signal of FAM (allele 2) detected in each sample. The cluster of dark blue squares represents samples carrying allele 2 (major A2 allele), the cluster of red circles indicates presence of allele 1 (minor A1 allele) and the cluster of green triangles describes heterozygous carriers of both alleles. The cluster of light blue squares on the bottom left are non-template controls showing no fluorescence signal.

4.5.3 Genomic DNA extraction

On screening day, a peripheral blood sample was drawn with an EDTA monovette (2.7 ml EDTA S-Monovette, SARSTEDT AG & Co. KG, Nümbrecht, Germany) and was stored, after a 15 min rest at room temperature, at -80 °C. For further processing all samples were sent to the laboratory of Prof. P. Kovacs (IFB AdiposityDiseases; Medical Research Center; Liebigstr. 21 in Leipzig). Genomic DNA was extracted from white blood cells of all 75 participants with the QuickGene DNA whole blood kit S (Kubaro Industries LTD., Osaka, Japan). In brief, according to the manufacturers protocol, blood cells were lysed with protease and lysis buffer and incubated at 56 °C in a Thermomixer compact (Eppendorf, Hamburg, Deutschland), mixed with absolute ethanol (JT Baker via Fisher Scientific GmbH, Schwerte, DE) and then transferred to columns with a silica membrane. Subsequently, bound DNA was cleaned with washing buffer and the solution was pushed through a filter (the system operates with pressure, centrifugation is not necessary). Lastly, DNA was removed from the filter with elution buffer (Tris-EDTA, pH 8.0), eluted in 180-200 µl volume and extracted gDNA was stored at -20 °C. DNA concentration and integrity were determined with a NanoDrop (Peqlab, Erlangen) at 260/280 nm.

4.5.4 Genotyping protocol

Genotyping of six SNPs was performed with Applied Biosystems TaqMan® Genotyping Assays and the TaqMan Genotyping Mastermix on a TaqMan®7500 Real Time PCR System (all supplied by Applied Biosystems, Foster, USA) according to the manufacturers protocol. The PCR program shown in Table 3 was used to amplify the DNA.

Table 3: SNP genotyping protocol.

Temperature	Duration	Function
95 °C	10 min	Denaturation
95 °C	15 sec	Amplification: 40 cycles
60 °C	1 min	
Fluorescence measurement		
10 °C	∞	Hold

The software ABI Prism 7500 (Applied Biosystems, Foster, USA) was used for read-out of detected alleles. Allele frequencies were tested for consistency with the Hardy-Weinberg equilibrium using an online tool for Hardy-Weinberg calculations (husdyr.kvl.dk). A p-value > 0.05 indicates that the allele distribution is consistent with the Hardy-Weinberg equilibrium but p-values > 0.01 are still acceptable due to the small sample size. Allele frequencies of the all SNPs were consistent with the Hardy-Weinberg equilibrium (for COMT: p = 0.02; all other p ≥ 0.15). The exact p-values are provided in Table S20 in section 9.1.5.

4.5.5 VNTR genotyping

To determine the repeat length of the VNTR in *DAT* a fragment length analysis was performed using PCR and gel electrophoresis. Qiagen's HotStar Taq Plus Mastermix Kit (Quiagen, Hilden, Germany) was used for gDNA amplification. According to the manufacturer's protocol, 10 µl HotStar Plus Master Mix 2x, 1 µl Q-Solution (replacing PCR-H2O), 2 µl Coral Load 10x and 1 µl of both forward and reverse primer were added to 5 µl of DNA template (≙ ca 100 ng) to reach a final reaction volume of 20 µl. The PCR protocol (Table 4) was programmed on a ThermalCycler (Applied Biosystems, Foster, USA).

Table 4: VNTR genotyping protocol.

Temperature	Duration	Function
95 °C	5 min	Denaturation
94 °C	30 sec	Amplification: 35 cycles
60 °C	30 sec	
72 °C	1 min	
72 °C	10 min	Hold

The primers were purchased from Biomers (Ulm/Donau, Germany) with the following sequences based on initial descriptions of the VNTR (Sano, Kondoh, Kakimoto, & Kondo, 1993; Vandenbergh et al., 1992):

- forward: 5'-TGCGGTGTAGGGAACGGCCTGAGA-3'
- reverse: 5'-TGTGGTCTGCAGGCTGCCTGCAT-3'

A base mismatch was detected in the third base of the forward primer and the third-last base of reverse primer when comparing the primer sequences given in the referenced papers with the Ensembl reference sequence ENSG00000142319 for *DAT*. These bases were therefore adapted.

PCR products were separated on 4 % agarose gels (Biozym Sieve GeneticPure Agarose, Biozym Scientific GmbH, Oldendorf, DE) by electrophoresis with a running time of 90 min at 110 V and visualized with UV light. The repeat length was quantified with the Serva Fast Load 50 bp ladder (SERVA Electrophoresis GmbH, Heidelberg, Germany) using a VWR® Imager2 (VWR International GmbH, Darmstadt, DE). A band at 410bp described the 9-repeats allele and a band at 450bp the 10-repeat carriers (see Fig. 10). Detected allele frequencies are consistent with the Hardy-Weinberg equilibrium (p = 0.22; see Table S20 in section 9.1.5). All materials used for DNA extraction and genotyping are listed Table S17 (section 9.1.2).

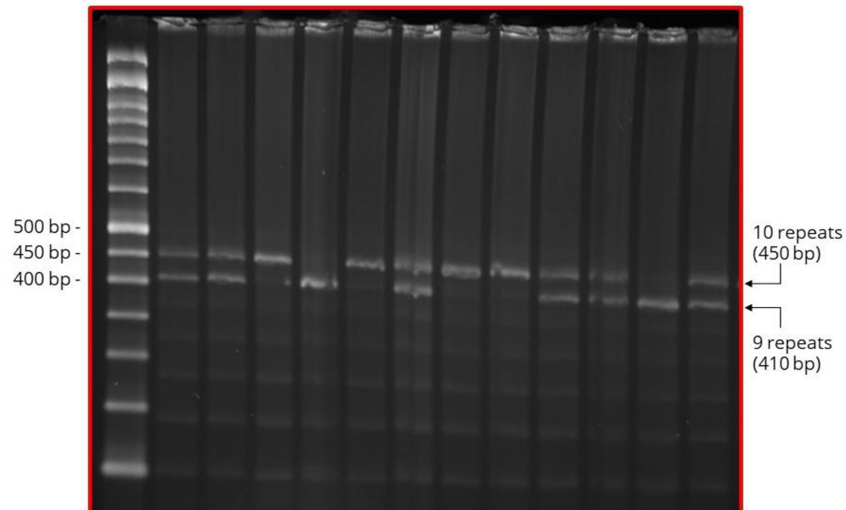


Figure 10: Gel electrophoresis of PCR products for determination of repeat length in the *DAT* VNTR. A band at 410bp described the 9-repeats allele and a band at 450 bp the 10-repeat carriers.

4.6 Metabolic, hormonal, and inflammatory blood parameters

Serum samples were used to characterize diet groups for the metabolic parameters glucose, HbA1c, insulin, leptin, triglycerides, cholesterol, LDL and HDL and the inflammation marker C-reactive protein (CRP) using a highly sensitive measurement (hsCRP). To obtain serum samples, blood was drawn using monovettes with clot activator (9 ml S-Monovette, SARSTEDT AG & Co., Nümbrecht, Germany). The sample was kept for 30 min at room temperature in an upright position, centrifuged with 3500 rpm for 10 min at 15 °C, and the supernatant (= serum) was separated from clotted blood cells and stored at -80 °C. The samples were sent to the laboratory of the University of Leipzig Medical Center for the measurements. Insulin resistance was calculated using the homeostasis model assessment (HOMA-IR):

$$\text{HOMA-IR} = \frac{\text{insulin [µU/ml] x fasted glucose [mmol/l]}}{22.5}$$

To fit the prerequisites of the formula, insulin values were converted from pmol/l to µU/ml using the factor 6. i.e. 1 µU/mL = 6 pmol/l (Knopp, Holder-Pearson, & Chase, 2019) and normal cut-off are based on laboratory recommendations (LaborDiagnostik Karlsruhe). Values for hsCRP and leptin that were below the detection threshold (< 0.15 and < 0.2, respectively) were set as the threshold value for statistical analyses.

4.7 Neuropsychological tests of executive functioning

Three well established neuropsychological tests (NP tests) were administered at the end of the first test day in the same order as they are described below.

4.7.1 Trail making test (TMT)

The TMT (Reitan & Wolfson, 1993) consists of two parts that are administered sequentially on two different paper sheets. In TMT A, which mainly captures visual attention and processing speed, encircled numbers from 1 to 25 need to be connected using a pen in ascending numerical order. The TMT B is a more direct measurement of executive abilities and adds information about set shifting and

cognitive/mental flexibility, as a series of numbers and letters needs to be connected alternating between ascending numerical and alphabetical order (Fig. 11). Both tests are to be completed as quickly as possible and the time in seconds (s) to complete each part, measured with the stopwatch function of the examiner’s smartphone, was used as the outcome variable. Group differences were tested for TMT A and TMT B, but as TMT B is more relevant in the context of executive functioning, further analyses were performed with TMT B only.

SAMPLE
Trail Making Test Part B

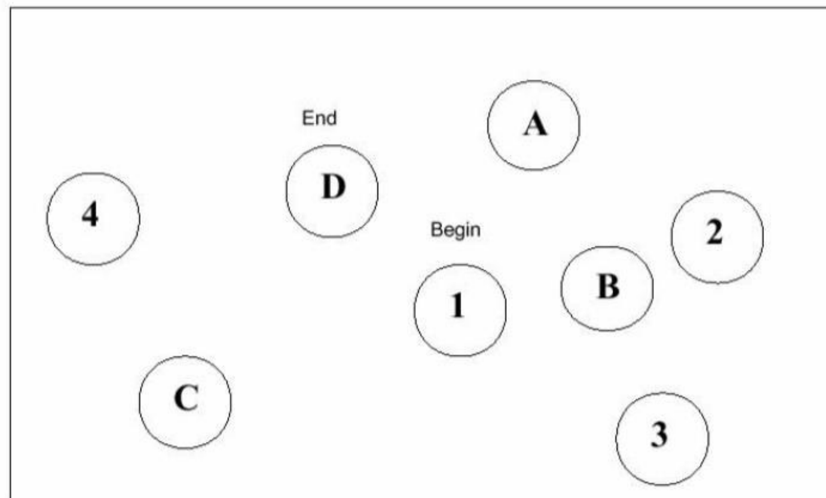


Figure 11: Trail making test part B. The figure depicts the introduction example of TMT B that was also provided on the front side of the test sheets for illustration and explanation purposes. The actual test is printed on the backside of the paper sheet and the test starts with turning the paper sheet.

4.7.2 Digit symbol substitution test (DSST)

The DSST is for example part of the Wechsler’s Adult Intelligence Scale WAIS-IV (Petermann & Wechsler, 2014) and can be administered as a pen and paper test. Executive abilities assigned to the DSST are sustained attention, processing speed and to a lesser degree working memory and associative abilities. The task requires to match abstract symbols to numbers according to a key grid given on top of the page (Fig. 12). Participants copied as many symbols as possible according to a provided key into a space below the corresponding numbers in reading direction within 120s stopped by the examiner. The number of correctly transferred symbols within 120s is the outcome score.

4.7.3 Digit span test (DS)

The DS is a working memory test and another subtest of Wechsler’s Adult Intelligence Scale (Petermann & Wechsler, 2014). The test comprises two subscales, the forward and the backward DS. Forward DS tests general short-term memory capacity and attention efficiency. The DS backwards is an actual working memory task as it requires manipulation of the information on hold of the short-term memory (Fink et al., 2014). For this study, the test was implemented in an Excel worksheet with

Microsoft Office 2016 (Fig. 13) on a Lenovo ThinkPad L570. When the participant indicated to be ready, the examiner pressed the "play"-button in the worksheet and the uppermost line of digits was read aloud by a computer voice with a speed of one digit per second. The participants were asked to repeat the digits they heard. In the forward DS test, the repeating order needs to be the same as the heard order, in the backward DS the heard digits needed to be recalled in reversed order. Each level consists of two trials and the number of digits to remember increases with every level, starting with three digits in the forward and two digits in the backward condition. To successfully complete a level, at least one trial needs to be reported correctly and the task ends, when the participant either reports both trials incorrectly or when the last level (9 digits in forward and 8 digits in backward condition) is successfully achieved. The number of completed levels forward and backward and a sum of both were the outcome variables in this test. Group differences were tested for DS test forward, backward and the total score, but as DS backward better reflects working memory (Diamond, 2013), further analyses were performed with DS backwards only.

Schlüssel:
1 2 3 4 5 6 7 8 9

Beispiel Übung
6 8 3 9 5 4 1 7 2 1 4 8 2 7 6 9 3 5
8 3 1 9 2 5 6 4 3 7 2 9 8 1 4 7 6 5
9 1 2 4 7 2 5 6 9 5 8 6 4 3 1 7 8 3
1 3 9 6 3 9 7 5 1 4 2 8 7 2 8 5 6 4
7 6 4 1 3 2 8 1 7 9 2 5 3 4 8 6 5 9
8 1 9 5 1 4 2 6 9 8 7 3 5 6 4 7 2 3
3 6 8 9 1 8 4 7 5 2 9 6 7 1 5 2 3 4
6 4 1 9 5 7 3 6 8 3 2 7 5 8 4 2 9 1

Figure 12: Digit symbol substitution test. The test is printed on a paper sheet as it is depicted in this figure. On top of the page a key ("Schlüssel") of numbers and corresponding symbols is given. According to this key, the symbols need to be transferred into the empty spaces below the numbers in reading direction. The first three numbers are an example ("Beispiel") for explanation of the task and the following six numbers are a practice ("Übung") for the examinee. When example and practice are finished, the task starts on the examiner's cue.

Level	Digit span forward (0/1)							Digits	Items
3	5	8	2					0	0
	6	9	4						0
4	6	4	3	9				0	0
	7	2	8	6					0
5	4	2	7	3	1			0	0
	7	5	8	3	6				0
6	6	1	9	4	7	3		0	0
	3	9	2	4	8	7			0
7	5	9	1	7	4	2	8	0	0
	4	1	7	9	3	8	6		0

Figure 13: Digit span test forward. The figure shows the Excel worksheet for the DS forward. On the left, the levels are indicated, starting with level 3 representing three digits. Each level consists of two trials. On cue, the examiner pressed the play button and a computer voice started reading out the digits of the respective trial. After each trial, the examiner ticked off the correct or incorrect responses and either started the next trial or, if two trials in one level were not correct, stopped the task.

4.8 Questionnaires

To characterize the diet groups in terms of eating behavior and personality, German versions of the following questionnaires were filled out by all participants using the online survey app LimeSurvey (LimeSurvey GmbH, Hamburg, Germany). Control of eating/restrained eating, emotional or situational perturbance of eating behavior and sensations of hunger were assessed with the Three-Factor Eating Questionnaire (FEV) (Pudel & Westenhöfer, 1989). The Yale Food Addiction Scale (YFAS) (Meule, Vögele, & Kübler, 2012) was used to test if participants had symptoms of food addiction and the Food Craving Questionnaire-Trait (FCQ-T) (Meule, Lutz, Vögele, & Kübler, 2012) measured feelings of craving for food. Basic personality traits, i.e. neuroticism, extraversion, openness, conscientiousness and agreeableness, were quantified with the NEO-Five Factor Inventory (NEO-FFI) (Körner et al., 2008) and the Behavioral approach and avoidance system questionnaire (BIS/BAS) (Strobel, Beauducel, Debener, & Brocke, 2001) was used to test for potential differences in inhibition and approach behavior. Impulsivity was evaluated with the Barrat Impulsiveness Scale (BIS 15) (Spinella, 2007) and with the Urgency, Premeditation, Perseverance, Sensation Seeking (UPPS) Impulsive Behavior Scale (Schmidt, Gay, d'Acremont, & van der Linden, 2008). The latter specifies four aspects of impulsive behavior in more detail, i.e. urgency (acting rashly under negative emotions), lack of premeditation (acting without overthinking the consequences), lack of perseverance (inability to stay focused on a task), and sensation seeking (seeking novel and exciting experiences).

4.9 Physical activity

Potential differences in physical activity were quantified using a pedometer (YAMASA Power-Walker lite PZ-270, YAMASA Tokei Keiki CO., LTD., Tokyo, Japan) and the International Physical Activity Questionnaire (IPAQ) (Lee, Macfarlane, Lam, & Stewart, 2011). The IPAQ assesses physical activity on three sub scales, i.e. walking, moderate activity (e.g. carrying a light weight, normal biking, swimming)

and vigorous activity (e.g. aerobic, running, quick swimming). A total score can be calculated in MET (metabolic equivalent of task)-minutes per week (Fan, Lyu, & He, 2014):

Total MET-min per week = Met-min Walking + Met-min Moderate + Met-min Vigorous

Met-min Walking = 3.3 * average minutes of walking per day * days with walking per week
Met-min Moderate = 4.0 * average minutes of moderate activity per day * days with moderate activity per week
Met-min Vigorous = 8.0 * average minutes of vigorous activity per day * days with vigorous activity per week

Step count was measured with the pedometer during everyday activities for seven continuous days after completion of the second test day. Participants were advised not to wear the pedometer during activities that could damage the pedometer, for example during activities in water or team sports with potential body contact. 37 participants completed the pedometer measurement in the HFS group and 30 in the LFS group as 4 participants in each group did not return the pedometer. The outcome measure is the average step count over 7 days or over the maximal number of values available.

4.10 Statistical analyses

A statistical analyses plan was preregistered as an OSF preregistration (https://osf.io/e3w8k). Statistical analyses were performed within R version 3.5.3 using R studio and the packages *Rcmdr* (R Commander), *stats*, *car, pastecs* and *emmeans.*

Descriptive statistics such as mean, standard deviation and minimal/maximal value for all measurements were computed with the *stat.desc* function. All data was tested for parametric assumptions of normality with the *Shapiro-Wilk test* and for equality of variance with *Levene's test*. Reciprocal or logarithmic transformations (natural logarithm) were applied if these assumptions were not met and if there was no non-parametric test alternative (i.e. *Welch's t-test* for data with unequal variances and *Two-samples Wilcoxon rank-sum test* for not normally distributed data).

Matching of HFS and LFS group for age, BMI, WHR, weight and IQ was confirmed with *independent-samples t-tests* or the non-parametric alternatives where appropriate.

Gene expression data was checked for outliers outside of the 2.2x interquartile range and outliers were removed from statistical analyses, as technical issues for these values cannot be excluded and therefore extreme values might not reflect true expression levels. Diet group differences in mRNA levels and in neuropsychological test performance were analyzed with *independent samples t-tests* or non-parametric alternatives where appropriate.

Associations between genotype and dietary preferences were tested with *Pearson's* χ^2*-test*. *Two-way analyses of variance* (*ANOVAs*) type "III" were used to analyze interacting effects of genotype and diet on gene expression and cognitive performance with the interaction term *diet x genotype* as predictor variable. If appropriate, post hoc tests were performed with the functions *lsmeans* and *contrast* to compare particular subgroups. *One-way ANOVAS* were used to explore effects of genotype in *FTO* SNPs on BMI.

Diet group differences in metabolic, hormonal, and inflammatory blood parameters were tested using descriptive statistics and *independent-samples t-tests* or *Wilcoxon rank-sum test*. To explore a potential involvement of metabolic or inflammatory state in mRNA expression regulation, correlations were tested between metabolic parameters (glucose, insulin, HOMA-IR, leptin, triglycerides, cholesterol, hsCRP) and mRNA expression levels of *COMT, DAT* and *DRD3*. Results are reported as *Pearson's r* for parametric data and *Spearman's rho* as non-parametric alternative.

The aim of this study was to assess dietary impact on the DA system independent of obesity. Therefore, to account for potential confounding effects of obesity on mRNA expression levels and to replicate previous findings of obesity-associated effects on DA-related gene expression, correlation

analyses were performed for BMI/WHR and mRNA expression levels of all genes of interest within the whole sample regardless of diet group. Correlations are reported as *Pearson's r* for parametric data and *Spearman's rho* as non-parametric alternative. For those pairs of WHR or BMI and mRNA expression where the correlation at least pointed towards a trend, the covariate WHR or BMI was added to an *analysis of covariance (ANCOVA)* to demonstrate that potential diet group differences in mRNA levels are not driven by the obesity measures.

Obtained measures of physical activity, the IPAQ MET minutes score and the average step count, were correlated in a linear regression to assess their consistency. Both measurements were tested for diet group differences with a *Wilcoxon rank-sum test* and an *independent samples t-test*, respectively. Effects of *DRD2*-related SNPs on the physical activity were analyzed in *one-way ANOVAs* with *genotype* as predicting variable and IPAQ score or step count as outcome.

To correct for potential inconsistencies in the participants' dietary reports, an estimate of DFS score reliability was added as a weighting variable to weighted *diet - mRNA* and *diet - NP test* ANOVAs. The reliability estimate is based on the difference in DFS scores between screening day and second test day. This "DFS difference" score was transformed to $\frac{1}{e^{|DFS\ difference|}}$. Transforming was mathematically necessary, as several participants scored equal on both test days and therefore would have a reliability estimate of 0, which cannot be used as a weighting variable. By using the e-function, small deviations in DFS scores are weighted less heavily than strong deviations.

Questionnaires assessing eating behavior and personality traits were analyzed using descriptive statistics and *independent-samples t-tests* or non-parametric alternatives where appropriate.

The significance level alpha was defined as 0.05 unless stated differently when correcting for multiple comparisons using the Bonferroni correction. Results with a p-value above the significance level but below 0.1 are regarded and discussed as potential trends. Effect sizes are reported as *Pearson's r* for the main analyses. Plots were created within the packages *ggplot2* and *ggpubr* except for the overview plots for group differences in neuropsychological tests (Fig. 15) that were plotted with GraphPad Prism version 7.

5. Results

5.1 Characteristics of study sample

Included participants were between 18 and 37 years old, had a BMI range from 18.5 to 36.4 kg/m², and a WHR between 0.65 and 0.97. For a complete demographical overview see Table 5. The groups were matched for age, BMI, WHR, weight, and additionally for IQ to remove effects of differences in general intelligence on cognitive performance (all p-values for group differences ≥ 0.31). DFS scores for the LFS group were between 33 and 52 (mean = 44 ± 4) and for the HFS group between 62 to 97 (mean = 71 ± 9).

Table 5: Demographical overview of study population. The table shows the descriptive statistics (minimal and maximal value, mean, and standard deviation) for age, BMI, WHR, weight, IQ score, and DFS score. The groups did not differ significantly in any of the demographical parameters as indicated by statistical analyses of group differences (all p ≥ 0.31). Tests used: independent-samples t-test for age, Welch's t-test for WHR, Two-sample Wilcoxon rank-sum test for BMI, weight, and IQ score. Abbreviations: Min: minimal value, Max: maximal values, SD: standard deviation, r: effect size (Pearson's r), BMI: body mass index, WHR: waist- to-hip-ratio, IQ: intelligence quotient, DFS: Dietary Fat and free Sugar – Short Questionnaire, n.a.: not applicable.

	HFS (n = 41)				LFS (n = 34)				Group difference	
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**	**Test statistics**	**p-value**
Age [years]	20	37	26.93	4.28	18	36	26.88	4.54	t(73) = 0.04	0.97
BMI [kg/m²]	18.62	36.42	23.78	2.94	18.46	29.96	24.16	2.74	W = 609	0.35
WHR	0.72	0.92	0.80	0.04	0.65	0.97	0.81	0.06	t(50.91) = -1.02	0.31
Weight [kg]	58.00	118.00	77.88	10.47	58.50	110.00	79.39	13.50	W = 649	0.88
IQ score	91	118	108.22	8.10	96	118	110.21	5.80	W = 672	0.79
DFS score	62	97	70.78	8.74	33	52	43.82	4.39	n.a.	n.a.

5.2 Main hypotheses: diet-associated differences in mRNA expression and cognitive performance

5.2.1 HFS diet-associated differences in mRNA expression of dopamine-related genes

One main research question of this project was if there are differences in mRNA expression patterns of genes within the DA pathway between diet groups. After correcting for multiple comparisons, results from quantitative PCR (Fig. 14) indicate no significant differences between diet groups in the mRNA expression of *DARPP-32* (W = 539, p = 0.25, r = - 0.15), *DRD2* (W = 225, p = 0.20, r = -0.19), and *DRD5* (W = 492, p = 0.27, r = -0.15) but potential trends for a group difference in the mRNA expression of *DRD3* (t(49.48) = -2.59, p = 0.013, r = 0.35), *DAT* (t(63) = 1.96, p = 0.054, r = 0.24), and *COMT* (t(96) = -1.81, p = 0.07, r = 0.21).

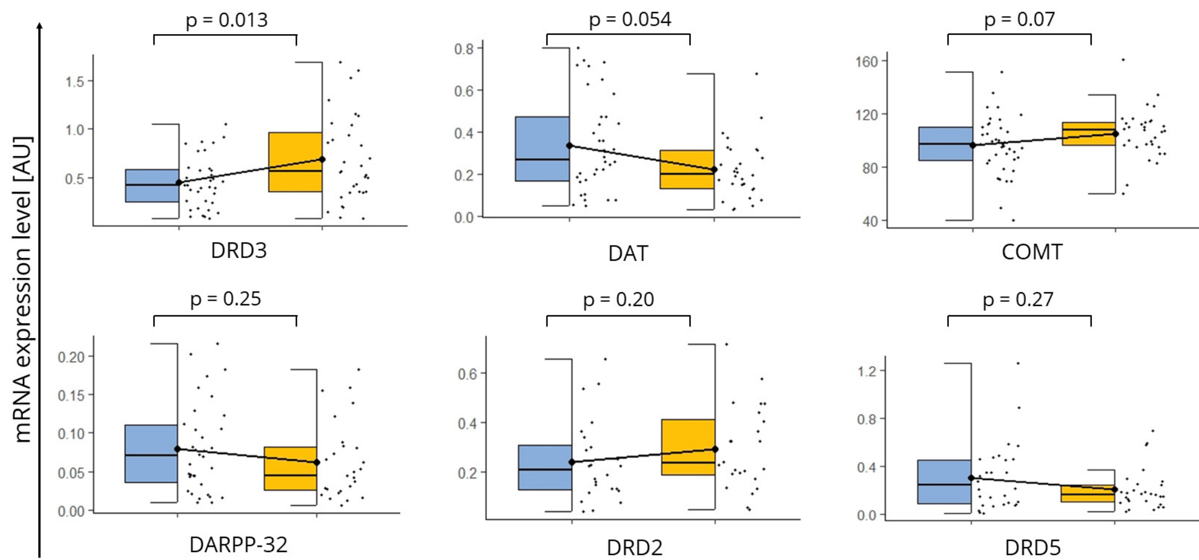


Figure 14: Peripheral mRNA expression of DA-related genes. After correction for multiple comparisons, there were no significant diet group differences in the mRNA expression of *DARPP-32* (W = 539, p = 0.25, r = - 0.15), *DRD2* (W = 225, p = 0.20, r = -0.19), and *DRD5* (W = 492, p = 0.27, r = -0.15) but trends potentially indicating group differences in *DRD3* (t(49.48) = -2.59, p = 0.013, r = 0.35), *DAT* (t(63) = 1.96, p = 0.054, r = 0.24), and *COMT* (t(96) = -1.81, p = 0.07, r = 0.21) mRNA expression in leukocytes. Symbols: the vertical bars in each box represent the median, the connected points indicate the statistical mean, the boxes show the interquartile range from first to third quartile, and the whiskers indicate the minimum and maximum of data points up to 2.2x IQR. Tests used: Welch's t-test for *DRD3,* independent-samples t-test for *COMT*, and *DAT* (after logarithmic transformation), Wilcoxon rank-sum test for *DARPP-32, DRD2* and *DRD5*. Abbreviations: AU: arbitrary units, COMT: catechol-O-methyltransferase, DARPP-32: dopamine-and-cAMP-regulated neuronal phosphoprotein 32, DAT: dopamine transporter, DRD: dopamine receptor, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, mRNA: messenger ribonucleic acid.

5.2.2 No diet group differences in cognitive performance

The second hypothesis of this study was to that HFS diet is associated with differences in DA-related cognitive performance. Therefore, participants completed three neuropsychological tests of executive functioning, but performance in all tests was not significantly different between groups (TMT A: t(73) = - 0.06, p = 0.95, r = 0.01; TMT B: W = 748, p = 0.59, r = - 0.06; DSST: W = 768, p = 0.45, r = - 0.09; DS forward: W = 631, p = 0.46, r = - 0.08; DS backward: W = 777, p = 0.38, r = - 0.10; DS total score: t(73) = 0.28, p = 0.78, r = 0.03; see Fig. 15).

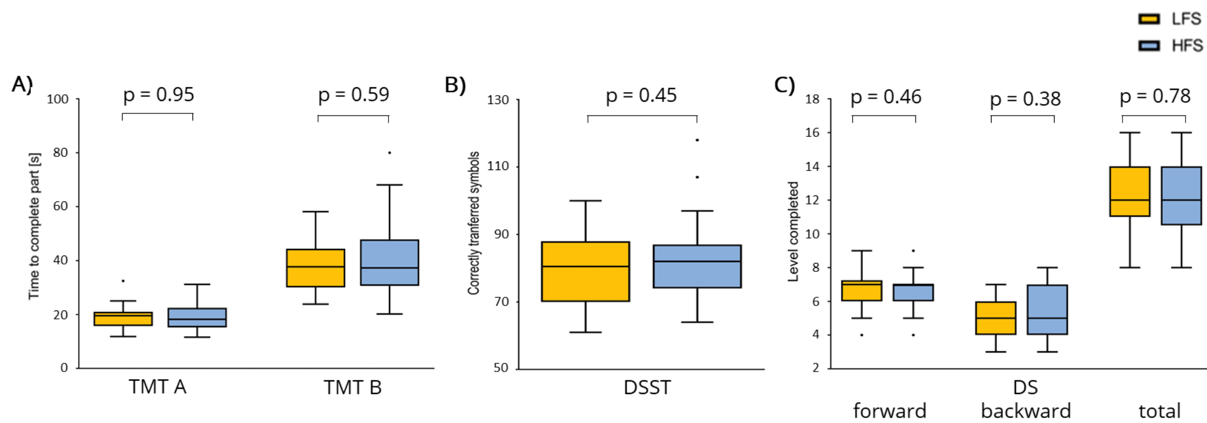


Figure 15: Performance in neuropsychological tests. Neuropsychological test performance did not differ significantly between diet groups. <u>**A)**</u> TMT A on the left (t(73) = - 0.06, p = 0.95, r = 0.01); TMT B on the right (W = 748, p = 0.59, r = - 0.06). <u>**B)**</u> DSST (W = 768, p = 0.45, r = - 0.09). <u>**C)**</u> DS forward on the left (W = 631, p = 0.46, r = - 0.08), DS backward in the middle (W = 777, p = 0.38, r = - 0.10); DS total score on the right (t(73) = 0.28, p = 0.78, r = 0.03). <u>Tests used:</u> independent-samples t-test for TMT A and DS total, Wilcoxon rank-sum test for TMT B, DSST, DS forward and backward. <u>Symbols:</u> the vertical bars in each box represent the median, the boxes show the interquartile range from first to third quartile, and the whiskers are drawn with the Tukey method. <u>Abbreviations:</u> DSST: Digit symbol substitution test, DS: Digit span test, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, s: seconds, TMT: Trail making test.

5.2.3 Mediation model: link between diet, gene expression and cognitive performance cannot be established

A mediation model was preregistered in the analyses plan for this study (see OSF preregistration: https://osf.io/e3w8k) to explore if potential diet-associated differences in cognitive performance are mediated by gene expression (see Fig. 16). However, the mediation analysis was not carried out since there were no significant diet group differences in any of the neuropsychological test performances (reflected in the c-path).

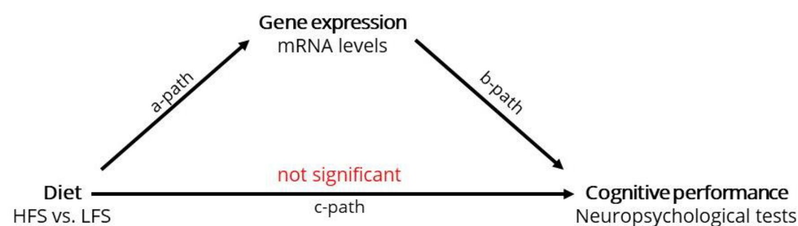


Figure 16: Mediation model of dietary effects on cognitive performance mediated by gene expression. A mediation model linking diet-associated differences in cognitive performance (c-path) with mRNA expression levels of DA-related genes (a-path and b-path) was not analyzed as the c-path reflecting the main effect was not significant.

5.3 Exploratory analysis: mRNA expression of uncoupling proteins *UCP-2* and *UCP-3*

Literature suggested that UCPs might play a role in central dopaminergic signal transmission and metabolic regulation. To see if any dietary effects in DA-related gene expression and cognition are accompanied by changes in the expression of *UCP*s, the mRNA expression levels of *UCP-2* and *UCP-3* were analysed exploratively for differences between diet groups. The results from the qPCR indicated

that *UCP-2* is expressed significantly higher in the HFS group (t(68) = 3.39, p = 0.0006, r = 0.38), whereas *UCP-3* mRNA levels are significantly lower in the HFS group compared to the LFS group (W = 339, p = 0.009, r = -0.32). Fig. 17 shows the data graphically.

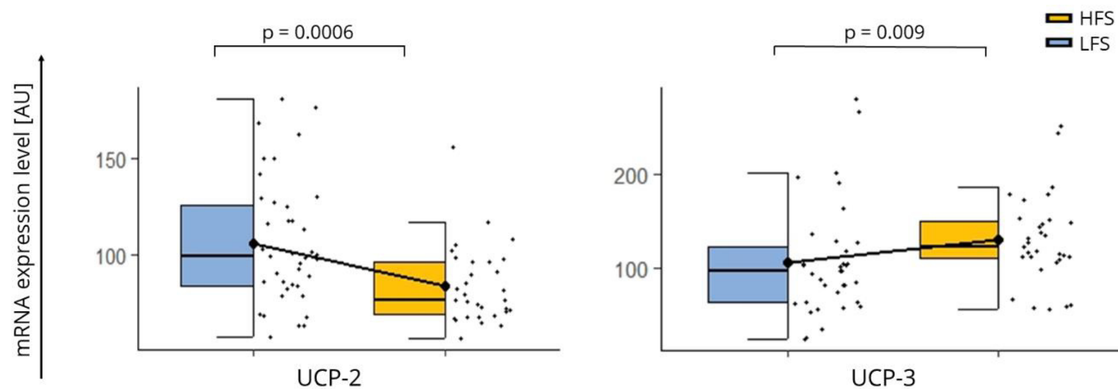


Figure 17: Gene expression of *UCP-2* and *UCP-3*. In the HFS group the mRNA expression levels of *UCP-2* were significantly higher (t(68) = 3.39, p = 0.0006, r = 0.38) and levels of *UCP-3* were significantly lower (W = 339, p = 0.009, r = -0.32) compared to the LFS group. Symbols: the vertical bars in each box represent the median, the connected points indicate the statistical mean, the boxes show the interquartile range from first to third quartile, and the whiskers indicate the minimum and maximum of data points up to 2.2x IQR. Tests used: independent-samples t-test for *UCP-2* and Wilcoxon rank-sum test for *UCP-3*. Abbreviations: AU: arbitrary units, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, mRNA: messenger ribonucleic acid, UCP: uncoupling protein.

5.4 Impact of genetic variability (SNPs)

5.4.1 Genotype in DA-related SNPs is not associated with high fat and sugar consumption

Genotype in DA-related SNPs might modify baseline dopaminergic signal transmission (see section 2.5.2) which could interfere with potential dietary effects. Therefore, in a first exploratory approach Pearson's χ^2-test was used to analyze if HFS or LFS diet consumption co-occurs with a particular SNP genotype in DA-related genes. The p-values of all χ^2-tests were > 0.1 indicating no significant association between SNP genotype and high or low fat and sugar consumption (Fig. 18).

COMT Val/Met rs4680		Allele group			Sum
		0	1	2	
Diet group	HFS	13	16	12	41
	LFS	12	11	11	34
	Sum	25	27	23	75
Pearson's χ2-test: p = 0.84					

DAT VNTR rs28363170		Allele group		Sum
		1	2	
Diet group	HFS	23	18	41
	LFS	16	18	34
	Sum	39	36	75
Pearson's χ2-test: p = 0.44				

DRD2 C957T rs6277		Allele group			Sum
		0	1	2	
Diet group	HFS	2	15	24	41
	LFS	3	5	26	34
	Sum	5	20	50	75
Pearson's χ2-test: p = 0.93					

DRD2 Taq1A rs1800497		Allele group		Sum
		1	2	
Diet group	HFS	17	24	41
	LFS	8	26	34
	Sum	25	50	75
Pearson's χ2-test: p = 0.101				

FTO rs8050136		Allele group			Sum
		0	1	2	
Diet group	HFS	9	22	10	41
	LFS	6	20	8	34
	Sum	15	42	18	75
Pearson's χ2-test: p = 0.88					

DARPP-32 rs907094		Allele group		Sum
		1	2	
Diet group	HFS	18	23	41
	LFS	20	14	34
	Sum	38	37	75
Pearson's χ2-test: p = 0.20				

FTO rs8053740		Allele group			Sum
		0	1	2	
Diet group	HFS	8	20	13	41
	LFS	7	16	11	34
	Sum	15	36	24	75
Pearson's χ2-test: p = 0.99					

Figure 18: *Diet x genotype* frequency tables and Pearson's χ^2-test. Frequency tables for each SNP of interest show the number of participants per *diet x genotype* subgroup. Pearson's χ^2-test indicated no co-occurrence of a alleles in any SNP with dietary intake of HFS or LFS diet, as all p-values were > 0.1. Abbreviations: COMT: catechol-O-methyltransferase, DARPP-32: dopamine-and-cAMP-regulated neuronal phosphoprotein 32, DAT: dopamine transporter, DRD: dopamine receptor, FTO: fat mass and obesity-associated protein, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, Met: methionine, Val: valine, VNTR: variable number tandem repeat.

5.4.2 Impact of SNP genotype on gene expression and cognitive performance, and interactions of genotype with diet

Two-way ANOVAs were used to test interactions between SNP genotypes and diet regarding gene expression and cognitive performance, as an interaction might drive or mask potential dietary influences. A complete overview of all ANOVA results can be found in Tables S21, S22, S23, and S24 (section 9.1.6).

Regarding gene expression, there was a trend level interaction between diet and genotype in the *FTO* SNP rs8050136 affecting the mRNA expression of *DRD2* ($F(2, 42) = 2.65$, $p = 0.08$). Apart from that there were no significant interactions between diet and any other genotype affecting the corresponding mRNA expression level ($p \geq 0.13$ for all interaction terms). The tested pairs were: rs4680 (Val/Met polymorphism) and *COMT* expression, rs28363170 (VNTR) and *DAT* expression, rs6277

(C957T polymorphism), rs1800497 (Taq1A polymorphism), rs8050136 (in *FTO*), and rs8053740 (in *FTO*) and *DRD2* expression, and rs907094 and *DARPP-32* expression. Regarding cognition, an interaction between genotype in the *DRD2* SNP rs6277 and dietary style significantly affected the performance of all three neuropsychological tests (TMT B: $F(2, 69) = 3.67$, $p = 0.03$; DSST: $F(2, 69) = 6.02$, $p = 0.004$; DS backwards: $F(2, 69) = 3.14$, $p = 0.049$). Post-hoc testing (Fig. 19) indicates that HFS dieters performed significantly worse than LFS dieters in the TMT B (Fig. 19A) only within the group of T/C carriers (allele group 1; $t(69) = 2.44$, $p = 0.02$). Within the groups of T/T-carriers (allele group 0) and C/C-carriers (allele group 2) the diet group differences were not significant (T/T: $t(69) = - 1.01$, $p = 0.27$; C/C: $t(69) = - 0.67$, $p = 0.45$). A significant dietary effect on DSST performance (Fig. 19B) is present only for C/C-carriers, where HFS dieters performed significantly better ($t(69) = - 3.12$, $p = 0.003$). Within the homozygous and heterozygous group of T-carriers there was no significant difference in performance between diet groups (T/T: $t(69) = - 0.71$, $p = 0.48$; T/C: $t(69) = 1.64$, $p = 0.11$). Performance in DS backwards (Fig. 19C) was significantly affected by diet only within the group of T/T-carriers, with HFS dieters performing better than LFS dieters ($t(69) = - 2.30$, $p = 0.02$). For heterozygous and homozygous carriers of the C-allele diet had no significant effect (T/C: $t (69) = 1.11$, $p = 0.27$; C/C: $t(69) = - 1.04$, $p = 0.30$).

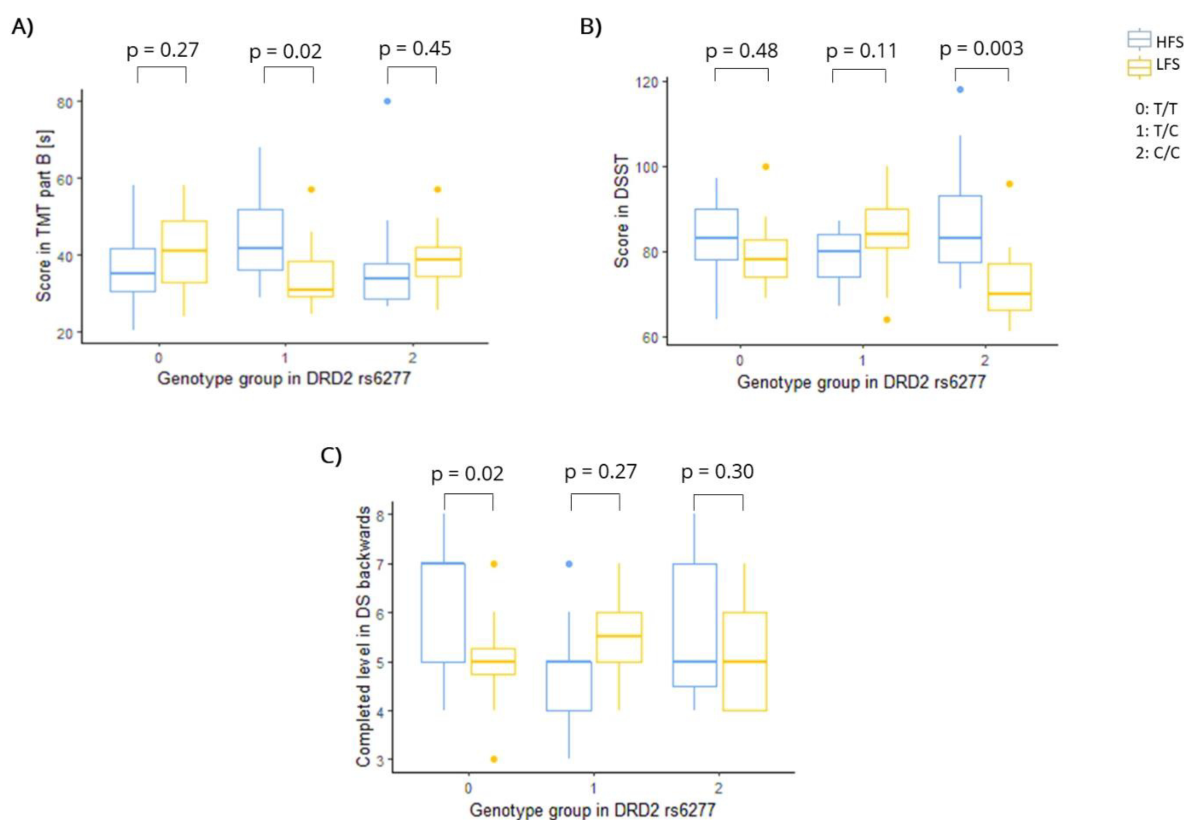


Figure 19: Interaction between genotype in rs6277 (*DRD2)* and diet affecting neuropsychological test performance. Within genotype subgroups of *DRD2* SNP rs6277 there were significant dietary effects on performance of the neuropsychological tests. **A)** A significant effect of diet on performance of the TMT B can be observed only in the heterozygous genotype group 1 reflecting T/C-carriers ($p = 0.02$). **B)** A significant dietary effect on DSST performance is present only in allele group 2 reflecting C/C-carriers ($p = 0.003$). **C)** A dietary effect on performance in the DS backwards is significant only in allele group 0 reflecting T/T-carriers ($p = 0.02$). Tests used: two-way ANOVAs followed by post hoc pair-wise comparisons within subgroups. Symbols: the vertical bars in each box represent the median, the boxes show the interquartile range from first to third quartile, and the whiskers indicate the minimum and maximum of data points up to 1.5x IQR. Abbreviations: DS: Digit span test, DSST: Digit symbol substitution test, DRD: dopamine receptor, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, TMT B: Trail making test part B.

Additionally, significant main effects of genotype or diet arising from the *diet x genotype* ANOVAs are reported in the following paragraph, as they might provide interesting hints for future research, even though this study was not designed to analyze the influence of SNP genotypes alone (sample size is putatively too low).

Diet had a significant main effect on *DAT* mRNA expression ($F(1, 61) = 4.07$, $p = 0.048$) and there is a potential trend for a dietary effect on *DRD2* mRNA expression ($F(1, 44) = 3.03$, $p = 0.09$) when including the genotypes (*DAT* VNTR length and genotype in the Taq1A SNP, respectively) in the ANOVA models. Further, there were significant genotype effects on performance in the Trail making test B independent of diet (Fig. 20, see also Table S22 in section 9.1.6). In detail, homozygous carriers of the 10-repeats variant (genotype group 2) in the *DAT* VNTR (Fig. 20A) performed significantly worse than homozygous and heterozygous carriers of the 9-repeats variant (genotype group 1; $F(1, 71) = 6.37$, $p = 0.014$) independent of diet (*diet x genotype* interaction: $F(1, 71) = 1.71$, $p = 0.20$). Additionally, genotype in *FTO* SNP rs8050136 had a significant effect on TMT B performance ($F(2,69) = 4.61$, $p = 0.013$). Post-hoc contrasts (Fig. 20B) indicate that C/C-carriers (allele group 2) performed significantly worse than A/C-carriers (group 1; $t(69) = 2.64$, $p = 0.01$) and there might be a similar trend between A/A-carriers (group 0) and A/C-carriers ($t(69) = 1.95$, $p = 0.06$), but A/A-carriers and C/C-carriers did not perform significantly different ($t(69) = -0.43$, $p = 0.66$). Finally, the Val/Met polymorphism in the *COMT* gene might also influence TMT B performance, but this effect was not statistically significant ($F(2, 69) = 2.84$, $p = 0.07$).

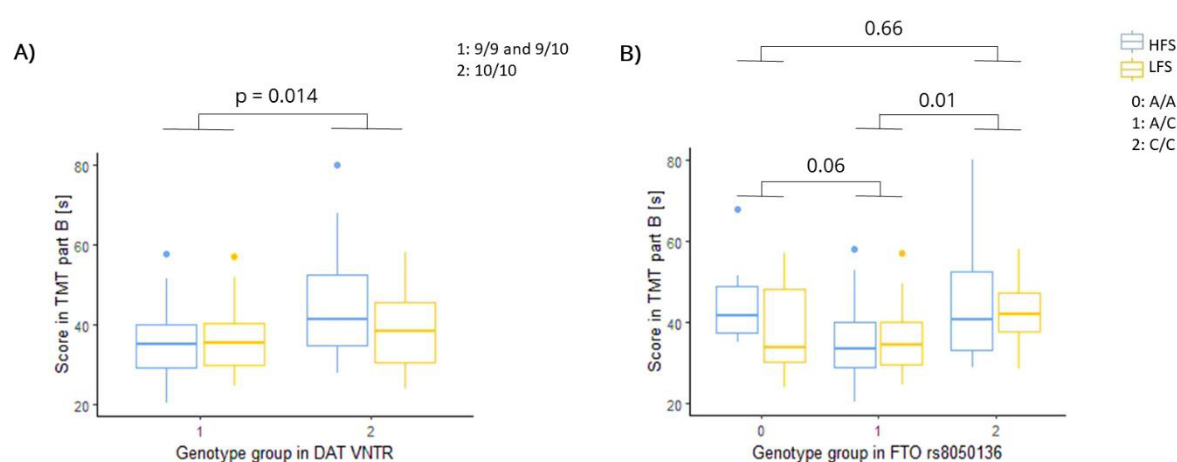


Figure 20: Effects of genotypes on performance of the Trail making test part B. **A)** Homozygous carriers of the 10-repeats variant in the *DAT* VNTR (genotype group 2) needed on average longer to complete the TMT than carriers of the 9-repeats variant (genotype group 1) and therefore had poorer performance in the TMT B (p = 0.014). **B)** C/C-carriers in the *FTO* SNP rs8050136 (allele group 2) performed significantly worse than A/C-carriers (allele group 1; p = 0.01) and A/A-carriers (allele group 0) also showed a trend to perform worse than the heterozygous A/C-carriers (p = 0.06), which was however not statistically significant. The allele group 0 and 2 did not performed significantly different (p = 0.66). Tests used: two-way ANOVA followed by post hoc pair-wise comparisons within subgroups. Symbols: the vertical bars in each box represent the median, the connected points indicate the statistical mean, the boxes show the interquartile range from first to third quartile, and the whiskers indicate the minimum and maximum of data points up to 1.5x IQR. Abbreviations: DAT: dopamine transporter, FTO: fat mass and obesity-associated protein, HFS: high fat and sugar diet group; LFS: low fat and sugar diet group, s: seconds TMT B: Trail making test part B, VNTR: variable number tandem repeat.

5.4.3 Exploratory replication analysis: no significant association between *FTO* SNPs and BMI

The SNPs in *FTO* originally gained attention due to their association with obesity and the predisposing effect of certain risk alleles for a higher BMI, i.e. the A allele in rs9939609 (proxy: rs8050136) and the

C allele in rs8053740 (Frayling et al., 2007; Tönjes et al., 2010). Therefore, one-way ANOVAs were performed to explore if this effect is replicable in this study, but the results indicate that there was no significant association between genotype in either SNP of *FTO* and BMI in the present sample (rs8050136: $F(2, 72) = 2.23$, $p = 0.12$; rs8053740: $F(2, 72) = 0.53$, $p = 0.56$). Fig. 21 shows the BMI distribution within *diet x genotype* groups for both SNPs. Including diet into the model as a second input variable has no relevant effect on this result (*diet x genotype* interaction for rs8050136: $F(2, 69) = 0.76$, $p = 0.47$; *diet x genotype* interaction for rs8053740: $F(2, 69) = 0.69$, $p = 0.51$).

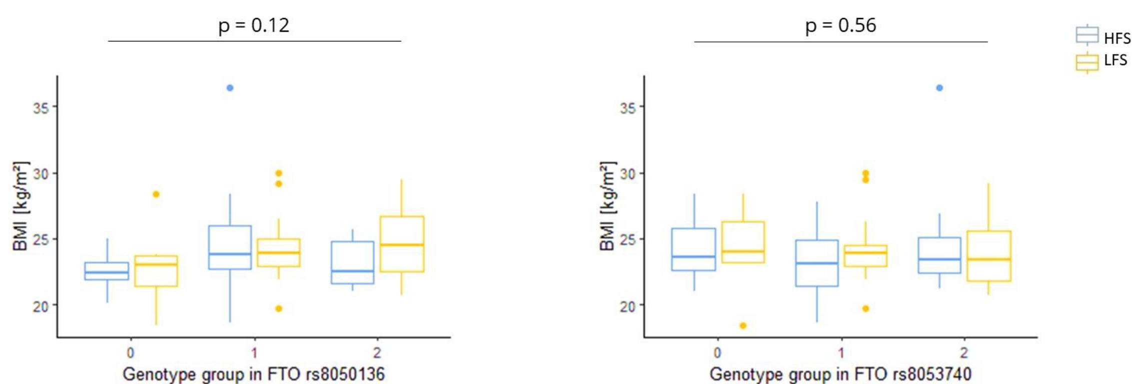


Figure 21: No effects of *FTO* SNPs on BMI. Genotype in *FTO*'s SNP rs8050136 as a proxy for rs9939609 and rs8053740 had no significant effects on BMI (p = 0.12 and p = 0.59, respectively). Test used: one-way ANOVA. Symbols: the vertical bars in each box represent the median, the boxes show the interquartile range from first to third quartile, and the whiskers indicate the minimum and maximum of data points up to 1.5x IQR. Abbreviations: BMI: body mass index; FTO: fat mass and obesity-associated protein; HFS: high fat and sugar diet group; LFS: low fat and sugar diet group.

5.5 Control analyses

5.5.1 Obesity measures: trends for diet-associated differences in mRNA expression are not affected by BMI and WHR

Diet groups were matched for the most relevant potential confounders regarding obesity, i.e. BMI and WHR, as mean, standard deviation and range were similar in both diet groups and there were no diet group differences in BMI and WHR (see section 5.1). Therefore, BMI or WHR should not influence observed diet group differences in mRNA expression. However, to ensure the exclusion of unexpected confounding effects of obesity and to replicate general effects of obesity on DA-related peripheral gene expression, correlation analyses were performed between BMI/WHR and mRNA expression levels of all genes of interest (Table 6). After correcting for multiple comparisons, the results indicate a significant diet-independent negative correlation between WHR and the mRNA expression of *DAT* ($r = -0.28$; $p = 0.004$) and potential trends for negative correlations between BMI and *DAT* expression ($r = -0.26$; $p = 0.04$) and BMI and *DRD3* expression ($r = -0.24$; $p = 0.053$) with similar directions of effects in both the HFS and LFS diet group (Fig. 22). All other correlations were not significant (all p-values ≥ 0.12).

As BMI and WHR showed potentially relevant correlations with mRNA expression levels of *DAT* and *DRD3*, the impact of BMI and WHR on the main analyses of dietary effects was tested in analyses of covariance (ANCOVAs) to explore if the observed trends for a dietary effect on *DAT* and *DRD3* mRNA expression (see section 5.2.1) are also present when controlling for BMI/WHR. The ANCOVA results (Table 7) suggest that although BMI and WHR had significant effects on the mRNA expression of *DRD3*

and *DAT* (WHR - DAT: p = 0.01; BMI - DRD3: p = 0.02; trend for BMI - DAT: p = 0.07) the main effects of diet were also significant or indicated a potential trend after including the covariates (Diet-DRD3 with covariate BMI: p = 0.004; Diet-DAT with covariate BMI: p = 0.049; Diet-DAT with covariate WHR: p = 0.07; see also Table 7 for comparisons between p-values for main effects of diet in the ANCOVA model and p-values for diet group differences without including covariates, taken from section 5.2.1).

Table 6: Correlations between mRNA expression levels and measures of obesity. Correlation analyses between mRNA expression of all genes of interest and BMI/WHR indicated a significant negative correlation between *DAT* mRNA expression and WHR (p = 0.004) and potential trends for negative correlations between *DAT* mRNA expression and BMI (p = 0.04), and *DRD3* mRNA expression and BMI (p = 0.053). Tests used: correlations with Pearson's r for *COMT* and *DRD3*, and Spearman's rho for *DAT, DARPP-32, DRD2, DRD5*, *UCP-2* and *UCP-3*. Abbreviations: BMI: body mass index, COMT: Catechol-O-methyltransferase, Cor. coef.: correlation coefficient, DARPP: dopamine-and-cAMP-regulated neuronal protein 32, DAT: dopamine transporter, DRD: dopamine receptor, UCP: uncoupling protein, WHR: waist-to-hip-ratio.

		mRNA expression							
		COMT	**DAT**	**DARPP**	**DRD2**	**DRD3**	**DRD5**	**UCP2**	**UCP3**
BMI	Cor. coef.	0.19	- 0.26	0.02	- 0.001	- 0.24	- 0.06	- 0.14	- 0.03
	p-values	0.12	**0.04**	0.89	0.99	**0.053**	0.68	0.24	0.82
WHR	Cor. coef.	- 0.10	- 0.35	- 0.03	0.11	- 0.02	- 0.09	0.09	- 0.09
	p-values	0.42	**0.004**	0.81	0.44	0.90	0.52	0.88	0.45

Table 7: Effects of measures of obesity on mRNA expression levels. The covariates BMI and WHR had considerable effects on the mRNA expression of *DAT* and *DRD3* as the main effects of these covariates in the ANCOVA models were significant or at least indicated a potential trend (p ≤ 0.07, see column "Covariate"). The main effects of diet after including the covariates into the model (see column "Diet") are also significant (Diet-DRD3 with covariate BMI: p = 0.004; Diet-DAT with covariate BMI: p = 0.049) or still point towards a trend for a dietary impact on gene expression (Diet-DAT with covariate WHR: p = 0.07). This is comparable to the results for diet group differences in mRNA expression before including covariates (see column "Diet group difference without covariate", p-values are taken from section 5.2.1). Test used: ANCOVA. Abbreviations: ANCOVA: analysis of covariance, BMI: body mass index, DAT: dopamine transporter, DRD: dopamine receptor, WHR: waist-to-hip ratio.

Model	Main effects of ANCOVA		**Diet group difference without covariate** (p-value)
	Covariate	**Diet**	
Diet - DAT mRNA; Covariate: **BMI**	F(1, 62) = 3.38, p = 0.07	F(1, 62) = 4.03, p = 0.049	0.054
Diet - DAT mRNA; Covariate: **WHR**	F(1, 62) = 6.89, p = 0.01	F(1, 62) = 3.45, p = 0.07	0.054
Diet - DRD3 mRNA; Covariate: **BMI**	F(1, 64) = 5.70, p = 0.02	F(1, 64) = 8.83, p = 0.004	0.013

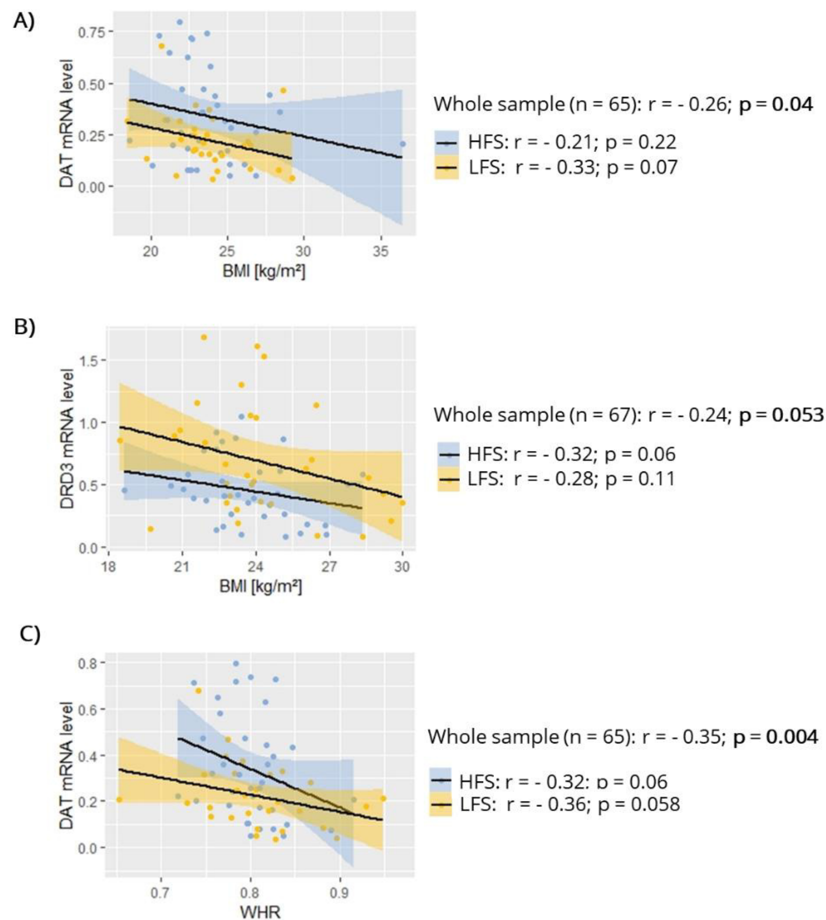


Figure 22: Correlations between measures of obesity and mRNA levels. Negative correlations between mRNA expression of *DAT* and *DRD3* and BMI/WHR were found in the whole sample as well as within both diet groups (HFS and LFS). **A)** There seems to be a trend for a negative correlation between BMI and *DAT* mRNA expression in the whole sample (p = 0.04; not significant due to the correction for multiple comparisons) and also in the LFS group (p = 0.07). **B)** There is a trend for a negative correlation between *DRD3* mRNA level and BMI in the total sample (p = 0.053) and in the HFS group (p = 0.06). **C)** The negative correlation between *DAT* mRNA level and WHR is significant in the full sample (p = 0.004) and also shows a similar trend in both diet groups (HFS: p = 0.06; LFS: p = 0.058). Tests used: correlations with Pearson's r for BMI - *DRD3* and Spearman's rho for BMI/WHR - *DAT*. Abbreviations: BMI: body mass index, DAT: dopamine transporter, DRD: dopamine receptor, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, n: sample size, whole sample: HFS + LFS (all available data points for respective mRNA, as there were drop-out during mRNA measurement, see section 4.4.7), WHR: waist-to-hip ratio.

5.5.2 No significant associations of diet or genotype in *DRD2*-related SNPs with physical activity

Weak correlation between average step count and MET minutes score in the IPAQ

Two different methods were used to quantify physical activity. The average step count over 7 continuous days (or in case of dropouts over the maximal number of data points available) during everyday activities was measured with a pedometer. Step count data is not available for 8 participants (4 per diet group) as the pedometer was not returned. Additionally, the IPAQ (International Physical Activity Questionnaire) was used to self-reportedly assess duration and energy expenditure of physical activities with a MET (metabolic equivalent of task) minutes score. The IPAQ was completed by all participants in the sample (HFS: n = 41, LFS: n = 34). To assess whether both tools are able to capture PA to the same degree, the average step count and the MET minutes score in the IPAQ were correlated regardless of diet group (Fig. 23A) where a positive but unexpectedly weak and only trend significant correlation was observed (r = 0.21, p = 0.09). Therefore, as the MET minutes score comprises three subscales separating MET minutes derived from walking, moderate activity and vigorous activity, and as the pedometer mainly captures walking activity, an additional correlation analysis was performed between the MET minutes score in the "walking" subscale of the IPAQ and average step count (Fig. 23B). This correlation was even weaker and not significant (r = 0.16, p = 0.21), indicating low overlap between activities captured by the pedometer and activities quantified with the MET minutes score in the IPAQ.

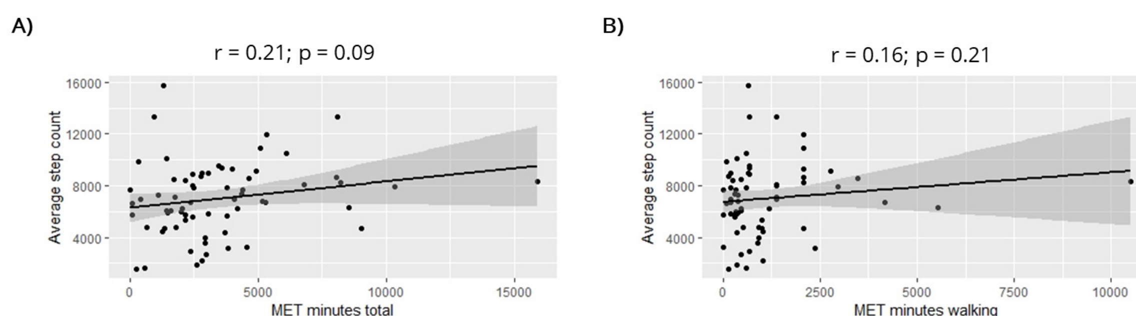


Figure 23: Correlation between MET minutes scores in the IPAQ and average step count. **A)** The total MET minutes score in the IPAQ showed a weak positive correlation with the average step count in the full sample, which did not reach statistical significance (r = 0.21, p = 0.09). **B)** The correlation between the MET minutes walking score in the IPAQ (capturing only MET minutes derived from walking activity) and average step count was not significant and even weaker than the correlation between total MET minutes score and average step count (r = 0.16, p = 0.21). Tests used: correlations with Pearson's correlation coefficient. Abbreviations: MET: metabolic equivalent of task, p: p-value, r: correlation coefficient.

No diet group differences in PA

Potential differences in PA could confound effects of diet in the dopaminergic system. Therefore, diet groups were tested for differences in average step count (independent-samples t-test) and in the total MET minutes score in the IPAQ (Wilcoxon rank-sum test). Results showed that the diet groups did not differ significantly in both parameters (Table 8).

Table 8: No diet group differences in measures of physical activity. Diet groups did not differ significantly in average step count ($p = 0.52$) or total MET minutes score ($p = 0.93$). Tests used: independent-samples t-test for step count, two-samples Wilcoxon rank-sum test for MET minutes total score. Abbreviations: HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, MET: metabolic equivalent of task, Min: minimal value, Max: maximal value, SD: standard deviation.

	Average step count		**MET minutes total**	
Group	HFS	LFS	HFS	LFS
Min	1844.71	1536.43	82.50	0.00
Max	11914.43	15767.71	9039.00	15887.40
Mean	6779.55	7234.06	3174.89	3734.42
SD	2547.82	3151.33	1927.61	3441.47
Group difference	$t(65) = -0.65, p = 0.52$		$W = 705.5, p = 0.93$	

No significant differences in PA associated with genotype in *DRD2*-related SNPs

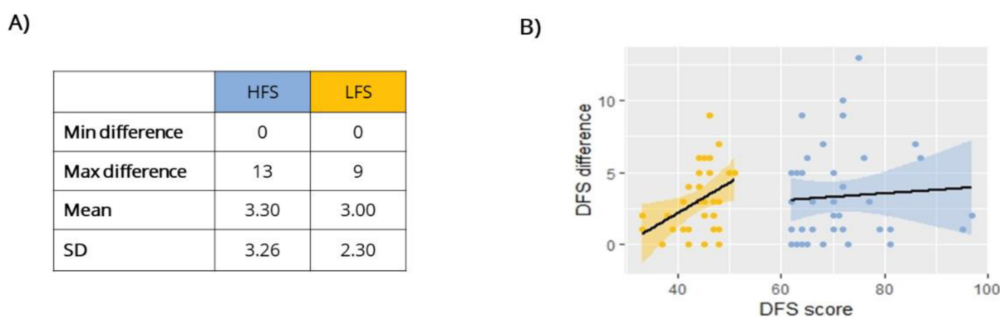
Baseline differences in the DA system, i.e. in genotype of SNPs affecting *DRD2* expression and function, might influence PA by affecting the motivation to move (Beeler et al., 2016; Friend et al., 2017). Thus, the effects of *DRD2*-related SNPs on PA were tested with one-way ANOVAs which showed no significant genotype effects on step count or MET minutes score (all $p \geq 0.31$; Table 9).

Table 9: No effect of genotype in *DRD2*-related SNPs on physical activity. The *DRD2*-related SNP variants in rs1800479 (Taq1A polymorphism) and rs6277 related to *DRD2* as well as the *FTO* SNPs rs8053740 and rs8050136 were tested for an association with average step count and total MET minutes score in the IPAQ using one-way ANOVAs. There was no significant association between any genotype and average step count/total MET minutes score (all $p > 0.31$). Abbreviations: DRD: dopamine receptor, FTO: fat mass and obesity-associated protein, MET: metabolic equivalent of task.

SNP name	**Effect of *genotype***	
	Step count	MET minutes score
DRD2 **rs1800497**	$F(1, 65) = 1.02, p = 0.31$	$F(1, 73) = 0.61, p = 0.44$
DRD2 **rs6277**	$F(2, 64) = 0.26, p = 0.77$	$F(2, 72) = 0.30, p = 0.74$
FTO **rs8053740**	$F(2, 64) = 0.33, p = 0.72$	$F(2, 72) = 0.57, p = 0.57$
FTO **rs8050136**	$F(2, 64) = 0.71, p = 0.50$	$F(2, 72) = 0.18, p = 0.84$

5.5.3 Unreliability in DFS scoring might influence the analysis of group differences

Participants were assigned to diet groups according to their DFS score, which is a self-reported measure and therefore a potential source of unreliability. In general, the DFS score was relatively stable as the mean difference between first and second measurement on screening day and second test day was 3.30 ± 3.26 in the HFS group and 3.00 ± 2.30 in the LFS group. However, for some participants the score obtained on the second measurement deviated strongly from the first measurement, the maximal difference between scores was 13 points in the HFS group and 9 points in the LFS group (see Fig. 24A). Within the HFS group the difference in scores between test days was independent of the actual DFS score, but strikingly in the LFS group the scores differed stronger for those participants having a higher baseline DFS score than for those having a low baseline DFS score (see Fig. 24B).


A)

	HFS	LFS
Min difference	0	0
Max difference	13	9
Mean	3.30	3.00
SD	3.26	2.30

Figure 24: Difference in DFS scores between screening day and second test day. **<u>A)</u>** The maximal difference in DFS scores was 13 points in the HFS group and 9 points in the LFS group. Mean differences in DFS scores were 3.30 ± 3.26 points in the HFS group and 3.00 ± 2.30 points in the LFS group. **<u>B)</u>** Within the HFS group (blue) the difference in DFS scores between measurements was unaffected by the baseline DFS score obtained on the screening day (X-axis "DFS score"). Within the LFS group (yellow) the difference between first and second score was higher when the baseline DFS score was higher and thus closer to the cut-off value for group definition. <u>Abbreviations</u>: DFS: Dietary Fat and free Sugar – Short Questionnaire, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, Max: maximal value, Min: minimal value, SD: standard deviation.

To account for this unreliability in DFS scoring, weighted ANOVAs were used to decrease the impact of data points that belong to participants with less consistent DFS scores. The weighting procedure is described in detail in section 4.10. Table 10 shows the newly obtained p-values for diet group differences after weighting for DFS score differences and for comparison also the initial p-values without weighting from section 5.2 and 5.3. The diet group differences in mRNA expression levels of *COMT, DAT*, and *DRD3* are significant when the data points are weighted for reliability compared to the trends before weighting (*COMT*: p = 0.006 vs. p = 0.07; *DAT*: p = 0.003 vs. p = 0.054; *DRD3*: p = < 0.001 vs. p = 0.013). The diet group difference in *UCP-3* expression remains significant (p = < 0.001 vs. p = 0.009). However, mRNA expression of *UCP-2* is not significantly different between diet groups when the DFS score difference is considered (before weighting: p < 0.001 vs. after weighting: p = 0.45). The p-values for the mRNA levels of *DARPP-32, DRD2,* and *DRD5* are different from the p-values before weighting but still indicate no significant group difference (*DARPP-32*: p = 0.14 vs. p = 0.25; *DRD2*: p = 0.24 vs. p = 0.20; *DRD5*: p = 0.61 vs. p = 0.27). Regarding cognition, performance in DSST differs significantly between diet groups (p = 0.004) and there might also be a trend difference in the performance of the DS backwards (p = 0.07) when including the weighting variable. Before weighting, there were no group differences in performance of these tests (p = 0.45 and p = 0.38, respectively). For the TMT B, there is no significant diet group difference independent of weighting (p = 0.59 vs. p = 0.75). This indicates that unreliability in grouping according to the DFS score does have a significant impact on some of the main outcome variables that needs to be considered when interpreting obtained results.

Table 10: Diet group differences in mRNA expression and cognition before and after weighting for DFS scoring deviations between test days. The table shows the comparison of p-values for diet group differences in main outcome parameters without weighting (see section 5.2.1, 5.2.2 and 5.3) and after weighting for unreliability in the DFS scoring. After weighting, the diet group differences for *COMT, DAT, DRD3, UCP-3*, and DSST are significant and there might also be a trend for a group difference in DS backwards performance, but the diet group difference in *UCP-2* expression is not significant anymore. Independent of weighting, there are no significant group differences in *DARPP-32, DRD2*, and *DRD5* expression as well as in TMT B performance. Abbreviations: COMT: catechol-O-methyltransferase, DARPP: dopamine-and-cAMP-regulated neuronal phosphoprotein 32, DAT: dopamine transporter, DRD: dopamine receptor, DS: Digit span test, DSST: Digit symbol substitution test, TMT: Trail making test, UCP: uncoupling protein.

p-value	COMT	DAT	DARPP	DRD2	DRD3	DRD5	UCP-2	UCP-3	TMT B	DSST	DS back
before weighting	0.07	0.054	0.25	0.20	0.013	0.27	< 0.001	0.009	0.59	0.45	0.38
after weighting	0.006	0.003	0.14	0.24	< 0.001	0.61	0.45	< 0.001	0.75	0.004	0.07

5.6 Metabolic, hormonal, and inflammatory parameters

5.6.1 No significant diet group differences in blood parameters

As increased HbA1c indicating diabetes mellitus type II and non-normal TSH reflecting thyroid dysfunction were predefined as post-hoc exclusion criteria, these blood parameters were checked for values outside of the normal range. For HbA1c, all participants were within normal range with HbA1c values < 5.7 %. Two participants per diet group had slightly elevated TSH values (the highest TSH value was 4.72 mU/l, the cut-off for normal range is 3.77 mU/l). As such small deviations in TSH levels would be an indication for repeating the TSH measurement in clinical diagnostics due to the high variability of TSH levels, the results were discussed with study physicians. It was concurringly agreed that the obtained TSH values are far below the clinical cut-off for a thyroid dysfunction, i.e. 10 mU/l and thus these participants were not excluded from the study sample as they show no indication for a severe thyroid dysfunction.

Diet groups did not differ significantly in any of the obtained metabolic, hormonal, and inflammatory parameters as all p-values for group differences were > 0.05 (Table 11). The formal trend for a group difference in fT3 ($t(73) = 1{,}70$, $p = 0.09$) was not elaborated on any further, as the other thyroid markers TSH and fT4 showed no similar tendency for a group difference. Additionally, the preregistered analyses plan (see OSF preregistration: https://osf.io/e3w8k) contained a mediation model to analyse if diet-associated differences in cognitive performance could be mediated by inflammation (CRP) or insulin resistance (HOMA-IR). In line with the approach in section 5.2.3, this mediation model was not carried out as there were no significant diet group differences in cognitive performance (reflecting the c-path of the model).

Table 11: Descriptive statistics and group differences for blood parameters. The table shows descriptive statistics (minimal and maximal value, mean and standard deviation) for the obtained blood parameters within diet groups. There were no significant differences between diet groups in any of the metabolic, hormonal, or inflammatory blood measurements (all $p > 0.05$). Tests used: independent-samples t-tests for glucose, HbA1c, cholesterol, LDL and fT3; Wilcoxon rank-sum test for insulin, HOMA-IR, leptin, triglycerides, HDL, hsCRP, and TSH. Abbreviations: fT3: free tri-iodothyronine, fT4: free thyroxine, HbA1c: glycated hemoglobin, HDL: high-density lipoprotein, HFS: high fat and sugar diet group, HOMA-IR: homeostasis model assessment, hsCRP: highly sensitive C-reactive protein, LDL: low-density lipoprotein, LFS: low fat and sugar diet group, Max: maximal value, Min: minimal value, SD: standard deviation.

	HFS = 41				LFS = 34				Group difference
	Min	Max	Mean	SD	Min	Max	Mean	SD	
Glucose [mmol/l]	4.49	6.67	5.29	0.43	4.54	6.10	5.18	0.33	t (73) = 1.19, p = 0.24
HbA1c [%]	4.74	5.62	5.21	0.22	4.86	5.55	5.20	0.18	t (73) = 0.26, p = 0.79
Insulin [pmol/l]	14.10	78.40	32.61	17.85	8.50	132.3	37.11	30.34	W = 704.5, p = 0.94
HOMA-IR	0.49	3.19	1.30	0.76	0.29	5.00	1.45	1.23	W = 719.0, p = 0.82
Leptin [ng/ml]	1.00	9.60	3.16	1.95	0.20	12.80	3.38	2.79	W = 730.0, p = 0.73
Triglycerides [mmol/l]	0.50	3.70	1.11	0.58	0.40	2.38	1.04	0.56	W = 794.5, p = 0.30
Cholesterol [mmol/l]	2.71	6.46	4.27	0.77	2.95	6.19	4.35	0.74	t(73) = -0.47, p = 0.64
LDL [mmol/l]	1.23	4.26	2.55	0.65	1.60	4.18	2.73	0.65	t(73) = -1.16, p = 0.25
HDL [mmol/l]	0.96	2.72	1.50	0.30	0.90	2.20	1.44	0.34	W = 766.5, p = 0.46
hsCRP [mg/l]	0.15	8.18	0.97	1.51	0.15	6.54	1.01	1.61	W = 756.0, p = 0.53
TSH [mU/l]	0.76	4.72	2.05	0.90	0.97	3.99	2.08	0.75	W = 657.5, p = 0.68
fT3 [pmol/l]	4.46	7.23	5.66	0.62	3.61	6.47	5.41	0.62	t(73) = 1,70, p = 0.09
fT4 [pmol/l]	12.20	23.80	16.38	2.65	12.40	21.20	16.39	2.10	W = 673.5, p = 0.81

5.6.2 Blood levels of cholesterol and CRP correlate with the peripheral mRNA expression of DA-related genes

Peripheral gene expression in the DA pathway might be related to metabolic or inflammatory pathways (see section 2.7). Thus, correlation analyses were performed between mRNA expression levels of those genes with potential diet-associated expression differences (i.e. *COMT, DAT, DRD3,* and exploratorily also for *UCP-2/3*) and metabolic (glucose, insulin, HbA1c, leptin, triglycerides and cholesterol) and inflammatory (CRP) blood markers. An overview of the results is shown in Table 12. After correction for multiple comparisons, there remain potential trends for a positive correlation between blood levels of cholesterol and the mRNA expression levels of *DAT* ($r = 0.30$, $p = 0.02$, Fig. 25A) as well as for a negative correlation between levels of hsCRP and *DRD3* expression ($r = -0.24$, $p = 0.048$, Fig. 25B). All other correlations were not significant (all $p \geq 0.1$). The correlation analyses between metabolic and inflammatory parameters and the expression levels of *UCP-2* and *UCP-3* also showed no significant results (data not shown).

Table 12: Correlations of metabolic and inflammatory blood parameters with mRNA expression levels. The table shows the results of the correlation analyses between metabolic (glucose, insulin, HbA1c, leptin, triglycerides, and cholesterol) and inflammatory (CRP) markers and mRNA expression levels of *COMT, DAT* and *DRD3* in the full sample regardless of diet group. After correction for multiple comparisons, there remain potential trends for a positive correlation between mRNA expression levels of *DAT* and blood levels of cholesterol (r = 0.30, p = 0.02) and for a negative correlation between levels of hsCRP and *DRD3* expression (r = - 0.24, p = 0.048). All other correlations were not significant (p ≥ 0.1). Tests used: correlations with Pearson's r for correlating glucose and cholesterol with *COMT* and *DRD3*, correlations with Spearman's rho for all other pairs. Abbreviations: COMT: catechol-O-methyltransferase, CRP: C-reactive protein, DAT: dopamine transporter, DRD: dopamine receptor, HbA1c: glycated hemoglobin.

	COMT	DAT	DRD3
Glucose	r = -0.07, p = 0.58	r = 0.09, p = 0.47	r = -0.41, p = 0.26
Insulin	r = -0.06, p = 0.61	r = -0.11, p = 0.38	r = -0.15, p = 0.23
HbA1c	r = 0.005, p = 0.97	r = 0.06, p = 0.64	r = 0.03, p = 0.81
Leptin	r = 0.07, p = 0.57	r = -0.12, p = 0.33	r = -0.08, p = 0.52
Triglycerides	r = -0.16, p = 0.18	r = 0.03, p = 0.79	r = -0.20, p = 0.10
Cholesterol	r = 0.02, p = 0.90	**r = 0.30, p = 0.02**	r = -0.01, p = 0.93
CRP	r = 0.17, p = 0.16	r = -0.19, p = 0.13	**r = -0.24, p = 0.048**

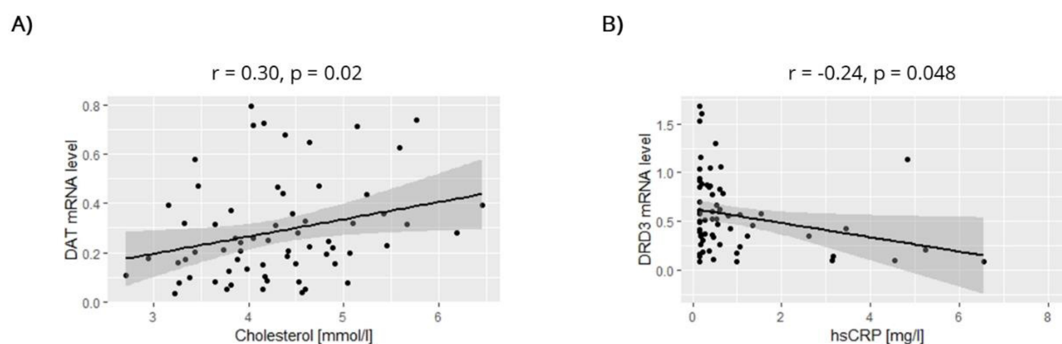


Figure 25: Correlations between blood parameters and mRNA expression levels. A) Blood cholesterol levels were positively correlated with mRNA expression of *DAT* (r = 0.30, p = 0.02). **B)** Levels of hsCRP were negatively correlated with mRNA expression of *DRD3* (r = -0.24, p = 0.048). Test used: correlations (Pearson's r for cholesterol-DAT and Spearman's rho for CRP-DRD3). Abbreviations: DAT: dopamine transporter, DRD: dopamine receptor, hsCRP: highly sensitive C-reactive protein, r: Spearman's rho.

5.7 Further sample characterization for personality traits and eating behavior

Questionnaire scores and subscores were analysed for diet group differences with independent-samples t-tests or a non-parametric alternative (Wilcoxon rank-sum tests for FCQ-T, YFAS, BIS, BAS Responsiveness/Fun, UPPS Perseverance, FEV, and NEO-FFI Conscientiousness) to characterize the groups for eating behaviour and personality traits. A complete overview of the results is provided in Table 13. The LFS group had on average significantly higher scores in the FEV subscale "Restrain" (p = 0.001), indicating stronger cognitive control of eating behaviour, and lower scores in the FEV subscale "Hunger" (p = 0.003), potentially suggesting lower subjective feelings of hunger in this group. In the FCQ-T subscore "Cues" the HFS group scored generally higher (p = 0.01) pointing towards higher reactivity to cues that trigger cravings to consume food. Additionally, there is a tendency for higher scores in the subscale "Reinforcement" in the HFS group (p = 0.08), which reflects higher anticipation of reinforcement when consuming food. Finally, the HFS group scored tendentially higher on the subscale "Neuroticism" in the NEO-FFI (p = 0.08). Due to multiple testing, only the group difference in the FEV subscale "Restrain" is significant and all other trends need to be interpreted with care.

Table 13: Group differences in questionnaires. The LFS group scored significantly higher in the FEV subscore "Restrain" ($p = 0.001$) and lower in the subscore "Hunger" ($p = 0.003$). The HFS group had on average tendentially higher scores in the FCQ-T subscore "Cues" ($p = 0.011$) and in the subscore "Reinforcement" ($p = 0.08$). Additionally, the HFS group shows a trend for higher scores in the NEO-FFI subscale "Neuroticism" ($p = 0.08$). Due to multiple testing, only the group difference in the FEV subscale "Restrain" is significant. Tests used: independent-samples t-test for BAS "Drive" and "Total", BIS-15, UPPS (except UPPS "Perseverance"), NEO-FFI (except NEO-FFI "Conscientiousness"); Wilcoxon rank-sum test for FCQ-T, YFAS, BIS, BAS "Responsiveness" and "Fun", UPPS "Perseverance", FEV, NEO-FFI "Conscientiousness". Abbreviations: BIS15: Barrat Impulsiveness Scale, BIS/BAS: Behavioral approach and avoidance system questionnaire, FCQ-T: Food Craving Questionnaire-Trait, FEV: Three-Factor Eating Questionnaire, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, NEO-FFI: NEO-Five Factor Inventory, UPPS: Urgency, Premeditation, Perseverance, Sensation Seeking Impulsive Behavior Scale, YFAS: Yale Food Addiction Scale.
[1] Sample size for FEV and NEO-FFI in the HFS group n = 40 and in the LFS group n = 33, due to dropouts.

Questionnaire	Subscales	HFS n = 41[1]				LFS n = 34[1]				Group difference
		Min	Max	Mean	SD	Min	Max	Mean	SD	p-value
FEV	Restrain	0.00	12.00	4.18	2.98	2.00	20.00	7.09	4.21	**0.001**
	Disinhibition	0.00	11.00	4.38	2.85	0.00	14.00	3.52	2.75	0.20
	Hunger	0.00	11.00	5.05	3.05	0.00	10.00	2.97	2.54	**0.003**
FCQ-T	Control	9.00	34.00	15.90	6.65	9.00	29.00	13.68	4.98	0.15
	Reinforcement	8.00	39.00	17.80	7.40	8.00	27.00	14.76	5.78	**0.08**
	Thoughts	10.00	33.00	14.59	6.37	10.00	30.00	13.03	4.16	0.70
	Emotions	4.00	20.00	6.88	3.78	4.00	15.00	6.09	2.38	0.85
	Cues	5.00	20.00	11.98	3.86	4.00	20.00	9.71	3.94	**0.01**
	Hunger	4.00	20.00	10.00	3.79	4.00	18.00	8.76	3.43	0.14
	Sum	46.00	146.0	77.15	27.10	39.00	128.0	66.03	19.88	**0.07**
YFAS		0.00	6.00	0.24	0.99	0.00	2.00	0.21	0.59	0.78
BIS/BAS	BIS	9.00	23.00	16.37	3.81	10.00	22.00	16.44	2.89	0.86
	BAS Drive	4.00	13.00	8.20	2.20	5.00	12.00	7.91	1.88	0.56
	BAS Responsiveness	5.00	13.00	8.95	1.91	6.00	13.00	8.62	1.86	0.39
	BAS Fun	5.00	13.00	8.10	1.92	4.00	12.00	7.82	2.11	0.56
	BAS Total	16.00	38.00	25.24	4.60	16.00	33.00	24.35	4.79	0.41
BIS15	Non planning impulsivity	5.00	16.00	10.93	3.14	5.00	16.00	10.50	3.01	0.55
	Motor impulsivity	6.00	18.00	10.80	2.65	6.00	15.00	10.68	2.51	0.83
	Attentional impulsivity	7.00	14.00	10.24	1.91	7.00	13.00	10.09	1.48	0.70
	Sum	20.00	47.00	31.98	5.72	23.00	41.00	31.26	4.55	0.56
UPPS	Urgency	15.00	34.00	24.78	4.76	14.00	31.00	23.26	4.13	0.15
	Premeditation	15.00	34.00	21.76	4.22	13.00	31.00	23.00	4.36	0.21
	Perseverance	10.00	31.00	19.46	4.71	12.00	33.00	18.44	4.83	0.24
	Sensation Seeking	21.00	47.00	35.88	6.27	21.00	45.00	34.74	6.92	0.46
NEO-FFI	Neuroticism	1.00	3.50	2.23	0.67	1.00	3.67	1.95	0.65	**0.08**
	Extraversion	2.50	4.33	3.55	0.50	2.00	4.83	3.52	0.58	0.76
	Openness	2.33	3.67	2.92	0.34	2.50	3.83	3.01	0.31	0.25
	Agreeableness	1.50	3.67	2.58	0.58	1.83	3.67	2.48	0.45	0.45
	Conscientiousness	2.83	4.33	3.63	0.42	2.50	4.17	3.60	0.37	0.92

6. Discussion

Obesity has been associated with impairments in dopamine (DA)-related cognitive functioning, i.e. reward-based decision-making, and inhibitory control of behavior, which might contribute to hedonic overeating observed in obese individuals. To date it is unclear whether this cognitive phenotype might be a consequence of obesity or could rather be the cause of excessive calorie intake and obesity development. This study aimed to explore if already a diet high in fat and sugar, which is usually considered as obesogenic when maintained chronically, is associated with differences in the DA system independent of established obesity. Thus, gene expression in the peripheral DA system and DA-related cognitive functions were studied in a sample of 75 healthy young men with a broad BMI range from 18.5 to 36.4 kg/m² that were separated into two groups of different dietary style. Participants were assigned to either a high-fat/sugar or a low-fat/sugar diet group (HFS vs. LFS group) according to their self-reported average intake of mainly saturated fat and refined sugar. The groups were matched for age, the obesity measures BMI, WHR, and weight as well as for IQ. The qPCR results indicated potential trends for differences between diet groups in the peripheral mRNA expression of dopamine receptor D3 (*DRD3*), dopamine transporter (*DAT)* and the DA degradation enzyme *COMT*. Even though these differences were not statistically significant, this might still point towards an association between HFS diet and peripheral DA-related gene expression, but further research is needed to validate the significance of such an association in humans. A link to DA-dependent cognition could not be established with the specific cognitive tasks used in this study, as diet groups did not perform significantly different in all tasks.

6.1 Differences in peripheral DA-related gene expression in association with HFS diet consumption

Gene expression analyses pointed towards potential trends for lower mRNA expression levels of *DRD3* and *COMT* as well as higher levels of *DAT* in the HFS diet group compared to the LFS group, whereas the expression of *DARPP-32, DRD2* and *DRD5* was not statistically different between diet groups.

Literature reported increased availability of DRD2/3 in the midbrain and striatum in PET scans of individuals with a higher BMI (Dang et al., 2016) or even a non-linear relationship between BMI and receptor availability with a positive association for normal weight and mildly obese individuals that turns into a negative association when reaching severe obesity with a BMI above 40kg/m² (Cosgrove et al., 2015). Based on these findings, higher expression levels of *DRD3* would have been expectable in HFS dieters analogous to those with a BMI in the overweight/mildly obese range if alterations related to HFS diet would precede obesity-associated changes in DA signaling. The results of this study, however, point towards lower peripheral mRNA expression of *DRD3* in association with HFS diet. If this trend reflects a true group difference, this speaks against the hypothesis that HFS diet drives the obesity-related changes. However, other reasons for this discrepancy could be that there is no diet-related difference in the expression of *DRD3* and the observed trend is a false positive or that the PET imaging of central DRD2/3 used in abovementioned studies cannot be compared to a peripheral gene expression measurement. Receptor availability, the outcome parameter of PET imaging, is not only determined by the actual number of target molecules, but also by various other influences, as tracer binding can occur unspecifically or be inhibited by endogenous ligands (Peng et al., 2013). Therefore, observed changes in receptor availability in PET scanning might not be accompanied by concordant changes in mRNA expression, especially in the periphery. *DRD3* gene expression in particular has seemingly not yet been studied in relation to obesity or HFS diet, neither in humans nor animals, and might thus be a valuable target for future research.

Obesity-associated changes in the mRNA expression of *DRD2* and *DRD5* have by contrast been reported frequently. Both central obesity assessed by waist circumference (Leite et al., 2016) as well as BMI (Mansur et al., 2018) were found to be associated with reduced *DRD2/5* expression in humans. Matching these findings, dietary intervention studies with rodents found similar effects of HFS diet exposure on *DRD2/5* gene expression (Adams et al., 2015; Alsiö et al., 2010; Barry et al., 2018; Hakim & Keay, 2019; Vucetic et al., 2012). Additionally, also DARPP-32 as a main player in dopaminergic downstream signaling was found to be altered in its expression in response to HFD (Carlin et al., 2013; Hryhorczuk et al., 2016). The results of this study do not support these findings, as there were no significant differences in mRNA expression of *DRD2*, *DRD5* and *DARPP-32* between diet groups. Even though it is conceivable that HFS diet actually does not have any effect on the peripheral expression of these genes in humans as indicated by these results, it is surely also possible that the effect size of grouping based on self-reported eating preferences is not strong enough to reliably detect a dietary impact. Studies with obese individuals might have produced clearer results as obesity, a condition with putatively high impact on many regulatory systems of the whole body, might have a stronger influence on DA-related gene expression than diet alone. Therefore, obesity-related effects might appear above unspecific background variation whereas potential diet-associated differences in this study sample might not be measurable with the methods used. Additionally, a trend for a dietary effect on *DRD2* gene expression appeared in the diet x genotype ANOVAs (see section 5.4.2) when controlling for genotype in the Taq1A SNP (rs1800497) in *DRD2.* This might support that there could be a dietary influence on *DRD2* expression that is masked by other influences, but as this interaction effect was not statistically significant it needs to be interpreted with care. Another explanation for not finding obesity-independent differences in *DRD2/5* and *DARPP-32* mRNA levels might be, however, that changes in the dopamine system might occur in response to developing obesity and not before obesity establishment and might thus not be preset in this study sample.

Regarding *DAT,* this study indicates a trend towards higher peripheral *DAT* mRNA expression in the HFS group compared to the LFS group. This is in line with the results of Vucetic et al. who also observed increased *DAT* mRNA levels after HFD exposure in the hypothalamus of mice (Vucetic et al., 2012). However, within the VTA animal literature reports relatively consistent downregulations of *DAT* expression (Carlin et al., 2013; Vucetic et al., 2012) and DAT activity (Baladi et al., 2015; Cone et al., 2013) in response to HFD. Studies with humans similarly found lower *DAT* mRNA expression in the SN and lower striatal DAT binding in association with a higher BMI (Wu et al., 2017). Thus, if there is a true HFS diet-related difference in *DAT* expression at all it is not clear to what extent the observed differences in peripheral levels of *DAT* mRNA are comparable to central *DAT* expression.

Finally, the expression of *COMT* in different brain regions, e.g. in the hypothalamus, VTA, PFC, NAc, and caudate putamen, was reported to be unaffected by HFD intake in some publications (Alsiö et al., 2010; Vucetic et al., 2012). However, Carlin et al. observed a downregulation of *COMT* mRNA expression in the VTA and NAc in animals after HFD (Carlin et al., 2013) which would be in line with the trend towards lower levels of *COMT* mRNA in the HFS group observed peripherally in this study. Carlin et al. further found that these expression changes were reversible and even overcompensated when switching to a LFD (Carlin et al., 2013). If a similar effect would be present in blood cells, this could be a potential reason why there was no significant diet group difference in *COMT* expression. It cannot be excluded that some participants, especially those with a DFS score close to the cut-off of their assigned diet group, did not follow an HFS or LFS diet strictly and might thus weaken the discriminatory power of the grouping variable. Nevertheless, it is not clear if this effect is actually transferable from the central to the peripheral level.

Another general reason why this study did not yield significant results regarding gene expression might be the experimental setting. With rodents it is possible to apply diets with very high

concentrations of the ingredient of interest and at the same time reduce external influences to a minimum, which is not equally possible in humans. The results from animal studies might therefore not always be replicable in studies with humans (Janssen et al., 2019). The commonly used percentage of calories from saturated fat or sugar in rodents' HFS diets is 60% (Adams et al., 2015; Barry et al., 2018; Cone et al., 2013) compared to 10% in the LFS counterpart. For instance, Carlin et al. exposed their experimental animals to an HFD consisting of 60% fat for as long as 12 weeks (Carlin et al., 2013). Contrastingly, the typical HFS diet in humans is the so-called "Western style" diet, which is rich in red meat, saturated fat (French fries, dairy, pizza etc.) and artificially sweetened high sugar snacks such as sweets or soft drinks (van Dam, Rimm, Willett, Stampfer, & Hu, 2002). However, the percentage of calories coming from mainly saturated fat in Western style diets is as low as 35% and the carbohydrate proportion, containing not only sugar, is around 50% (Last & Wilson, 2006). A high fat diet with 60% of calories from saturated fat can rarely be achieved in studies with humans. Thus, changes in the DA system induced by a Western style diet, if present at all, would surely be less pronounced than effects observed in animal studies.

As a final note it is to mention that it was not possible to analyze peripheral *TH* and *DRD1* mRNA expression even though many human and animal studies indicate *TH* and *DRD1* as potentially promising targets for diet-associated changes in gene expression (Alsiö et al., 2010; Carlin et al., 2013; Leite et al., 2016; Li et al., 2009; Vucetic et al., 2012; Wu et al., 2017). This is because *DRD1* is generally not expressed in blood cells (Gladkevich et al., 2004; Kirillova et al., 2008) and *TH* expression was either too low for a reliable detection in the qPCR measurement or the purchased assay did not work sufficiently.

Validity of peripheral markers as a surrogate for the central dopaminergic system

Regarding the literature discussed above it should also be considered that some findings reported in association with obesity and in animal studies are related to specific tissues or even brain regions. For instance, Leite et al. studied mRNA expression of blood cells in obese humans (Leite et al., 2016), Mansur et al. analyzed human post-mortem samples of the dlPFC (Mansur et al., 2018), Adams et al. found effects in protein expression of *DRD2* only in the NAc (Adams et al., 2015) and Vucetic et al. found a dietary impact on the expression of dopamine receptors and *DARPP-32* only in the reward circuitry but not in hypothalamus (Vucetic et al., 2012). Related to *DAT*, Vucetic. et al observed a downregulation of *DAT* mRNA expression in the VTA but found approximately tripled *DAT* levels in the hypothalamus (Vucetic et al., 2012). This suggests that gene expression changes in association with HFS diet or obesity could be at least to a certain extent specific to particular tissues and even brain regions. In line, some authors emphasize the tissue- and brain region specificity of epigenetic processes like methylation (Bakulski et al., 2016; Davies et al., 2012). Due to this potential region specificity of a dietary impact on gene expression regulation it is possible that both up- and downregulation of a gene might be present simultaneously in different brain regions (see for instance Vucetic et al., 2012). This would obviously not be reflected in blood as a surrogate tissue. It is even conceivable that diet-associated differences in gene expression could be specific to particular nutrients like saturated fatty acids (Hryhorczuk et al., 2016) and intake of other nutrients might counteract their influence. Thus, the effects of a mixed-style diet that is typically consumed by humans might be weaker. Tissue- and nutrient-specificity of dietary effects might be another potential reason why there were no differences in the mRNA expression of *DRD2/5* and *DARPP-32* and only trends for differences in the expression of *DRD3, COMT* and *DAT* in this study.

In this context it needs to be pointed out that the concept of this study is based on evidence in favor of using peripheral surrogate markers for the brain (see Table S14 in 9.1.1). However, there are several limitations and refutations to this approach that are discussed hereafter (for a literature

overview see Table S15 in section 9.1.1). This study's rationale hypothesized a general applicability of the idea that gene expression patterns observed in peripheral blood cells might be transferable to patterns in the brain. However, not for all genes of interest there is supporting evidence for a correlation between peripheral and central patterns yet. For instance, there are seemingly no studies available that directly assessed this correlation for *COMT* and *DARPP-32* and furthermore, there is even evidence speaking against a correlation between peripheral and central expression for *DAT* (Buttarelli et al., 2009) and *DRD2/5* (Kirillova et al., 2008). This might limit the comparability of peripheral and central findings. Further, the majority of studies supporting the use of peripheral markers are based on specific circumstances. Many study samples consisted of clinical subpopulations, e.g. patients with DA-related diseases like schizophrenia, Parkinson's disease, or addictions (see Table S14 in 9.1.1, evidence category 1). These studies provide good hints that the central DA system is at least to some extent reflected in the periphery but there is no direct mechanistic and generalizable proof for this idea. Additionally, a study analyzing correlations between the peripheral expression of genes in the DA and serotonin pathway (serotonin is another representative of the monoamine transmitter family) and central levels of monoamine metabolites found strong evidence for a correlation in the serotonin pathway whereas the correlation in the DA pathway was less strong (Luykx et al., 2016). This also speaks against a generalizability of findings from specific conditions to a broader framework.

Probably the most important limitation to consider in this context is that blood leukocytes itself are a heterogenous tissue with different cell types, i.e. granulocytes, lymphocytes, and monocytes, that further comprise several cell subtypes like T- and B-lymphocytes. Leukocytes are the cellular basis of the immune system and each cell subtype is enrolled in different functional processes of immunity like phagocytotic activity, antigen presentation, release of antimicrobial toxins or production of antibodies and cytokines. Further, DA especially in lymphocytes plays an important role in immune system regulation, e.g. of T-cell function and differentiation (Buttarelli et al., 2011; Levite, 2016) which is completely distinct from the function of DA as a neurotransmitter. For instance, DRD expression on T-lymphocytes seems to depend on resting or activity state of the T-cells (Levite, 2016). The immune activity of leukocytes in general might therefore affect the expression of DA-related genes independent of diet and these effects would thus not reflect the conditions in the brain. It might even be possible that the observed diet-related expression differences in the DA system of leukocytes might influence the inflammatory state of the body and that obesity-associated low-grade inflammation might be attributable to a dietary influence on leukocytes, but this remains speculative and cannot be answered within the framework of this study.

To what extent the DA system in peripheral blood cells is therefore actually comparable to the central DA status and if the assumption of similarity might only be applicable to particular genes or specific brain regions is not clear and needs careful validation in follow-up studies. For instance, post-mortem studies that confirm a correlation between diet-associated gene expression patterns in leukocytes and in the brain or preferably even in specific brain regions would help to overcome this limitation and establish reliable evidence. However, a post-mortem study with dietary context in healthy subjects will be exceptionally difficult to realize as it is seemingly impossible to find a suitable study sample of significant sample size. Another indirect but more feasible approach might be using imaging techniques like PET and SPECT scanning with specific markers for the genes of interest that would allow a comparison between centrally detected abundance levels of the gene product with the corresponding peripheral expression.

Transferability to epigenetics, proteins, and functionality

This study focused on the measurement of mRNA levels for gene expression analyses as a well-accepted standard procedure. However, mRNA is not always fully translated to the final protein and

thus amounts of mRNA do not necessarily correlate with corresponding protein expression (Levite, 2016; Pecqueur et al., 2001). This might be due to regulation processes on translational level or mRNA decay. Gene expression patterns measured in terms of mRNA levels should thus be compared to the actual protein level in future studies, e.g. with western blot analyses, to confirm transferability. Furthermore, protein abundance is not the only feature that determines functionality. For instance, COMT enzyme activity is affected by the genetic polymorphism rs4680 independent of the actual amount of COMT protein and therefore cognitive alterations associated with this SNP are usually not attributed to gene expression changes (Chen et al., 2004; Lotta et al., 1995). Another example is DARPP-32 that is part of a complex signaling network with various activators and inhibitors (see section 2.5.1). Thus, DARPP-32 protein levels alone are not conclusive about the functional effects of DARPP-32 in the complex downstream signaling pathway. It might further be interesting to expand analyses to the DNA level and study epigenetic correlates of observed gene expression patterns, e.g. if promotor methylation profiles are in line with observed up- or downregulations of the respective gene (similarly done in these studies: Carlin et al., 2013; Frieling et al., 2010; Vucetic et al., 2012. In sum, diet-related differences in the dopaminergic system might not necessarily appear on the level of mRNA expression and future studies combining epigenetic analyses with measurements of mRNA and protein expression as well as cognitive assessments would create a broader picture of the interplay between diet and the dopaminergic system.

6.2 No significant biasing influences of obesity and genotype on mRNA expression analyses

As described in section 2.5.2, there are several genetic polymorphisms (SNPs) that might influence dopaminergic signal transmission. Thus, an impact of genotype in DA-related SNPs on mRNA expression and potential interactions with diet needed to be excluded. ANOVAs controlling for such an effect indicated no significant interactions and thus genotype did seemingly not have highly disturbing influences on the mRNA expression analyses. The trend level interaction appearing between diet and genotype in the *FTO* SNP rs9939609 (surrogate: rs8050136) affecting the expression of *DRD2* might be worth following up on in a future study but cannot be addressed here any further, as the sample size is most likely too low for reliable analyses. However, a recent study found no association between variants in *FTO* SNP rs9939609 and availability of DRD2 measured with [18F]fallypride PET (Dang et al., 2018) which could also indicate that the trend might be false positive.

BMI and WHR were shown to be mediators or at least moderators of obesity-associated differences in gene expression within the DA system (Leite et al., 2016; Mansur et al., 2018; Ramos-Lopez, Riezu-Boj, Milagro, Martinez, & MENA Project, 2018c; Wu et al., 2017). Even though the groups were matched for BMI and WHR, correlation analyzes between measures of obesity and mRNA expression levels were carried out as control analyses independent of diet group. They suggested that BMI and WHR were negatively correlated with mRNA expression levels of both *DAT* (correlation with BMI and WHR) and *DRD3* (correlation with BMI only). To ensure that the trends for gene expression differences in *DAT* and *DRD3* between diet groups are not driven by obesity rather than diet, covariation analyses (ANCOVAs) were added which indicated that the trends are still present when including BMI or WHR as covariates. This supports that the observed trends for diet group differences in the mRNA expression of *DAT* and *DRD3* might be relevant and should be addressed in future studies.

6.3 Exploring links between DA-related gene expression and metabolic and inflammatory blood parameters

Links to metabolic blood parameters

Metabolic hormones such as insulin and leptin or fat- and sugar-related nutrients could potentially influence the DA system (for details see section 2.7.1) and might thus provide a potential mechanistic link between diet and the observed differences in DA-related gene expression. To approach this hypothesis, blood levels of glucose, insulin, HbA1c, leptin, triglycerides, and cholesterol were exploratorily correlated with mRNA expression levels of those genes that might be expressed differently between diet groups, i.e. *COMT, DAT* and *DRD3,* even though there were no diet group differences in all blood parameters.

The results indicated a potential trend for a positive correlation between blood levels of cholesterol and the mRNA expression of *DAT.* Cholesterol is an essential structural element of cell membranes and can thus be found almost everywhere in the body. Though cholesterol can generally be absorbed from the gut after food intake, the vast proportion of cholesterol is synthesized internally. Cholesterol abundance in the brain can be attributed almost exclusively to synthesis capacity and efficient recycling processes, as circulating cholesterol cannot cross the blood-brain-barrier (Björkhem & Meaney, 2004). Nevertheless, several studies found an association between peripheral cholesterol levels and the risk of Parkinson's disease, a disorder that is related to loss of dopaminergic neurons in the midbrain (Hu, Antikainen, Jousilahti, Kivipelto, & Tuomilehto, 2008; Huang et al., 2019; Lau, Koudstaal, Hofman, & Breteler, 2006). It is therefore not clear if and how peripheral cholesterol levels might have a relevant direct or indirect impact on central DA-related processes. A direct link between cholesterol and peripheral or central gene expression of *DAT* has seemingly not been shown yet, and so far cholesterol as an integral cell membrane component has been only theoretically found to affect DAT functioning (Jones, Zhen, & Reith, 2012; Rahbek-Clemmensen et al., 2017; Zeppelin, Ladefoged, Sinning, Periole, & Schiøtt, 2018). The physiological role that cholesterol might play in *DAT* mRNA expression therefore remains elusive and might be targeted in future studies, especially as this finding is only a trend and could therefore also be false positive.

For markers of glucose metabolism, i.e. plasma glucose levels, insulin levels, and HbA1c there were no significant correlations with mRNA expression of *COMT*, *DAT* or *DRD3*. This indicates that either glucose metabolism might not play a role in the regulation of mRNA expression of these genes or an effect is not detectable with the applied methods. In line, relationships between COMT and glucose metabolism, specifically glucose intolerance, have mainly been studied for *COMT* genotype (Hall. et al., 2016) or for COMT function in organs other than brain and blood cells, as the enzymatic degradation function of COMT is not restricted to DA in brain and blood (Kanasaki et al., 2017). An association with *COMT* gene expression has seemingly not been reported so far. Contrarily, *DAT* mRNA expression was found to be affected by insulin specifically. Elevated insulin levels were associated with increased central *DAT* mRNA expression (Figlewicz, Szot, Chavez, Woods, & Veith, 1994) as well as protein expression on the cell surface (Jones et al., 2017; Kleinridders & Pothos, 2019) and central DAT availability (Pak et al., 2020) and activity (Baladi et al., 2015). The observation in this study sample, however, points if at all in the opposite direction with a weak and not significant negative correlation between insulin levels and *DAT* expression. It is conceivable that insulin action in the periphery has a different effect on *DAT* gene expression than central insulin signaling or that *DAT* mRNA expression in peripheral blood cells is not comparable with the central *DAT* status, but a meaningful conclusion cannot yet be drawn here. Regarding dopamine receptors, associations with glucose metabolism were found before, e.g. a negative association between insulin sensitivity and DRD2 availability in the ventral striatum (Dunn et al., 2012) or a negative correlation of peripheral *DRD4* mRNA expression with HbA1c.

However, there is seemingly no literature showing such a direct link for *DRD3* in particular and a study even suggested no association between HbA1c and the expression of *DRD3* (Leite et al., 2016) which would be in line with the lack of correlation regarding *DRD3* in this study.

There were also no significant correlations between plasma levels of leptin or triglycerides and the expression of *COMT, DAT* or *DRD3* in this sample. For *DRD3*, this is in line with Leite et al. who reported associations of leptin levels with peripheral expression of *DRD2* and *DRD5*, but not with the expression of *DRD3* (Leite et al., 2016). However, as there is literature supporting an association between fat metabolism and the regulation of DA-related gene expression (Dunn et al., 2012; Leite et al., 2016) this relationship needs further exploration in the future.

Link to low-grade inflammation

Besides the potential direct metabolic influence of HFS diet on the DA system, literature indicates that low-grade inflammation in association with HFS diet intake (Cândido et al., 2018) might affect DA-related cognitive functions (Felger et al., 2013; Felger & Miller, 2012; Treadway et al., 2017) and thus may also impact peripheral gene expression. To explore this relationship, correlations between blood levels of the inflammatory marker CRP and mRNA expression levels of *COMT, DAT* and *DRD3* were analyzed. The results partly support the idea of a potential influence of inflammation on DA-related gene expression, as blood levels of CRP correlated negatively with the mRNA expression of *DRD3* on a trend level. However, this correlation needs to be interpreted with care as firstly it did not reach statistical significance and secondly the observed CRP levels were generally very low or even below the detection threshold, although a highly sensitive CRP measurement was applied. This leads to an accumulation of most data points around the level of the detection threshold and the correlations are therefore strongly driven by a few data points reflecting slightly higher CRP levels. The correlations could thereby be biased by single cases and might not show a general effect. Additionally, this association is not in line with the results of a study with centrally obese individuals where mRNA levels of *DRD2* and *DRD5* but not of *DRD3* were associated with inflammatory leukocyte patterns (Leite et al., 2016). This could however also be explained by methodological differences as Leite et al. did not analyze inflammatory serum markers and therefore their finding might not reflect the same framework that was assessed here. A negative effect of inflammatory cytokines in the serum on striatal availability of *DRD2* (PET imaging) was found both in non-human primates (Felger et al., 2013) and in obese patients (Felger & Treadway, 2017) but a direct link with *DRD3* expression in particular seems not to be addressed yet. This link between diet, inflammation and DA might be worth exploring in follow-up studies even though it cannot be excluded that the potential association between *DRD3* and CRP might be attributable to the involvement of DA in general inflammatory regulation unrelated to neurotransmission. This is especially relevant as CRP levels were not significantly different between the HFS and the LFS diet group indicating that diet did not have a strong impact on inflammatory state in the HFS group as far as it is reflected by CRP.

Further, there were no correlations between the mRNA expression of *COMT* or *DAT* and CRP levels. Findings of other studies nevertheless pointed towards an association of inflammation with the expression of these genes as for instance the central protein expression of *COMT* was increased after application of inflammatory stimuli (Helkamaa et al., 2007). However, the comparability of this finding with peripheral *COMT* mRNA expression might be highly limited by methodological differences as Helkamaa et al. firstly measured protein levels and secondly analyzed gene expression centrally in the substantia nigra of rodents after central stimulus application. Inflammatory effects on *DAT* expression were reported but the direction of effects is not clear. Recent reviews either suggest increased expression of *DAT* associated with neuroinflammation (Felger & Treadway, 2017) or argue that obesity-associated decreases in *DAT* expression (Wu et al., 2017) might be due to obesity-related inflammatory

signals (Leite & Ribeiro, 2019). More research is thus necessary to create a reliable picture of a potential relationship between low-grade inflammation and the DA system, a convincing conclusion cannot be drawn here.

6.4 Exploratory analysis of diet group differences in UCPs

UCP-1 was found to be involved in the regulation of energy expenditure and to influence the susceptibility for diet-induced obesity in mice (Labouesse et al., 2018) but only *UCP-2* and *UCP-3* are expressed in blood cells. Thus, the expression of *UCP-2* and *UCP-3* was exploratory analysed for differences related to HFS diet consumption, even though the UCPs are not really comparable (see section 2.10). And indeed, the HFS diet group showed significantly higher mRNA levels of *UCP-2* compared to LFS dieters, whereas the expression of *UCP-3* was significantly lower. The translation of these results to a functional relevance in leukocytes is difficult though, as both *UCP-2* and *UCP-3* mRNA is not translated into a functional protein in blood cells (Pecqueur et al., 2001; Tissue expression of UCP2 - Summary - The Human Protein Atlas, 2015; Tissue expression of UCP3 - Summary - The Human Protein Atlas, 2015; Uhlén et al., 2015). If the regulation of mRNA expression of UCPs in blood cells would be transferrable to the expression regulation in other tissues, it could be speculated that *UCP-2* might be generally upregulated in response to high fat and sugar intake as this diet might cause oxidative stress (Alzoubi et al., 2018) and UCP-2 seems to be capable of counteracting oxidative stress by decreasing the production of reactive oxygen species (Esteves & Brand, 2005). Similarly, a general downregulation of *UCP-3* could be explained by an abundance of energy due to high fat and sugar consumption that decreases the necessity of fatty acid turnover for energy supply. If less fatty acid turnover is needed, the transportation of fatty acids via UCP-3 out of the mitochondria, which allows higher rates of fatty-acid oxidation (Brand & Esteves, 2005), might be less necessary and *UCP-3* could in turn be downregulated. These findings might provide hints for an interaction between HFS diet and expression regulation of UCPs, but the speculations about a functional relevance and other relationships maybe even in the CNS would surely need further approval.

6.5 Cognitive phenotype related to HFS diet intake

6.5.1 Diet groups are not different in neuropsychological test performance

Analyzing mRNA expression of DA-related genes in peripheral blood cells can provide, if at all, only indirect hints about the conditions in the CNS. It was therefore planned to link observed peripheral gene expression patterns with a cognitive phenotype. However, the diet groups were relatively similar in performance of all administered cognitive tests of executive functioning. Therefore, this link between peripheral mRNA expression and cognition could not be established.

Higher task sensitivity might increase the chance of detecting potential diet-related cognitive differences

Even though the results suggest that HFS diet actually does not influence executive functioning, the putatively most likely explanation for not finding differences in test performance is that the sensitivity of the administered tasks was too low to detect potential subtle diet-related cognitive differences in otherwise healthy young subjects. In this context it needs to be considered what the neuropsychological tests were originally intended for. The Trail making test (TMT) is part of the Halstead-Reitan Neuropsychological Battery which was designed to assess brain injuries. It is reliably detecting Alzheimer’s disease or dementia but is not sufficient to detect mild cognitive impairment

(Wei et al., 2018). Similarly, the Digit symbol substitution test (DSST) was found to be a highly sensitive task to detect cognitive dysfunction in clinical patients, e.g. with psychiatric diseases, and can also detected fluctuations in cognitive functions (Jaeger, 2018), but it is difficult to tell how big a potential impact of diet might be on the performance in the TMT and the DSST compared to the impact of clinical disorders and brain injuries. The Digit span test (DS) is one subtest of the Wechsler Adult Intelligence Scale (WAIS), a complex IQ test battery, and thus the DS might be most meaningful only in combination with other subtests of the WAIS. In line, a study testing sensitivity of different working memory tasks found that working memory is best assessed when tasks are combined that add a visual component and require more complex working memory manipulation than the DS alone (Egeland, 2015).

Nevertheless, different studies found impairments in the neuropsychological tests used in this study in association with obesity. Gunstad et al. found a significant correlation between BMI and performance in the DS subscale forward and the TMT (Gunstad et al., 2007). Further, a significant difference between lean and obese adolescents was found for performance in both part A and part B of the TMT (Maayan et al., 2011) as well as in the TMT difference score B-A (Verdejo-García et al., 2010). Yau et al. detected at least a trend level difference between obese and lean adolescents without metabolic syndrome in both the TMT and the DSST (Yau et al., 2014) and the effect of obesity on all three tests also applies for older adults (Cohen et al., 2011). It might thus be possible that obesity-associated cognitive impairments (Yang et al., 2018) are not caused by diet but by other traits of this condition that develop later during obesity-establishment or that the cognitive impairments are actually the reason for acquiring excessive weight. When analyzing mainly normal-weight or mildly obese subjects as in this study, cognitive impairments might not be present yet. However, it seems also plausible that obesity as a complex metabolic condition has a higher impact on cognition than HFS diet alone and studies with obese subjects are more likely to find cognitive impairments also in the rather unsensitive neuropsychological tests. In line, there are examples in the literature that indicate a detectable effect of HFS diet on cognitive functions when using other cognitive assessments. For instance, a review suggested that consumption of “Western style” diet impairs hippocampal function and hippocampus-related learning and memory (Kanoski & Davidson, 2011) and one study even showed that HFS diet, determined by the DFS score as in this study, is related to specific memory-related dysfunctions in generally healthy subjects consuming HFS diet (Francis & Stevenson, 2011). It can therefore be assumed that diet has a relevant impact on cognitive performance at least in the working memory domain. Other more complex and putatively more sensitive tasks are available to assess executive functioning, e.g. the Wisconsin card sorting task for cognitive flexibility (Yang et al., 2018), the N-back task as an alternative to the DS for working memory (Gonzales et al., 2010), and the Test of Everyday Attention as a complex measure of attention that might be more informative than the DSST (Robertson, Ward, Ridgeway, & Nimmo-Smith, 1996). Future studies should consider using these or other highly sensitive tasks of executive functioning to increase the probability of detecting subtle cognitive changes related to HFS diet if differences are actually present.

External and internal influences might overwrite a dietary effect

It needs to be considered that influences other than diet might have had an impact on cognitive performance of some participants that might overwrite potential dietary effects. There may be unknown or unquantifiable influences on the experimental conditions during cognitive testing that cannot always be excluded or controlled for, e.g. occupational or emotional stress (Feuerhahn, Stamov-Roßnagel, Wolfram, Bellingrath, & Kudielka, 2013; Özdemir et al., 2013) or distraction due to noise. These factors could cause unspecific variability in obtained cognitive measures and further decrease the chance of detecting diet-related differences with already putatively low effect size.

Additionally, genetic polymorphisms might cause baseline differences in dopaminergic signal transmission and thus affect cognitive performance (see section 2.5.2). ANOVAs were therefore used to test potential genetic influences of SNPs on NP test performance. The results showed significant interactions between diet and genotype in *DRD2* SNP rs6277 for all three neuropsychological tests. In detail, within the heterozygous allele group of T/C-carriers, HFS dieters performed worse in all three neuropsychological tests than LFS dieters but this is significant only for the TMT B. Within the groups of both homozygous T/T- and C/C-carriers, diet had the opposite effect such that HFS dieters performed partly better, but this was significant only for C/C-carriers in the DSST and for T/T carriers in the DS backwards. This would mean that HFS diet would have a disadvantageous effect on executive functioning but only in combination with heterozygous presence of both the T and the C allele in the SNP rs6277. When carrying either two T alleles or two C alleles, HFS diet would improve executive performance. It is striking that this interaction with diet is present only for this SNP and then again for all neuropsychological tests, speaking against a false positive finding, but especially in the light of the previously described “gene dose effect” (Smith et al., 2017) these observations are hard to interpret. Nevertheless, it might be worth further studying the influence of this SNP on cognition in the dietary context.

6.5.2 Diet-independent main effects of DA-related SNPs on TMT B performance

This study was not designed to analyze isolated effects of DA-related SNPs on neuropsychological test performance, as the size of the study sample is most likely not sufficient to reliably detect those effects. Genetic studies usually have much bigger samples with more than 100 participants (Berryhill et al., 2013; Cardel et al., 2019). Nevertheless, potentially interesting significant genotype effects on TMT B performance appeared in the *diet x genotype* ANOVAs which were planned only as control analyses and these effects might therefore still be worth mentioning.

Firstly, repeat length of the DAT *VNTR* had a significant diet-independent effect on performance of the TMT B. Homozygous carriers of the 10-repeats variant on average needed more time to complete the test than carriers of the 9-repeats variant which generally fits with the previous finding that homozygous carriers of the 9-repeat variant have better cognitive flexibility (Fagundo et al., 2014). However, Fagundo et al. used both the Wisconsin card sorting task (WCST) and the TMT to assess cognitive flexibility and found a significant effect of the VNTR repeat length only in the WCST and not in the TMT, but as their study sample consisted of patients diagnosed with pathological gambling, the transferability of this finding could be limited. Furthermore, TMT B performance was additionally affected by genotype in *FTO* SNP rs9939609 (surrogate: rs8050136) such that A/T-carriers (surrogate: A/C) performed significantly better than T/T-carriers (surrogate: C/C). This connection between genotypes in the *FTO* SNP rs9939609 and cognitive flexibility has seemingly not been described before and might be a target for further exploration, especially as it is unclear what impact the A and T allele alone might have on cognitive functioning, as the homozygous groups (A/A- and T/T-carriers) did not differ significantly in TMT B performance. Nevertheless, also a false positive effect needs to be considered in that context.

6.6 Diet group characterization for personality traits and eating behavior

Self-reported questionnaires were used to further characterize the diet groups. Regarding eating behavior, the HFS group scored lower in the subscale “restrain” and higher in the subscale “hunger” of the Three-Factor Eating Questionnaire (Pokorny, 2011; Pudel & Westenhöfer, 1989) indicating less

cognitive control over food consumption and more prominent subjective feelings of hunger in the HFS group. These observations replicate previous findings that were obtained in a study with healthy young women and similar study design (Hartmann et al., 2019). Additionally, the HFS group had higher scores in the subscale "cues" of the Food Craving Questionnaire-Trait (Meule, Lutz et al., 2012). This subscale describes the reactivity to environmental cues that trigger cravings to consume food and the results therefore suggest that HFS dieters are actually more vulnerable to external stimuli that disturb their eating behavior. Finally, the HFS group showed a tendency for higher scores in the "reinforcement" subscale of the Food Craving Questionnaire-Trait that represents higher anticipation of reward-related reinforcement when consuming food. In sum, these characteristics of eating behaviour in the HFS group would fit with the expectation that HFS dieters have lower abilities to constantly control their food consumption. This could in turn lead to increased intake of palatable food items which are typically those high in fat and/or sugar. For interpretation it needs to be considered that due to multiple testing only the group difference in the FEV subscale "Restrain" is significant and all other potential group differences can only be regarded as trends.

Questionnaires assessing personality traits revealed no significant difference between diet groups regarding inhibition and approach behavior (BIS/BAS), impulsivity (BIS15 and UPPS), and basic personality traits, i.e. extraversion, openness, conscientiousness, and agreeableness (NEO-FFI). Similar to the previous study by Hartmann et al. the HFS group scored tendentially higher in the NEO-FFI subscale "neuroticism" (Hartmann et al., 2019), but this difference was not significant. Taken together, this implicates that the study sample is relatively homogenous on a behavioral and personality level and the main difference between the groups can be found in the factor diet/eating behavior.

6.7 No indications for a connection between the dopaminergic system and physical activity

Animal studies indicated an influence of dopaminergic signaling on PA as for instance lower abundance of DRD2 decreased the motivation to move in studies with *DRD2* knockout mice (Beeler et al., 2016; Friend et al., 2017). To explore this potential connection, the *DRD2*-related SNPs rs1800497 (Gluskin & Mickey, 2016; Jönsson et al., 1999b) and rs6277 (Duan et al., 2003) as well as the *FTO* SNPs rs9939609 and rs8053740 (Sun et al., 2017) were tested for an association with step count or the IPAQ score, as they might influence DRD2 abundance and function, but there was no significant association between any genotype and the PA outcomes. This might, however, also be attributable to the low sample size or the experimental conditions of the PA measurement. PA in a human sample might be highly confounded by social obligations and activities, such as the level of activity during work, regular exercise in sports clubs, the amount of free-time besides social duties and personal interests in sports and movement. PA measured in freely moving animals by contrast putatively mainly captures spontaneous PA driven by the intrinsic motivation to move. Further, the variability in activity levels was restricted due to the exclusion criterion which could be another reason why potential influences of SNP genotypes might not be detectable in this sample.

Noticeably, step count and the MET minutes score did not correlate as well as expected. It is thus possible that the measurements capture different activities as for instance the pedometer was not suitable for carrying during team sports or water activities which should be comprised in the MET minutes score. Nevertheless, it is also conceivable that self-reporting of activities and the use of a simple pedometer that is activated also by shaking or biking and not only by actual walking might lead to inaccuracy of the measurement that could in turn even reduce observable effects. Future studies should consider more accurate measures of PA that capture a wide range of different activity types and maybe even combine different measures to one score to obtain more reliable evidence for or against interactions between PA and dopaminergic signaling.

6.8 Limitations and implications for further human research

6.8.1 Transferability of findings from this study sample to a general population

There are several features in the design and sample acquisition process of this study that might limit generalizability and transferability of the obtained results. Firstly, the study sample comprised disproportionally many students compared to the average population (~56% in the HFS group and ~74% in the LFS group), most likely because students are most responsive to study advertisements. However, serious difficulties in recruiting especially participants for the LFS diet group did not allow slowing down recruitment of students for the sake of a more heterogenous sample as this would have threatened the feasibility of reaching the already tightly planned sample size. Additionally, earlier studies indicated potentially interesting effects of age (Dang et al., 2016) and gender (Carlin et al., 2013) that cannot be addressed in this study as the sample consisted of relatively young (18 and 37 years) male participants. This approach aimed to minimize unspecific variability in the DA system as both the menstrual cycle (Hidalgo-Lopez & Pletzer, 2017) and age (Volkow et al., 1998) were found to impact dopaminergic signaling. Further, with the cross-sectional study design it is not possible to analyze the interesting impact of long-term vs. short-term exposure to HFS diet (Cone et al., 2013). Finally, a known impact especially on the reward system is the use of illicit drugs. Drug intake was thus an exclusion criterion for the study, but it might still be possible that some participants did not truly report recent drug intake, e.g. due to shame or fear of consequences when admitting the consumption of illegal drugs and even intentional false reporting might be conceivable to not be excluded from the study. This is supported by the LEMON dataset (Babayan et al., 2019) where 12 out of 227 participants had urine samples positive for illicit drugs (7 for THC, 4 for benzodiazepines and 1 for morphine/heroine) even though all participants reported to not take any drugs. There is no proof for the occurrence of such cases within the sample of this study, but it can also not be excluded. Therefore, it might be worth using an estimate of drug use such as urine samples in future studies and analyze it as a confounder to quantify the yet unknown and most likely highly individual impact of drug use on the DA system and with that attenuate this unknown source of variation.

6.8.2 Validity of the DFS questionnaire as a grouping variable

Using a self-reported measure of food intake for grouping of participants needs to be discussed as a potential source of undesired variability in the data. Some participants had highly deviating DFS scores between the first and last test day even though the DFS questionnaire was shown to have sufficient test-retest reliability (Fromm & Horstmann, 2019). Within the LFS group the reliability of DFS scoring even decreased when participants scored higher and thus closer to the cut-off for the LFS group. Even though the DFS score is based on recall of average food consumption frequencies over the last 12 months, it might naturally be highly influenced by food consumption within the last few months, weeks or even days. This could potentially explain uncertainties in recall of consumed food items or consume frequencies. It might also be possible that reflecting about consuming frequencies on the screening day leads to higher awareness for food intake during the following days and weeks and thus participants might give different answers in the repeated measurement. To account for this unreliability, the analyses of diet-associated differences in the main outcome parameters, i.e. mRNA expression and cognition, were repeated with weighted data points according to the consistency of DFS scores of the respective participant. In line with the results before weighting, there is still no significant diet group difference in *DARPP-32*, *DRD2,* and *DRD5* expression, but the trends in the expression of *COMT, DAT*, and *DRD3* as well as the significant difference in *UCP-3* expression become more pronounced after weighting. The significant group difference for *UCP-2* mRNA expression

however disappears. Regarding cognitive performance, when including weighting HFS diet has a significant effect on performance of the DSST and a trend might be present for DS backwards, whereas performance of the TMT B is still not significantly different between diet groups. It is possible that these results reflect the true effects of diet on DA-related mRNA expression and cognition better than the unweighted results as the input parameter should be more reliable, but a more consistent measure of food intake is necessary to confirm the reproducibility of the observed dietary effects after weighting.

Furthermore, the DFS questionnaire is only a rough estimate of fat and sugar intake. It only assesses 26 specific food items, e.g. beef, nachos, bolognaise, bacon, pizza, cookies, chocolate, or energy drinks and it might well be that particular food items with high fat or sugar content are not comprised in the DFS score. An exceptional preference for such food items or very high intake of only a few food items that are part of the score would lead to an underestimation of fat/sugar consumption. Additionally, one would expect differences in metabolic blood parameters such as HbA1c, triglycerides or cholesterol (Moreno-Fernández et al., 2018) between the HFS and the LFS diet group, if the diets were substantially different, which is not the case in this study sample. A more comprehensive questionnaire, a food diary or a dietary intervention study with controlled food application could increase precision of the HFS vs. LFS grouping and should be considered in future studies.

Another striking feature of the study population regards the obesity measures. BMI, WHR and weight were matched between diet groups to be able to analyze dietary effects independent of actual obesity. Nevertheless, when considering the increased proportion of fat, sugar and therefore potentially also calories in the HFS diet, one would assume at least a tendency for higher BMI/WHR/weight values in the HFS group. However, the LFS group actually exhibits slightly higher values in these measures. There are several potential explanations for this observation. One would be that the DFS questionnaire assesses only consumption frequency of specific food items but not portion size or general food intake. It might be possible that at least some participants in the LFS group have a high total calorie intake leading to a higher weight. Additionally, it is conceivable that there are subjects within the LFS sample that were formerly obese and lost weight due to LFS dieting more than one year ago but still have a higher weight set point despite LFS dieting. In case that this explanation would be applicable for some participants, the DFS grouping would still be valid as the LFS diet was maintained for more than one year and potential LFS diet effects should thus be stabilized. Finally, BMI and weight are not directly conclusive about the contribution of fat and muscle mass to the actual weight. It is possible that, even though the groups are not different in their physical activity, at least some participants in the LFS group have a higher percentage of muscle mass leading to a higher weight/BMI that is not attributable to body fat. A more complex assessment of obesity measures including not only BMI, weight, and WHR but also a measurement of body fat mass or fat percentage might be able to add valuable information about HFS and LFS diet group characteristics regarding body composition.

6.8.3 Limitations of measuring gene expression with qPCR

The first important limitation of the mRNA expression measurement in this study is the generally high fluctuation of obtained mRNA expression values between participants. The highest and lowest raw cT values deviated up to approximately six units from the corresponding mean cT value and even for the standardized mRNA levels, strong deviations of obtained minimal or maximal values from the mean were present. This is in line with the wide between-sample variation that another study observed for mRNA expression of DRDs in peripheral blood cells (Kirillova et al., 2008). These deviations might be explained by natural fluctuations as blood is a biological tissue that is subject to unspecific external influences. Additionally, heterogeneity and inaccuracies in acquisition and processing of blood samples

and in the mRNA expression measurement itself cannot be excluded, even though standard operating procedures were used to optimally standardize the process. The normalization procedure described in section 4.4.6 aims to reduce the impact of these external influences, but some residual inhomogeneity might remain. Another issue regarding the qPCR measurement is the considerable number of dropouts for some genes of interest. For instance, for *DRD2* there was a particularly high number of dropouts with in total 24 undetermined values. A potential explanation for this could be that DA-related genes like *DRD2* are in general expressed relatively low in peripheral blood cells (Gene page - DRD, GTEx Portal, 2016) and some expression levels might therefore be below the detection threshold. This not only decreases statistical power of the gene expression analyses but could additionally induce a bias. Based on the assumption that many of the undetermined values might be those below the detection threshold, a relatively huge amount of low mRNA values would be missing. If there is an imbalance of undetectable values between diet groups, this might influence the statistical analysis. For instance, five values for *DAT* expression are missing in the LFS group and only two in the HFS group. Given the trend for lower *DAT* levels in the LFS group, it seems possible that if five low values in the LFS group would be missing, the detection of these values would have led to more a pronounced diet group difference. Nevertheless, this is pure speculation and there is no proof that no other issues like technical failure caused the dropouts. Furthermore, the low mRNA expression levels of the genes of interest made it necessary to use relatively high amounts of template cDNA for the qPCR measurement. This left not enough material for double or even triple measurements which would have reduced the fluctuation in measured values due to technical issues. Taken together, despite utmost care in standardizing blood processing procedures and the mRNA expression measurement, the high variability of measured mRNA levels might mask subtle diet-associated differences in gene expression. A higher sample size and anticipation of the need for a considerable amount of template RNA might improve the statistical power of future studies.

6.8.4 Causality

As this study has a cross-sectional design, it is not possible to draw evidence-based causal conclusions about an association between HFS diet and DA-related gene expression. Two main causal mechanisms potentially linking HFS diet, obesity and DA are most but not exclusively conceivable. On the one hand, HFS diet could induce changes in the DA system that lead to maintenance of obesity-promoting behavior, thus causing long-term weight gain and obesity establishment. Reversely, diet-independent baseline differences in the DA system e.g. due to genetic variability, might lead to differences in cognitive control, reward sensitivity and motivational features and in turn increase or decrease the individual susceptibility for diet-induced obesity. Longitudinal studies with dietary interventions including cross-over conditions between HFS and LFS diet or long-term follow-up studies potentially even from childhood on could narrow down causal chains and unveil underlying mechanisms for potential diet- and obesity-associated alterations in the DA system.

6.9 Conclusion

In summary, this study provides some hints that HFS diet intake might be associated with alterations in the dopaminergic signaling system, i.e. in the expression of *DRD3, DAT* and *COMT,* but to what extent these findings actually reflect true dietary effects, are transferable to the brain and might be relevant for cognitive functions and which mechanistic processes might link HFS diet with gene expression in the DA system remains elusive. Nevertheless, this study offers several targets for future research to further explore the interplay between diet, obesity, and dopamine. A better understanding of this interplay might have implications for the prevention and treatment of obesity in the future.

7. Summary

Dissertation zur Erlangung des akademischen Grades Dr. med.

Titel: The interplay between a dietary preference for fat and sugar, gene expression in the dopaminergic system and executive cognition in humans

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Obesity is a public as well as individual health issue of increasing importance. The severity of obesity-associated comorbidities such as cardiovascular diseases, diabetes mellitus type II, cancer or even major depression is well established by now. In fact, individuals with obesity are often aware of these impending negative consequences and feel the wish to lose weight by dieting or doing sports, but many fail to incorporate their intent into everyday life. This neglect of long-term goals for the benefit of rewarding short-termed pleasures such as palatable food may be at least partly attributable to deficits in inhibitory control of behavior and alterations in reward-based decision-making that were found in association with obesity. The cognitive processes allowing goal-directed behavioral control are called executive functions and include e.g. selective attention, working memory, and cognitive flexibility. These are modulated through influences from the neurotransmitter dopamine. Accumulating evidence from rodent literature indicates that changes in the dopaminergic system, e.g. in gene expression, are in fact already present after exposure to a diet high in fat and sugar independent of actual body weight. Diet might thus provide a mechanistic basis linking obesity with the observed differences in dopaminergic signaling. However, evidence for this link in humans is scarce and by far not extensive enough to show a direct connection or even draw causal conclusions.

The aim of this study is thus to contribute to filling this gap in literature by exploring associations between dietary fat and sugar intake and dopamine-related gene expression and cognition in a human sample (n = 75). Based on a cross-sectional study design, the participants were grouped according to their self-reported dietary fat and sugar consumption (high fat and sugar vs. low fat and sugar diet group) and the groups were matched for measures of obesity (weight, BMI and waist-to-hip ratio).
Using quantitative polymerase chain reaction (qPCR), the mRNA expression of the following genes within the dopaminergic system was analyzed in peripheral blood cells, which have been suggested as a potential surrogate for the brain:

- *dopamine receptors D2/D3/D5* (*DRD 2/3/5*), G-protein coupled receptors for dopaminergic signal transmission,
- *dopamine-and cAMP-regulated neuronal phosphoprotein 32* (*DARPP-32*), a central player in postsynaptic downstream signaling,
- *dopamine transporter* (*DAT*) which is relevant for dopamine reuptake from the synaptic cleft and thus signal termination,
- and *catechol-O-methyltransferase* (*COMT*), a dopamine degradation enzyme.

For the assessment of executive functions, three basic neuropsychological tests were administered:

- the *Trail making test part A and B* assessing e.g. cognitive flexibility (especially part B),
- the *Digit symbol substitution test* capturing mainly processing speed and attention,
- and the *Digit span tests forward and backward* as a working memory assessment.

Further, the following single nucleotide polymorphisms (SNPs) and a various number tandem repeat (VNTR) in DA-related genes were genotyped and added as covariates to the analyses to control for potential confounding effects of genotype on mRNA expression and cognitive performance:

- rs4680 in *COMT,*
- rs1800497 (Taq1A polymorphism) and rs6277, both related to *DRD2,*
- rs9939609 and rs8053740 in *FTO,*
- rs907094 in *DARPP-32,*
- and rs28363170, a VNTR in *DAT*/*SLC6A3*.

Additionally, metabolic parameters, i.e. glucose, HbA1c, insulin, leptin, triglycerides, cholesterol, and the inflammatory marker C-reactive protein (CRP) were tested for differences between diet groups and for correlations with mRNA expression to explore potential underlying mechanistic connections between diet, metabolism/inflammation, and gene expression.

Results & Discussion

The qPCR data showed no significant diet group differences in the mRNA expression of *DARPP-32, DRD2*, and *DRD5* and only potential non-significant trends for group differences in *DRD3, COMT* and *DAT*. Genetic polymorphisms also had no significant impact on the corresponding mRNA expression levels. The hypothesized diet-related differences in gene expression are therefore not supported by the present data. Thus, it needs to be considered that changes in the dopaminergic system described in association with obesity could instead occur in response to developing obesity and might not be driven by a high fat and sugar diet already before obesity establishment. However, the trends might still point towards a relevant dietary influence. It is conceivable that the discriminatory power and accuracy of grouping based on self-reported eating preferences is not strong enough to reliably detect a dietary impact. In fact, studies with alternative designs were able to report clearer results: gene expression differences might have emerged more pronouncedly in samples comparing obese with normal weight individuals, as obesity is a condition affecting the whole body and might have stronger effects on gene expression than diet alone and further, in experiments with animals, it is possible to

apply diets with very high concentrations of fat/sugar and at the same time reduce external (confounding) influences to a minimum. Potential diet-associated effects in this study sample might therefore be masked by high unspecific background variation. Additionally, it is to mention that the observed trends only partly fit with the findings in rodents that were exposed to a high fat/sugar diet. Such conflicts might be explained by methodological differences between the studies, as gene expression in the animals was mainly assessed directly in the brain and not in blood cells. Although there are good hints for a correlation between peripheral and central DA-related gene expression patterns at least to some extent, the validity of blood cells as a surrogate for the brain is not unequivocally supported by the literature. The main objection is that gene expression regulation might be specific to tissues and even different brain regions and might therefore not be comparable between different tissues types. Additionally, leukocytes are involved in the body's immune response and thus the function of dopamine in leukocytes might be completely distinct from its function as a neurotransmitter in the brain. Thus, the use of surrogate tissue is a major uncertainty of this study and the abovementioned indications for an association between diet and gene expression need careful validation in future studies. For instance, the use of post-mortem human brain samples or imaging techniques like PET scanning with specific markers for the dopaminergic system might help to confirm or disprove the findings reported here.

Blood parameters regarding glucose or fat metabolism (glucose, insulin, HbA1c, leptin, triglycerides, and cholesterol) and inflammation (CRP) did not differ significantly between the diet groups. The correlation analyses between these parameters and mRNA expression of *COMT, DAT*, and *DRD3* independent of diet group, however, showed trends for a positive correlation between blood levels of cholesterol and the mRNA expression of *DAT* as well as for a negative correlation between CRP and the expression of *DRD3*. Even though the correlations were rather weak and not statistically significant, these associations might still indicate an involvement of metabolic and inflammatory state in gene expression regulation within the dopaminergic system. Studies with a comparable design, however, are too scarce to sufficiently underpin or disprove this hypothesis. Thus, future projects with a higher sample size, a longitudinal design or even dietary interventions including cross-over conditions between high-fat/sugar and low-fat/sugar diet are necessary to disentangle if glucose or fat metabolism and inflammation might be involved in mechanistically connecting diet and the dopaminergic system.

A link to cognition could not be established with the tasks used in this study as the performance in all neuropsychological tests was not significantly different between diet groups. This may be due to the low sensitivity of the applied tasks but could also indicate that the intake of fat and sugar does not have an impact on executive functioning. The use of more sensitive tasks might thus increase the probability of detecting subtle cognitive changes related to diet if such differences actually exist.

Taken together, this study provides some hints that high dietary fat and sugar intake might be associated with alterations in the dopaminergic signaling system on a gene expression level. However, the diet group differences were not statistically significant, and it further remains uncertain to what extent these findings are translatable to the brain and might be relevant for cognitive functions. Yet, these preliminary results offer several targets for future research to further explore the interplay between diet, obesity, and the dopaminergic system with potential impact for the prevention and treatment of obesity.

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9. Attachments

9.1 Supplementary materials

9.1.1 Evidence for and against the use of peripheral surrogate markers

Table S14: Evidence for the use of peripheral surrogate markers. Leukocytes from a peripheral blood sample were used in this study as a surrogate tissue for the brain to study dietary effects on the dopaminergic system that might apply for the central physiological processes as well. This table summarizes evidence in favor of this approach. Abbreviations: CSF = cerebrospinal fluid, PANSS = Positive and Negative Syndrome Scale, PBL = peripheral blood lymphocytes, PD = Parkinson's disease. Evidence category: 1 = related to neuropsychiatric disorder, 2 = direct comparison between brain and blood (post-mortem or indirect), 3 = similar methodology/reasoning.
* = most relevant studies

*Evidence **for** the validity of a peripheral proxy*				
Evidence category	**Publication**	**Sample**	**Method**	**Finding**
1	(Barbanti et al., 1999)	De novo **Parkinson's disease** (PD) patients (n = 50) vs. control patients with other neurodegenerative disease (n = 36) vs. healthy controls (n = 26)	Radioligand binding assay	Higher density of D1-like and D2-like receptors on PBL in PD patients vs. both controls
1	(Barbanti et al., 2000)	**Migraine** patients (n = 25) vs. controls (n = 20)	Radioligand binding assays	Higher density of DRD3 and DRD4 on peripheral blood lymphocytes (PBL) in patients vs. controls
1	(Caronti et al., 1999)	**Parkinson's disease** patients (n = 25) vs. controls (n = 12)	DA quantification + TH immunoreactivity	Reduced DA content and tyrosine hydroxylase immunoreactivity in PBL of PD patients vs. controls
1	(Cui et al., 2015)	Patients with currently diagnosed **psychosis** (n = 32) vs. controls (n = 31)	mRNA expression	Higher *DRD3* mRNA expression in T-lymphocytes of patients vs. controls; no significant difference in *DRD2* expression but positive correlation between the *DRD2* mRNA level and excited factor score of the Positive and Negative Syndrome Scale (PANSS).
1	(Czermak, Lehofer, Wagner et al., 2004)	Abstinent alcohol **addicts** (n = 19) + abstinent heroin addicts (n = 20) vs. control (n = 29)	mRNA expression	50% reduction in *DRD4* mRNA in PBL in addict group vs. controls
1	(Frieling et al., 2010)	Patients with **eating disorders** (22 with anorexia nervosa, 24 with bulimia) vs. controls (n = 30)	mRNA expression + DNA methylation	Higher *DAT* mRNA + promotor hyper-methylation; lower *DRD2* mRNA + promotor hypermethylation in PBLs of patients vs. controls
1	(Goodarzi et al., 2009)	Opioid **addicts**, methadone maintained, long-term abstinent vs. controls (n = 30 per group)	mRNA expression	Higher *DRD3* mRNA expression in addicted + methadone maintained vs. abstinent + controls; lower *DRD4* mRNA in abstinent + addicted vs. methadone maintained + controls; lower *DRD5* mRNA in abstinent vs. controls
1	(Ilani et al., 2001)	Patients with **schizophrenia** (n = 14) vs. controls (n = 11)	mRNA expression	Higher *DRD3* mRNA in patients vs. controls
1	(Kwak et al., 2001)	**Schizophrenia** patients (drug-medicated n = 44,	mRNA expression	Decreased *DRD3* mRNA in lymphocytes of drug-free vs. controls + drug-medicated;

		drug-free n = 28, drug-naïve n = 15) vs. controls (n = 31)		increased *DRD5* mRNA in drug-free vs. drug-medicated, mRNA levels modulated by antipsychotic drugs
1	(Liu et al., 2013) *	**Schizophrenia** (acute phase n = 5, chronic state n = 27) vs. controls (n = 30)	mRNA expression	No difference in *DRD2* mRNA in leukocytes, but correlation with positive symptom points of schizophrenia in acute phase (this fits with the dopamine hypothesis of schizophrenia); higher *DAT* mRNA levels in leukocytes in chronic patients vs. controls
1	(Nishihara et al., 2011) *	**Schizophrenia** patients (n = 11) vs. controls (n = 12)	mRNA expression	No difference in *DRD3/4* mRNA in leukocytes in patients vs. controls, but correlation of *DRD3* mRNA with symptom severity (PANSS total score) and *DRD3* mRNA with working memory scale
1	(Pellicano et al., 2007)	**Parkinson's disease** patients (n = 14) vs. patients with essential tremor (n = 11)	DAT immunoreactivity	*DAT* immunoreactivity in PBL discriminates between PD patients and patients with essential tremor
1	(Urhan-Kucuk et al., 2011)	**Schizophrenia** patients (n = 55) vs. controls (n = 51)	mRNA expression	*DRD3* mRNA expression can differentiate between schizophrenia subtypes, but not between patients and controls
1	(Vousooghi et al., 2015)	**Computer** game addicts (n = 20) vs. controls (n = 20)	mRNA expression	*DRD5* mRNA lower in PBL of computer game addicts vs. controls
1	(Bakulski et al., 2016)	**Review**: advantages and disadvantages of brain and blood samples in neuropsychiatric research	n.a.	Limitations of brain samples: • Very rare (small sample sizes and difficulties in matching) • Available only post-mortem => unknown influence of dying, samples mostly of elderly individuals with comorbidities • No longitudinal studies or replications within one individual possible • Compiling complete datasets including clinical, demographic, risk factor and environmental exposure data is challenging
1	(Buttarelli et al., 2011)	**Review**: peripheral blood cells in neuropsychiatric research	n.a.	The authors argue in favor of the use of peripheral markers in neuropsychiatric diseases
1	(Gladkevich et al., 2004)	**Review**: potential of lymphocytes in studying neuropsychiatric disorders	n.a.	The authors argue in favor of the use of lymphocytes for studying neuropsychiatric disorders with cDNA-microarray techniques
2	(Davies et al., 2012) *	**Post-mortem** brain samples + blood samples of healthy elderly individuals (n = 9), matched samples from frontal cortex and cerebellum (n = 42)	DNA methylation in different tissues	Differences found between individuals are correlated across brain and blood tissue within one individual => when differences appear in blood cells, they are likely to be observed in brain tissue as well
2	(Kirillova et al., 2008) *	**Blood samples** from patients in a substance use disorder study (n =	mRNA expression	Comparable expression between brain and blood: DRD3 and DRD4

		38) + **human brain reference** RNA		
2	(Luykx et al., 2016)	Patients for elective minor surgery -> **healthy** individuals (n = 240)	Monoamine metabolites in cerebrospinal fluid (CSF) + whole-genome gene expression in blood	Gene expression profiles in blood correlate with monoamine metabolite levels in CSF (mainly serotonin metabolite 5-HIAA)
2	(Masliah et al., 2013) *	**Post-mortem** brain and leukocyte samples from Parkinson's disease patients (n = 5) vs. controls (n = 6)	DNA methylation profile	DNA methylation differences characteristic for Parkinson's disease in patients vs. controls were concordant in blood and in brain samples
3	(Czermak, Lehofer, Renger et al., 2004)	**Healthy** adults (n = 50)	mRNA expression	Negative correlation between the trait of persistence and *DRD3* mRNA expression in PB
3	(Leite et al., 2016) *	Healthy blood donors with (n = 17) vs. without (n = 13) **central obesity** (CO)	mRNA expression	Lower *DRD2/4/5* and *TH* mRNA in mononuclear cells in CO vs. non-CO; Correlation between mRNA expression and obesity markers (weight, BMI, waist circumference, leptin, inflammation, HbA1c)
3	(Ramos-Lopez et al., 2018b) *	**Healthy** adults (n = 473)	DNA methylation	Methodological similarity: association between DNA methylation in blood cells and markers of obesity, metabolic profiles, and dietary style

Table S15: Evidence against the use of peripheral surrogate markers. Leukocytes from a peripheral blood sample were used in this study as a surrogate tissue for the brain to study dietary effects on the dopaminergic system that might apply for the central physiological processes as well. This table summarizes evidence against this approach. Abbreviations: CSF: cerebrospinal fluid, HVA: homovanillic acid, PANSS: Positive and Negative Syndrome Scale, PBL: peripheral blood lymphocytes, PD: Parkinson's disease. Evidence category: 1 = related to neuropsychiatric diseases, 2 = direct comparison between brain and blood (post-mortem or indirect).

* = most relevant studies

*Evidence **against** the validity of a peripheral proxy*				
Evidence category	**Publication**	**Sample**	**Method**	**Finding**
1	(Bakulski et al., 2016)	**Review**: advantages and disadvantages of brain and blood samples in neuropsychiatric research	n.a.	Advantages of brain samples: • brain is the primary origin of neuropsychiatric disorders => brain tissue is most appropriate • tissue-specificity of physiological epigenetic processes
1	(Buttarelli et al., 2009)	**Parkinson's disease** patients (n = 11)	Comparison of DAT immunoreactivity between blood lymphocytes (antibody binding) and caudate/ putamen (SPECT)	No correlation between peripheral and central DAT immunoreactivity
1	(Buttarelli et al., 2011)	**Review**: peripheral blood cells in neuropsychiatric research	n.a.	• Involvement of DA signaling in immune system functioning: DRD activation modulates T-cell function • Lack of correlation between peripheral and central DAT activity
2	(Davies et al., 2012)	**Post-mortem** brain samples + blood samples of healthy elderly individuals (n = 9), matched samples from frontal cortex and cerebellum (n = 42)	DNA methylation in different tissues	DNA methylation in highly specific for cortex, cerebellum, and blood tissue
2	(Kirillova et al., 2008) *	**Blood samples** from patients in a substance use disorder study (n = 38) + **human brain reference** RNA	mRNA expression	Gene expression not comparable between brain and blood for *DRD2* and *DRD5*
2	(Luykx et al., 2016)	Patients for elective minor surgery -> **healthy** individuals (n = 240)	Monoamine metabolites in cerebrospinal fluid + whole-genome gene expression in blood	Gene expression profiles in blood correlate with monoamine metabolite levels in CSF, but best for serotonin metabolites, only nominally significant evidence for DA metabolite HVA

9.1.2 Material specifications

Table S16: Materials for mRNA quantification.

Method	Name	Supplier
Sample acquisition & RNA extraction	Tempus™ Blood RNA Tube	Thermo Fisher Scientific, Waltham, Massachusetts, USA
	Norgen's Preserved Blood RNA Purification Kit I	Norgen, Biotec Corp., Thorold, ON, Canada
	RiboRuler High Range RNA Ladder, ready-to use	Thermo Scientific , Waltham, Massachusetts, USA
	SeaKem® LE Agarose	Lonza, Rockland, ME, USA
Reverse transcription	QuantiTect® Reverse Transcription Kit	Qiagen, Hilden, Germany
Quantitative PCR	TaqMan® Gene Expression Assays (specified for each gene in Table S18)	Thermo Fisher Scientific, Waltham, Massachusetts, USA
	Applied Biosystems™ TaqMan® Gene Expression Master Mix	
	Applied Biosystems TaqMan®7500 Real Time PCR System	
	Universal Human Reference RNA	
Reagents	Aqua distilled	B. Braun Melsungen AG, Melsungen, DE
	Ethanol 96 %	JT Baker via Fisher Scientific GmbH, Schwerte, DE
Consumables	PCR plates, 96 well	Sapphire Mikroplatte, 96 Well, Greiner Bio-One, Frickenhausen, DE
	Covering foil, thermostable	Sarstedt, Nürnbrecht, DE
	Low Binding Tubes	Eppendorf, Hamburg, DE
	Pipette tip	Sarstedt, Nürnbrecht, DE
	Gloves Peha-Soft Nitrile	Hartmann, Heidenheim, DE
Devices	Pipettes	Eppendorf, Hamburg, DE
	Multipipette Stream	Eppendorf, Hamburg, DE
	VortexGenie 2	Scientific Industries, New York, USA
	Heraeus Fresco 17 Centrifuge	Thermo Fisher Scientific, Waltham, Massachusetts, USA
	Eppendorf Centrifuge 5804 R	Eppendorf, Hamburg, DE
	Thermal Cycler	Applied Biosystems, Foster, USA
	Themomixer compact	Eppendorf, Hamburg, DE
	Nanodrop 2000 Spectrometer	Thermo Fisher Scientific, Waltham, Massachusetts, USA
	VWR® Imager2	VWR International GmbH, Darmstadt, DE
Software	ABI 7500 Real Time PCR Software	Applied Biosystems, Foster, USA

Table S17: Materials for DNA extraction and genotyping.

Category	Designation	Supplier
DNA extraction	EDTA monovette 2.7 ml EDTA	S-Monovette, SARSTEDT AG & Co. KG, Nümbrecht, Germany
	Quick Gene DNA whole blood kit S	Kubaro Industries LTD., Osaka, Japan
Genotyping	Applied Biosystems TaqMan®7500 Real Time PCR System	Applied Biosystems, Foster, USA
	Applied Biosystems TaqMan® Genotyping Assays	
	TaqMan Genotyping Mastermix	
VNTR genotyping	Qiagen's HotStar Taq Plus Mastermix Kit	Quiagen, Hilden, Germany
	Primers • forward: 5'-TGCGGTGTAGGGAACGGCCTGAGA-3' • reverse: 5'-TGTGGTCTGCAGGCTGCCTGCAT-3'	Biomers (Ulm/Donau, Germany)
	Biozym Sieve GeneticPure Agarose	Biozym Scientific GmbH, Oldendorf, DE
	Serva Fast Load 50 bp ladder	SERVA Electrophoresis GmbH, Heidelberg, Germany
Consumables	PCR plates, 96 well	Greiner Bio-One, Frickenhausen, DE
	Covering foil, thermostable	Sarstedt, Nürnbrecht, DE
	Low Binding Tubes	Eppendorf, Hamburg, DE
	Pipette tip	Sarstedt, Nürnbrecht, DE
	Gloves Peha-Soft Nitrile	Hartmann, Heidenheim, DE
Reagents	Aqua distilled	B. Braun Melsungen AG, Melsungen, DE
	Ethanol 100 %	JT Baker via Fisher Scientific GmbH, Schwerte, DE
Devices	Pipettes	Eppendorf, Hamburg, DE
	Multipipette Stream	Eppendorf, Hamburg, DE
	VortexGenie 2	Scientific Industries, New York, USA
	Plattenzentrifuge 2-6E	Sigma Laborzentrifugen GmbH, Osterode am Harz, DE
	Multifuge X1R Heraeus	Thermo Scientific, Bremen, DE
	Thermal Cycler	Applied Biosystems, Foster, USA
	Themomixer compact	Eppendorf, Hamburg, DE
	NanoDrop	Peqlab Co., Shanghai, China
	VWR® Imager2	VWR International GmbH, Darmstadt, DE
Software	ABI Prism 7500	Applied Biosystems, Foster, USA
	Genetics Calculations	www.husdyr.kvl.dk

Table S18: TaqMan Gene Expression Assays. The necessary amounts of template per sample for a sufficient qPCR detection rate (due to low expression levels) were experimentally determined during establishment of the experiment.

Gene	Assay	Template amount per sample
DRD2	Hs00241436_m1	4 µl UV ≙ 100 ng
DRD3	Hs00364455_m1	3 µl UV ≙ 75 ng
DRD4	Hs00609526_m1	4 µl UV ≙ 100 ng
DRD5	Hs00361234_s1	3 µl UV ≙ 75 ng
DDC	Hs01105048_m1	1 µl UV ≙ 25 ng
TH	Hs00165941_m1	Assay did not work
DAT	Hs00997374_m1	4.5 µl UV ≙ 112.5 ng
DARPP-32	Hs00259967_m1	4 µl UV ≙ 100 ng
COMT	Hs00241349_m1	4 µl 1:5 ≙ 20 ng
UCP-2	Hs01075227_m1	1 µl 1:5 ≙ 5 ng
UCP-3	Hs01106052_m1	3 µl UV ≙ 75 ng
TBP	Hs00427620_m1	1 µl 1:5 ≙ 5 ng
HPRT1	Hs02800695_m1	1 µl 1:5 ≙ 5 ng
RPLP0 (36B4)	Hs00420895_gH	1 µl 1:5 ≙ 5 ng

9.1.3 Housekeeping gene software

Selection of housekeeping genes

The HKGs are selected based on experience of the group of Prof. Kovacs, who frequently work with TBP, RPLP0 and HPRT1. HKGs should be stably expressed independent of environmental circumstances and experimental conditions. Therefore, diet group were checked for differences in the expression of TBP, RPLP0 and HPRT1. As expected, the independent-samples t-tests indicated no differences for TBP expression (p = 0.11), for RPLP0 (p = 0.82) or for HPRT1 (p = 0.23). The best HKG was then determined with two independent software tools.

BestKeeper software

The software "BestKeeper" is an excel-based tool provided by (Pfaffl et al., 2004). It calculates several descriptive statistics for all HKGs, such as the arithmetic mean, standard deviation (Std dev) and coefficient of variance (CV). Stability of expression is defined as a Std dev < 1. From all stably expressed HKGs the BestKeeper Index is calculated as the geometric mean of the cT values of all HKGs for each sample individually and pair-wise correlations are run between the BestKeeper Index and each HKG. The closer the correlation coefficient (coeff. of corr. [r]) is to 1, the higher is the correlation between the BestKeeper and the HKG and that HKG that correlates best with BestKeeper Index is defined as the best HKG for standardization. Alternatively, the BestKeeper Index itself can be used for standardization. Regarding standardization in this study, according to Table S19 the Std dev of the expression of HPRT1 is equal to the Std dev of RPLP0 and the BestKeeper Index and HPRT1 shows the lowest CV. Additionally, HPRT1 correlates best with the BestKeeper Index and can thus be considered as the best HKG. As the BestKeeper Index cannot be corrected for efficiency it was not considered for standardization.

Table S19: Output BestKeeper Software. BestKeeper indicates *HPRT1* as the best HKG, as the Std dev is < 1 (stable expression) and its expression shows the best correlation with the BestKeeper Index (coeff. Of corr. = 0.975).

	TBP	RPLP0	HPRT1	BestKeeper
n	69	69	69	**69**
Ar. mean [CP]	**31.60**	**24.38**	**31.41**	**28.92**
Min [CP]	29.48	22.82	29.89	**27.23**
Max [CP]	33.64	27.14	33.24	**30.88**
Std dev [± CP]	**0.69**	**0.61**	**0.61**	**0.61**
CV [% CP]	**2.19**	**2.50**	**1.95**	**2.10**
BestKeeper vs.				
coeff. of corr. [r]	**0.938**	**0.934**	**0.975**	
p-value	0.001	0.001	0.001	

Normfinder software

Another excel-based software to find the best HKG of several potential ones was proposed by (Andersen et al., 2004). Using the cT values corrected for efficiency (E) by E^{-cT} as input data the tool calculates intra- and intergroup variations based on variances. Including experimental groups into the calculation is a valuable feature of this tool. From the calculated variabilities a stability value is derived that indicates the best HKG or combination of HKGs. Fig. S26 shows that HPRT1 has the lowest stability value, the lowest intergroup variation and the lowest intragroup variation in the HFS group. TBP shows a better variation value in the LFS group but higher variation in HFS group and a higher intergroup variation. Thus, HPRT1 is the most stably expressed HKG for standardization, which is in line with the result of the BestKeeper Software.

Intergroup variation		
Group identifier	1 (LFS)	2 (HFS)
TBP	-0.083	0.083
RPLP0	0.082	-0.082
HPRT1	0.001	-0.001

Intragroup variation		
Group identifier	1 (LFS)	2 (HFS)
TBP	0.006	0.080
RPLP0	0.155	0.425
HPRT1	0.056	0.008

Best gene	HPRT1
Stability value	0.039
Best combination	TBP and RPLP0
Stability value for best combination of two genes	0.081

Gene name	**Stability value**
TBP	0.116
RPLP0	0.157
HPRT1	0.039

Figure S26: Output Normfinder Software. *HPRT1* has the lowest stability value (0.039), the lowest intergroup variation (0.001) and the lowest intragroup variation in the HFS group (0.008). *TBP* shows a better intragroup variation value in the LFS group (0.006) than *HPRT1* (0.056) but higher variation in HFS group (0.08) and a higher intergroup variation (0.083). Thus, *HPRT1* is the most stably expressed HKG for standardization.

9.1.4 Verification of the equation for the arbitrary units (AU) calculation

Equation proposed by Pfaffl. (2004) for efficiency correction:

$$\text{Ratio} = \frac{Etar^{cTtar(c)-cTtar(i)}}{Eref^{cTref(c)-cTref(i)}}$$

E_{tar}: efficiency of qPCR of target assay

E_{ref}: efficiency of qPCR of reference assay

$cT_{tar(c)}$: threshold cycle of target assay of each control sample

$cT_{tar(i)}$: threshold cycle of target assay of each intervention sample

$cT_{ref(c)}$: threshold cycle of reference assay of control sample

$cT_{ref(i)}$: threshold cycle of reference assay of intervention sample

Basic commonly accepted equations:

1. $\Delta cT = cT_{Target} - cT_{Reference}$
2. $\Delta\Delta cT = \Delta cT_{Intervention} - \Delta cT_{Control}$
3. Ratio of x-fold change = $2^{-\Delta\Delta cT}$ or $2^{-\Delta cT}$ depending on experimental design

The equation of Pfaffl (2004) can be transformed to basic equation 3 assuming best efficiencies for both target and reference qPCR and therefore $E_{tar} = E_{ref} = 2$. This assumption is commonly accepted and equations 1-3 assume E = 2.

$$\text{Ratio} = \frac{Etar^{cTtar(c)-cTtar(i)}}{Eref^{cTref(c)-cTref(i)}} = \frac{2^{cTtar(c)-cTtar(i)}}{2^{cTref(c)-cTref(i)}} =$$

$$2^{cTtar(c)-cTtar(i)-cTref(c)-cTref(i)} =$$

$$= 2^{cTtar(c)-cTref(c)-cTtar(i)-cTref(i)} = 2^{\Delta cT(c)-\Delta cT(i)} = \frac{2^{\Delta cT(c)}}{2^{\Delta cT(i)}}$$

$$= \frac{1}{\frac{2^{\Delta cT(i)}}{2^{\Delta cT(c)}}} = \frac{1}{2^{\Delta cT(i)-\Delta cT(c)}} = 2^{-\Delta\Delta cT}$$

Based on the mathematic model proposed by Pfaffl et al. it can be assumed that mathematically replacing E = 2 with the obtained efficiencies using a standard curve during qPCR is legitimate. Therefore, the equation $2^{-\Delta cT}$ was transformed so that the qPCR efficiencies of both the reference and target assay can be incorporated:

$$2^{-\Delta cT} = 2^{-(cTtar-cTref)} = 2^{-cTtar+cTref} = 2^{cTref-cTtar} = \frac{2^{cTref}}{2^{cTtar}} =$$

$$= \frac{Eref^{cTref}}{Etar^{cTtar}} = \frac{Etar^{-cTtar}}{Eref^{-cTref}}$$

$\text{AU} = \frac{Etar^{-cTtar}}{Eref^{-cTref}}$ was used to normalize cT values in this study.

9.1.5 Hardy-Weinberg equilibrium

Table S20: Hardy-Weinberg equilibrium. The allele distributions of all SNPs except for COMT rs4680 were consistent with the Hardy-Weinberg equilibrium (indicated by p-values > 0.05 in Pearson's X^2- Test). The low p-value for COMT rs4680 is most likely due to low sample size and as p is > 0.01 it can still be considered as consistent with the Hardy-Weinberg equilibrium.

SNP	X^2	p-value
DARPP-32 (PPP1R1B) rs907094	0.01	0.93
COMT rs4680	5.86	0.02
DRD2 rs1800497	2.08	0.15
DRD2 rs6277	2.12	0.15
FTO rs8053740	0.05	0.82
FTO rs8050136	1.11	0.29
DAT (SLC6A3) VNTR	1.49	0.22

9.1.6 *Diet x genotype* interactions

Table S21: *Diet x genotype* interactions affecting the mRNA expression of corresponding genes. The table shows the analyzed SNP and the corresponding gene/mRNA, the number of participants (n) in each *diet x allele* subgroup, descriptive statistics (mean and standard deviation) for mRNA expression levels in each subgroup and the main results of the *diet x genotype* ANOVAs (main effect of genotype, main effect of diet and interaction). There was a trend level interaction between diet and genotype in the *FTO* SNP rs8050136 affecting the mRNA expression of *DRD2* ($F(2, 42) = 2.65$, $p = 0.08$)**.** Additionally, there was a significant main effect of diet on *DAT* mRNA expression ($F(1, 61) = 4.07$, $p = 0.048$) as well as a trend for a dietary effect on *DRD2* mRNA expression ($F(1, 44) = 3.03$, $p = 0.09$) when including genotype in the model (*DAT* VNTR length and genotype in the Taq1A SNP, respectively). All other interactions and main effects were not significant ($p \geq 0.11$; the significant main effect of diet in the ANOVA model of rs8050136 cannot be interpreted when the interaction is significant). Abbreviations: COMT: catechol-O-methyltransferase, DARPP-32: dopamine-and-cAMP-regulated neuronal phosphoprotein 32, DAT: dopamine transporter, DRD: dopamine receptor, FTO: fat mass and obesity-associated protein, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, Met: methionine, n: sample size, VNTR: variable number tandem repeat, SD: standard deviation, SNP: single nucleotide polymorphism, Val: valine.

				Descriptive statistics			***Diet x genotype* ANOVA**		
SNP	**Gene**	**Diet group**	**Allele group**	**n**	**Mean mRNA level**	**SD**	**Genotype**	**Diet**	**Interaction**
***COMT* rs4680 (Val/Met)**	*COMT*	HFS	0	13	94.88	25.26	F(2, 65) = 0.19, p = 0.83	F(1, 65) = 1.62, p = 0.21	F(2, 65) = 0.26, p = 0.77
			1	16	95.55	19.53			
			2	10	99.98	22.68			
		LFS	0	12	105.64	17.32			
			1	9	107.42	28.26			
			2	11	103.35	11.02			
***DAT* rs28363170 VNTR**	*DAT*	HFS	1	21	0.33	0.24	F(1, 61) = 0.28), p = 0.60	**F(1, 61) = 4.07, p = 0.048**	F(1, 61) = 0.50, p = 0.48
			2	15	0.35	0.22			
		LFS	1	13	0.18	0.11			
			2	16	0.25	0.16			
***DARPP-32* rs907094**	*DARPP-32*	HFS	1	15	0.07	0.05	F(1, 57) = 2.33, p = 0.13	F(1, 57) = 0.11, p = 0.74	F(1, 57) = 2.41, p = 0.13
			2	19	0.09	0.06			
		LFS	1	16	0.07	0.05			
			2	11	0.06	0.05			
***DRD2* rs1800497 (Taq1A)**	*DRD2*	HFS	1	12	0.28	0.18	F(1, 44) = 1.38, p = 0.25	**F(1, 44) = 3.03, p = 0.09**	F(1, 44) = 0.86, p = 0.36
			2	12	0.20	0.14			
		LFS	1	7	0.41	0.19			
			2	17	0.24	0.14			
***DRD2* rs6277**		HFS	0	8	0.16	0.07	F(2, 42) = 2.33, p = 0.11	F(1, 42) = 0.13, p = 0.72	F(2, 42) = 0.15, p = 0.86
			1	10	0.32	0.17			
			2	6	0.22	0.21			
		LFS	0	9	0.19	0.14			
			1	9	0.40	0.17			
			2	6	0.30	0.12			
***FTO* rs8050136**		HFS	0	6	0.18	0.09	F(2, 42) = 0.3, p = 0.74	**F(1, 42) = 3.85, P = 0.06**	**F(2, 42) = 2.65, p = 0.08**
			1	12	0.26	0.19			
			2	6	0.27	0.17			
		LFS	0	6	0.38	0.19			
			1	12	0.30	0.13			
			2	6	0.19	0.20			
***FTO* rs8053740**		HFS	0	4	0.18	0.15	F(2, 42) = 1.65, p = 0.20	F(1, 42) = 0.95, p = 0.33	F(2, 42) = 0.09, p = 0.92
			1	12	0.28	0.17			
			2	8	0.21	0.17			
		LFS	0	6	0.25	0.11			
			1	10	0.36	0.19			
			2	8	0.24	0.17			

Table S22: *Diet x genotype* interactions affecting performance in the Trail making test part B. The table shows the number of participants (n) in each *diet x allele* subgroup, descriptive statistics (mean and standard deviation) for the performance in the Trail making test part B in the respective subgroup and the results of the *diet x genotype* ANOVAs (main effect of genotype, main effect of diet and interaction). There was a significant interaction between diet and genotype in *DRD2* SNP rs6277 affecting performance of the Trail making test part B ($F(2, 69 = 3.67$, $p = 0.03$). Significant main effects of genotype independent of diet are present for repeat length of the VNTR in *DAT* ($F(1, 71) = 6.37$, $p = 0.014$; interaction: $p = 0.20$) and genotype in *FTO* SNP rs8050136 ($F(2, 69) = 4.61$, $p = 0.013$; interaction: $p = 0.44$). Further, there was a trend level effect of genotype in the Val/Met polymorphism in *COMT* ($F(2, 69) = 2.84$, $p = 0.07$; interaction: $p = 0.52$). All other interactions and main effects were not significant ($p \geq 0.20$; the trend main effect for genotype in rs6277 cannot be interpreted when the interaction is significant). Abbreviations: COMT: catechol-O-methyltransferase, DARPP-32: dopamine-and-cAMP-regulated neuronal phosphoprotein 32, DAT: dopamine transporter, DRD: dopamine receptor, FTO: fat mass and obesity-associated protein, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, Met: methionine, n: sample size, VNTR: variable number tandem repeat, s: seconds, SD: standard deviation, SNP: single nucleotide polymorphism, Val: valine.

			Descriptive statistics			***Diet x genotype* ANOVA**		
SNP	**Diet group**	**Allele group**	**n**	**Mean score [time in s]**	**SD**	**Genotype**	**Diet**	**Interaction**
***COMT* rs4680 (Val/Met)**	HFS	0	13	43.73	11.59	**$F(2, 69) = 2.84$, $p = 0.07$**	$F(1, 69) = 0.08$, $p = 0.78$	$F(2, 69) = 0.66$, $p = 0.52$
		1	16	35.00	8.81			
		2	12	42.36	14.83			
	LFS	0	12	42.08	9.03			
		1	11	36.24	12.03			
		2	11	35.38	7.48			
***DAT* rs28363170 VNTR**	HFS	1	23	36.16	8.84	**$F(1, 71) = 6.37$, $p = 0.014$**	$F(1, 71) = 0.05$, $p = 0.82$	$F(1, 71) = 1.71$, $p = 0.20$
		2	18	44.74	14.10			
	LFS	1	16	36.96	9.59			
		2	18	38.96	10.29			
***DARPP-32* rs907094**	HFS	1	18	38.77	10.05	$F(1, 71) = 0.12$, $p = 0.73$	$F(1,71) = 0.004$, $p = 0.95$	$F(1, 71) = 0.63$, $p = 0.43$
		2	20	39.23	10.60			
	LFS	1	20	39.23	10.60			
		2	23	40.83	13.62			
***DRD2* rs1800497 (Taq1A)**	HFS	1	17	39.81	13.33	$F(1, 71) = 0.01$, $p = 0.90$	$F(1, 71) = 0.25$, $p = 0.62$	$F(1, 71) = 0.89$, $p = 0.35$
		2	24	40.00	11.41			
	LFS	1	8	41.01	7.88			
		2	26	37.10	10.36			
***DRD2* rs6277**	HFS	0	13	36.45	9.52	**$F(2, 69) = 2.50$, $p = 0.09$**	$F(1, 69) = 1.22$, $p = 0.27$	**$F(2, 69) = 3.67$, $p = 0.03$**
		1	17	43.88	10.99			
		2	11	37.92	15.33			
	LFS	0	12	40.91	10.51			
		1	14	34.70	8.98			
		2	8	39.52	9.83			
***FTO* rs8050136**	HFS	0	9	45.66	10.21	**$F(2, 69) = 4.61$, $p = 0.013$**	$F(1, 69) = 1.66$, $p= 0.20$	$F(2, 69) = 0.83$, $p = 0.44$
		1	22	35.24	9.01			
		2	10	45.06	15.84			
	LFS	0	6	38.43	13.23			
		1	20	36.20	8.76			
		2	8	42.28	9.75			
***FTO* rs8053740**	HFS	0	8	41.32	18.46	$F(2, 69) = 0.04$, $p = 0.95$	$F(1, 69) = 0.0006$, $p = 0.98$	$F(2, 69) = 0.07$, $p = 0.93$
		1	20	39.11	10.49			
		2	13	40.31	10.54			
	LFS	0	7	39.67	11.25			
		1	16	37.28	9.35			
		2	11	38.06	10.55			

Table S23: **Diet x genotype *interactions affecting performance in the Digit symbol substitution test.*** The table shows the number of participants (n) in each diet x allele group, descriptive statistics (mean and standard deviation) for Digit symbol substitution test performance in the respective subgroup and the results of the diet x genotype ANOVAs (main effect of genotype, main effect of diet and interaction). There was a significant interaction between diet and genotype in *DRD2* SNP rs6277 on Digit symbol substitution test performance (interaction: F(2, 69) = 6.02, p = 0.004). All other interactions and main effects were not significant (p ≥ 0.12; the trend main effect of genotype in the ANOVA model of rs6277 cannot be interpreted when the interaction is significant). Abbreviations: COMT: catechol-O-methyltransferase, DARPP-32: dopamine-and-cAMP-regulated neuronal phosphoprotein 32, DAT: dopamine transporter, DRD: dopamine receptor, FTO: fat mass and obesity-associated protein, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, Met: methionine, n: sample size, VNTR: variable number tandem repeat, SD: standard deviation, SNP: single nucleotide polymorphism, Val: valine.

			Descriptive statistics			***Diet x genotype* ANOVA**		
SNP	**Diet group**	**Allele group**	**n**	**Mean score [transferred symbols]**	**SD**	**Genotype**	**Diet**	**Interaction**
***COMT* rs4680 (Val/Met)**	HFS	0	13	79.46	8.25	F(2, 69) = 1.25, p = 0.29	F(1, 69) = 0.03, p = 0.87	F(2, 69) = 0.07, p = 0.93
		1	16	85.44	10.01			
		2	12	81.08	13.49			
	LFS	0	12	79.25	12.05			
		1	11	82.36	8.62			
		2	11	78.82	11.03			
***DAT* rs28363170 VNTR**	HFS	1	23	84.35	12.24	F(1, 71) = 2.01, p = 0.16	F(1, 71) = 1,92, p = 0.17	F(1, 71) = 1.37, p = 0.25
		2	18	79.61	7.97			
	LFS	1	16	79.56	12.03			
		2	18	80.61	9.26			
***DARPP-32* rs907094**	HFS	1	18	79.33	7.82	F(1, 71) = 2.46, p = 0.12	F(1, 71) = 0.03, p = 0.87	F(1, 71) = 0.89, p = 0.35
		2	23	84.57	12.22			
	LFS	1	20	79.90	9.15			
		2	14	80.43	12.55			
***DRD2* rs1800497 (Taq1A)**	HFS	1	17	82.88	11.40	F(1, 71) = 0.09, p = 0.76	F(1, 71) = 2.03, p = 0.16	F(1, 71) = 1.14, p = 0.29
		2	24	81.83	10.45			
	LFS	1	8	77.13	14.30			
		2	26	81.04	9.20			
***DRD2* rs6277**	HFS	0	13	82.46	9.93	**F(2, 69) = 2.84, p = 0.07**	F(1, 69) = 0.51, p = 0.48	**F(2, 69) = 6.02, p = 0.004**
		1	17	78.53	6.60			
		2	11	87.82	14.65			
	LFS	0	12	79.58	8.88			
		1	14	84.50	9.85			
		2	8	73.25	11.09			
***FTO* rs8050136**	HFS	0	9	79.89	7.10	F(2, 69) = 0.56, p = 0.57	F(1, 69) = 0.95, p = 0.33	F(2, 69) = 1.25, p = 0.29
		1	22	84.00	11.89			
		2	10	80.60	11.00			
	LFS	0	6	85.83	12.59			
		1	20	79.20	10.48			
		2	8	78.13	8.58			
***FTO* rs8053740**	HFS	0	8	86.13	12.59	F(2, 69) = 0.86, p = 0.43	F(1, 69) = 1.27, p = 0.26	F(2, 69) = 0.78, p = 0.46
		1	20	82.50	11.58			
		2	13	79.54	7.80			
	LFS	0	7	80.00	11.70			
		1	16	83.31	10.40			
		2	11	75.55	8.95			

Table S24: *Diet x genotype* interactions affecting performance in the Digit span test backwards. The table shows the number of participants (n) in each *diet x allele* group, descriptive statistics (mean and standard deviation) for Digit span test performance in the respective subgroup and the results of the *diet x genotype* ANOVAs *(*main effect of genotype, main effect of diet and interaction*)*. There was a significant interaction between diet and genotype in *DRD2* SNP rs6277 affecting performance in the Digit span test backwards (F(2, 69) = 3.14, p = 0.049). All other interactions and main effects were not significant (p ≥ 0.11; the significant main effects in the ANOVA model of rs6277 cannot be interpreted when the interaction is significant). Abbreviations: COMT: catechol-O-methyltransferase, DARPP-32: dopamine-and-cAMP-regulated neuronal phosphoprotein 32, DAT: dopamine transporter, DRD: dopamine receptor, FTO: fat mass and obesity-associated protein, HFS: high fat and sugar diet group, LFS: low fat and sugar diet group, Met: methionine, n: sample size, VNTR: variable number tandem repeat, SD: standard deviation, SNP: single nucleotide polymorphism, Val: valine.

			Descriptive statistics			***Diet x genotype* ANOVA**		
SNP	**Diet group**	**Allele group**	**n**	**Mean score [levels]**	**SD**	**Genotype**	**Diet**	**Interaction**
***COMT* rs4680 (Val/Met)**	HFS	0	13	5.08	1.04	F82, 69) = 1.92, p = 0.15	F(1, 69) = 0.10, p = 0.75	F(2, 69) = 0.92, p = 0.40
		1	16	6.00	1.37			
		2	12	5.50	1.62			
	LFS	0	12	4.92	1.31			
		1	11	5.18	1.08			
		2	11	5.64	1.03			
***DAT* rs28363170 VNTR**	HFS	1	23	5.61	1.34	F(1, 71) = 0.07, p = 0.79	F(1, 71) = 0.01, p = 0.91	F(1, 71) = 0.73, p = 0.40
		2	18	5.50	1.47			
	LFS	1	16	5.56	1.15			
		2	18	4.94	1.11			
***DARPP-32* rs907094**	HFS	1	18	5.61	1.33	F(1, 71) = 0.05, p = 0.83	F(1, 71) = 0.17, p = 0.28	F(1, 71) = 0.24, p = 0.63
		2	23	5.52	1.44			
	LFS	1	20	5.15	1.18			
		2	14	5.36	1.15			
***DRD2* rs1800497 (Taq1A)**	HFS	1	17	5.65	1.41	F(1, 71) = 0.13, p = 0.72	F(1, 71) = 0.88, p = 0.35	F(1, 71) = 0.19, p = 0.66
		2	24	5.50	1.38			
	LFS	1	8	5.13	0.99			
		2	26	5.27	1.22			
***DRD2* rs6277**	HFS	0	13	6.15	1.28	**F(2, 69) = 3.26, p = 0.04**	**F(1, 69) = 5.30, p = 0.02**	**F(2, 69) = 3.14, p = 0.049**
		1	17	5.00	1.17			
		2	11	5.73	1.56			
	LFS	0	12	5.00	1.28			
		1	14	5.50	1.09			
		2	8	5.13	1.13			
***FTO* rs8050136**	HFS	0	9	5.22	1.39	F(2, 69) = 2.33, p = 0.11	F(1, 69) = 0.007, p = 0.93	F(2, 69) = 0.70, p = 0.50
		1	22	5.95	1.25			
		2	10	5.00	1.49			
	LFS	0	6	5.17	1.33			
		1	20	5.30	1.22			
		2	8	5.13	0.99			
***FTO* rs8053740**	HFS	0	8	5.50	1.31	F(2, 69) = 0.02, p = 0.98	F(1, 69) = 0.56, p = 0.46	F(2, 69) = 1.05, p = 0.35
		1	20	5.55	1.39			
		2	13	5.62	1.50			
	LFS	0	7	6.00	1.00			
		1	16	5.13	1.31			
		2	11	4.91	0.83			

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10. Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

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12. Publications

Poster

Rausch, F., Hartmann, H., Janssen, L., Schleinitz, D., Kovacs, P., & Horstmann, A. (2020). *Diet-associated differences in peripheral dopamine gene expression and their interaction with cognitive performance in humans*. Poster presented at the 16th Leipzig Research Festival of Life Sciences, Studienzentrum der Medizinischen Fakultät, University Leipzig, Germany.

Rausch, F., Hartmann, H., Janssen, L., Schleinitz, D., Kovacs, P., & Horstmann, A. (2019). *Diet-associated differences in peripheral dopamine gene expression and their interaction with cognitive performance in humans.* Poster presented at the Autumn School of the SFB 1052 "Obesity mechanisms", Heidelberg, Germany.

Rausch, F., Hartmann, H., Janssen, L., Schleinitz, D., Kovacs, P., & Horstmann, A. (2019). *Diet-associated differences in peripheral dopamine gene expression and their interaction with cognitive performance in humans*. Poster presented at 3rd Nordic Neuroscience Meeting, Helsinki, Finland.

Hartmann, H., **Rausch, F.,** & Kobel, M. (2019). *Investigating the interplay between diet, dopamine and cognitive performance*. Poster presented at the Retreat of the Collaborative Research Council 1052 "Obesity Mechanism", Grimma, Germany.

Review

Janssen, L., Herzog, N., Waltmann, M., Breuer, N., Wiencke, K., **Rausch, F.**, Hartmann, H., Poessel, M., & Horstmann, A. (2019). Lost in translation?: On the need for convergence in animal and human studies on the role of dopamine in diet-induced obesity. *Current Addiction Reports, 6*(3), 229-257. doi:10.1007/s40429-019-00268-w.

The results of this project will be published in a peer-reviewed journal.

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