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PRE-CLINICAL INVESTIGATION OF BRAIN MECHANISMS ASSOCIATED WITH PARKINSON'S DISEASE:
THE IMPACT OF DIET

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Pre-clinical investigation of brain mechanisms associated with Parkinson's disease: The impact of diet

Luiza Reali Nazario

This thesis is valid for a double degree as a collaboration between the University of Groningen, the University Medical Center Groningen (UMCG), and the Pontifical Catholic University of Rio Grande do Sul (PUCRS). The work on this thesis has been carried out in the Department of Nuclear Medicine and Molecular Imaging of the UMCG, as well as in the Laboratory of Neurochemistry and Psychopharmacology of PUCRS.

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ENGLISH SUMMARY

Attempts are being made to find a cure or at least a better treatment that can slow down the progression of Parkinson's disease, but recently, no intervention has been discovered. A better understanding of the basic mechanisms that underlie this disease is of great importance for the development of new drugs. Lifestyle factors, such diet, can influence different biological mechanisms involved in PD, such as the purinergic system, the dopaminergic system, neuroinflammation and microbiota. In this thesis, these factors will be explored in different animal models.

In Chapter 2, the interaction of adenosine receptors with dopaminergic receptors and the potential role of adenosine receptor ligands in the treatment of PD are reviewed. In this chapter, the potential relation of adenosine with lifestyle and diabetes is discussed as well.

In Chapter 3, we investigated the interaction of purinergic and dopaminergic receptors in a zebrafish model of PD. After intra-encephalic injection of 6-OHDA, zebrafish presented behavioral changes and slight effect on the dopaminergic system.

In Chapter 4, we investigated the feasibility of *in vivo* PET imaging in living healthy adult zebrafish and in a zebrafish model of inflammation. This study demonstrated that *in vivo* PET imaging with ^{18}F-FDG and ^{18}F-NaF in living zebrafish is feasible and differences in uptake can be seen in a model of inflammation.

In Chapter 5, we analyzed the impact of a cafeteria diet on the reward system in rats. Our study suggests that D_2 receptors play a role in obesity and consumption of a cafeteria diet, but no alterations after challenge was observed.

To elucidate the influence of a high fat diet (HFD) on PD progression, we investigated the effect of a HFD on the availability of D_2 receptors and behavioral parameters in rats, in Chapter 6. The HFD aggravated the damage in the PD model, suggesting a detrimental role of the HFD on the onset or progression of PD.

In Chapter 7, we suggest that the documented impact of HFD on neuroinflammation may be mediated by the gut microbiome in the 6-OHDA rat model of PD and it is independent of peripheral inflammation.

The knowledge of the basic mechanisms underlying PD and their relationship with changes in lifestyle can help scientists to better understand the factors that trigger the onset of the disease and aid the development of new treatments and diagnostic tools. These studies can help reveal of the interaction between behavior, microbiota, dopaminergic and purinergic response, and inflammation in the gut and the brain.

Keywords: Zebrafish, Purinergic System, PET imaging, High-fat diet, Parkinson's disease.

RESUMO EM PORTUGUÊS

Tentativas estão sendo feitas para encontrar uma cura ou pelo menos um tratamento melhor que possa retardar a progressão da doença de Parkinson (DP), mas, recentemente, nenhuma intervenção foi descoberta. Uma melhor compreensão dos mecanismos básicos que estão por trás dessa doença é de grande importância para o desenvolvimento de novos medicamentos. Fatores do estilo de vida, como a dieta, podem influenciar diversos mecanismos biológicos envolvidos na DP, como o sistema purinérgico, o sistema dopaminérgico, a neuroinflamação e a microbiota. Nesta tese, esses fatores serão explorados em diferentes modelos animais.

No Capítulo 2, a interação dos receptores de adenosina com os receptores dopaminérgicos e o papel potencial dos ligantes do receptor de adenosina no tratamento da DP são revisados. Neste capítulo, a relação potencial da adenosina com o estilo de vida e o diabetes também é discutida.

No Capítulo 3, investigamos a interação de receptores purinérgicos e dopaminérgicos em um modelo de DP em peixe-zebra. Após injeção intra-encefálica de 6-OHDA, o peixe-zebra apresentou alterações comportamentais e ligeiro efeito no sistema dopaminérgico.

No Capítulo 4, investigamos a viabilidade da imagem PET *in vivo* em peixes-zebra adultos saudáveis e em um modelo de inflamação do peixe-zebra. Este estudo demonstrou que a imagem PET *in vivo* com ^{18}F-FDG e ^{18}F-NaF em peixes-zebra vivos é viável e diferenças na captação podem ser vistas em um modelo de inflamação.

No Capítulo 5, analisamos o impacto de uma dieta de cafeteria no sistema de recompensa em ratos. Nosso estudo sugere que os receptores D_2 desempenham um papel na obesidade e no consumo de uma dieta de cafeteria, mas nenhuma alteração após o desafio foi observada.

Para elucidar a influência de uma dieta rica em gordura (HFD) na progressão da DP, investigamos o efeito de uma HFD sobre a disponibilidade de receptores D_2 e parâmetros comportamentais em ratos, no Capítulo 6. A HFD agravou o dano no modelo de DP, sugerindo um papel prejudicial do HFD no início ou na progressão da DP.

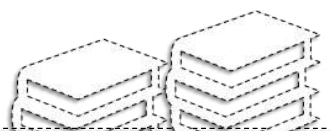
No Capítulo 7, sugerimos que o impacto documentado da HFD na neuroinflamação pode ser mediado pela microbiota intestinal no modelo de DP utilizando 6-OHDA em ratos e este é independente da inflamação periférica.

O conhecimento dos mecanismos básicos subjacentes à DP e sua relação com as mudanças no estilo de vida pode ajudar os cientistas a entender melhor os fatores que desencadeiam o aparecimento da doença e no desenvolvimento de novos tratamentos e ferramentas de diagnóstico. Esses estudos podem ajudar a revelar a interação entre comportamento, microbiota, resposta dopaminérgica e purinérgica e inflamação no intestino e no cérebro.

Palavras chave: Zebrafish, Sistema Purinérgico, Imagem PET, dieta rica em gordura, Doença de Parkinson.

CHAPTER 1

General Introduction



Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder (Dorsey et al. 2007), with the highest incidence in countries like Canada, United States and Argentina (Figure 1). According to the Parkinson`s Foundation, around 10 million people are currently suffering from this disease and this number is increasing, with around 6.1 million people being diagnosed with PD in 2016, as compared to 2.5 million in 1990 (Ray Dorsey et al. 2018). This progressive disease is typically diagnosed between the ages of 55 and 65, and globally affects 1% of the population over 60 years old, with an increased risk with age (Driver et al. 2008; de Lau and Breteler 2006).

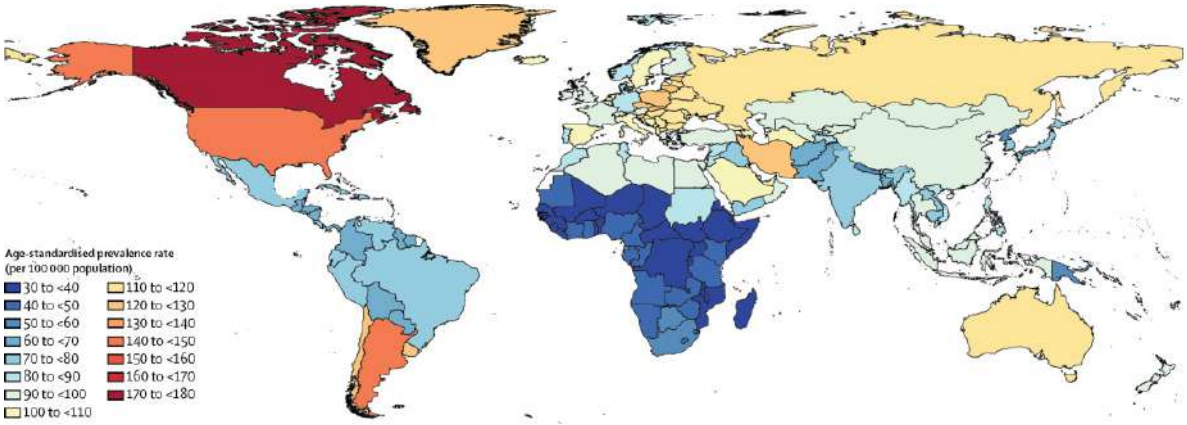


Figure 1: Age-standardized prevalence of Parkinson's Disease per 100,000 inhabitants by location for both sexes, 2016 (adapted from Ray Dorsey et al. 2018)

The hallmark of PD is the loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNc), leading to a deficit in dopamine signaling in the projection areas, like the striatum. The dopamine signaling reduction causes the typical motor symptoms of the disease, such as gait disturbances, rigidity and a resting tremor (Rizek, Kumar, and Jog 2016). Diagnosis of PD mostly occurs when 70%-80% of dopaminergic neurons in the striatum are already lost (de Rijk et al. 2000). Although the dopaminergic nigrostriatal neurons seem to be particularly vulnerable to degeneration (Hung and Lee 1996), deficits beyond the dopaminergic neurons in the basal ganglia, like in the serotonergic and noradrenergic systems, occur as well. These deficits can lead to non-motor symptoms (NMS), like cognitive decline, anxiety, depression, gastrointestinal dysfunctions, behavioral changes, sleep disturbances and fatigue (Pfeiffer 2016).

The degeneration of neurons in both motor and non-motor circuits is associated with the accumulation of Lewy bodies. H. Braak and colleagues (2003) developed a staging system based on Lewy bodies deposition. Lewy bodies consist of misfolded and aggregated α-synuclein protein deposits (Heiko Braak et al. 2003a). These toxic aggregates accumulate in the nerve cells and eventually lead to cell death (Spillantini et al. 1997). The stepwise degeneration of neurons leads to a corresponding increase in clinical symptoms (reviewed by Schapira, Chaudhuri, and Jenner 2017) (Figure 2). Cellular malfunction, like disruption of the lysosomal autophagy system, mitochondrial dysfunction, endoplasmic reticulum stress, and calcium homeostasis dysregulation may contribute to the progressive deterioration of the dopaminergic neurons in *substantia nigra* (reviewed by Michel, Hirsch, and Hunot 2016), however the exact mechanism underlying the neurodegeneration remains unclear so far (Zeng, Geng, and Jia 2018).

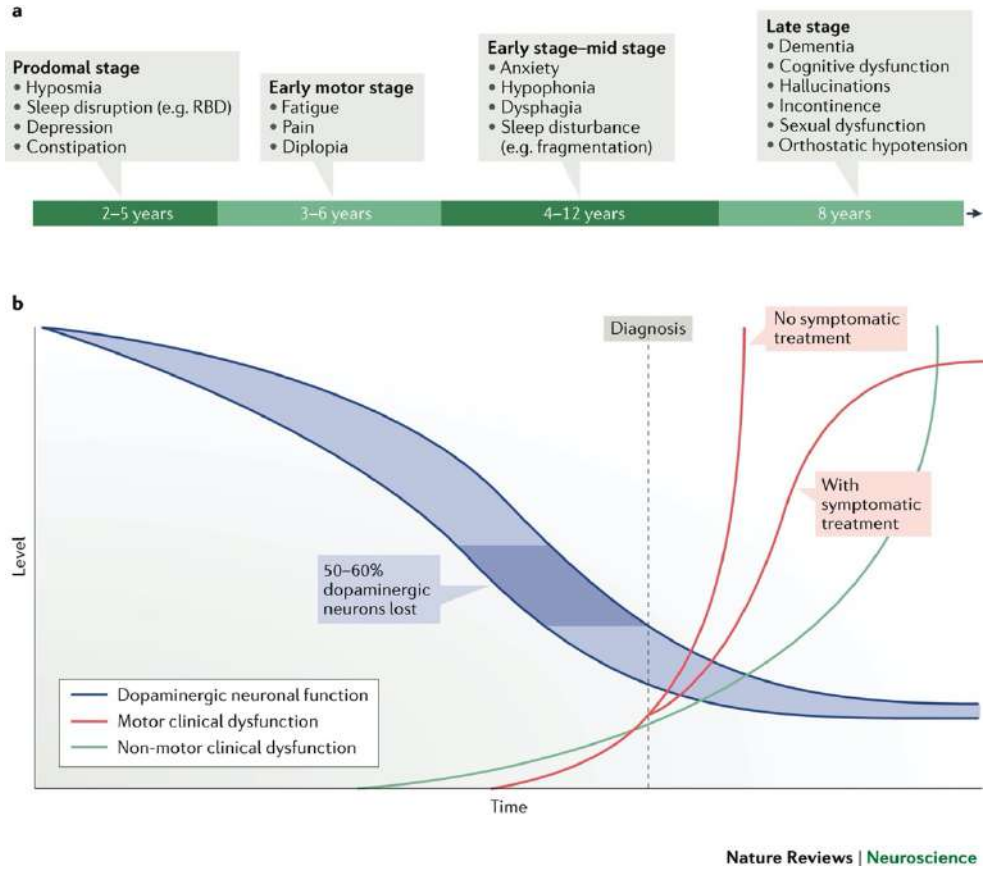


Figure 2: a) Schematic representation of the timeline for the manifestation of the non-motor symptoms of Parkinson's Disease. b) Graphic depiction of the decline in dopaminergic neuronal function and the corresponding development of the motor and non-motor symptoms of Parkinson's disease (from Schapira et al. 2017).

Heiko Braak and colleagues investigated the spread of the Lewy bodies in more than 150 patients diagnosed with PD post-mortem, incidental Lewy body disease and healthy controls (Heiko Braak et al. 2003b). They found that α-synuclein pathology propagates from the dorsal motor nucleus of the *nervus vagus* along to caudo-rostral axis in the brain (Figure 3).

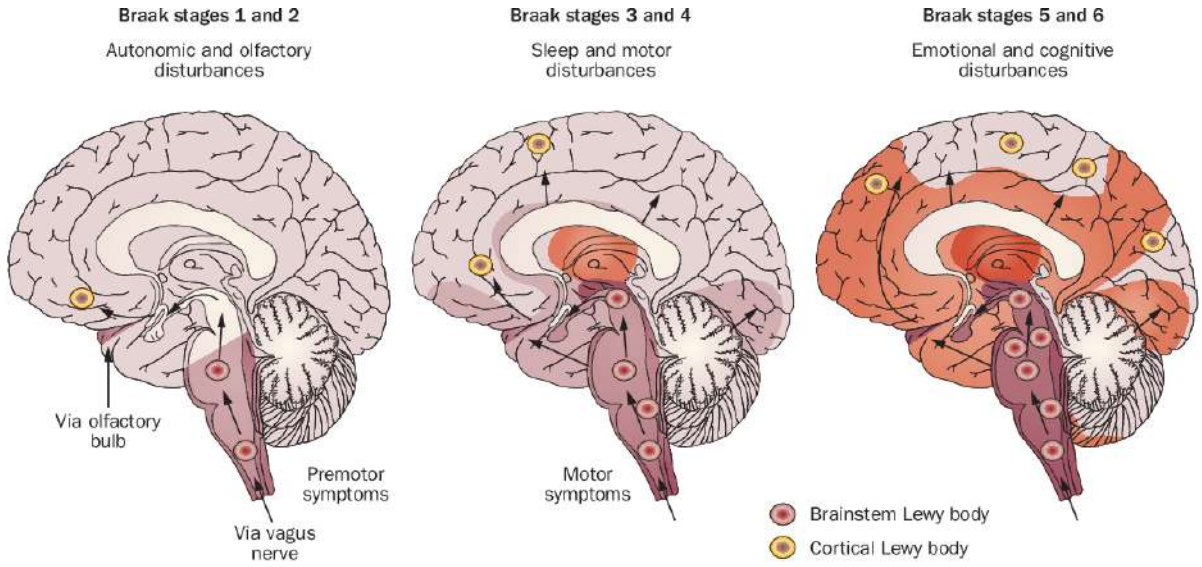


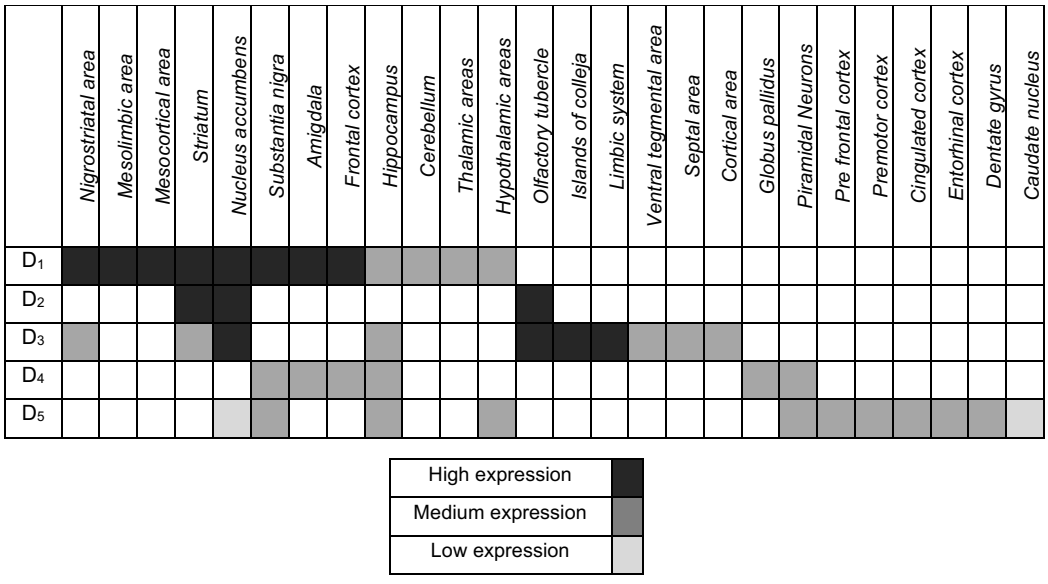
Figure 3: The H. Braak staging system of Parkinson's disease, showing that Lewy bodies deposition starts in the olfactory bulb and the medulla oblongata, followed by infiltration of Lewy pathology into cortical regions (from Doty 2012).

The majority of PD cases are sporadic. In sporadic PD, a combination of genetic susceptibility and environmental factors is thought to contribute to the development of the disease (Sulzer 2007). Familial PD with specific genetic defects may account for less than 10% of all cases (Gasser 2001), but has helped to gain insight into the pathogenesis of PD. The *SCNA* gene, encoding the α-synuclein protein, was the first gene to be associated with PD (Polymeropoulos et al. 1997). Mutations in the *SCNA* and *LRRK2* genes have been linked to late-onset PD (Corti, Lesage, and Brice 2011). *Parkin*, *PINK1* and probably *DJ-1* have also been associated with PD. Mutations in these genes are associated with mitochondrial dysfunctions and are important risk factors for the development of early-onset autosomal recessive PD (Corti et al. 2011).

The dopaminergic system

As mentioned above, dysfunction of the dopaminergic system plays an important role in PD. The neurotransmitter of the dopaminergic system, dopamine (DA), is a member of the catecholamine family. DA is involved in different physiological processes, including locomotor activity and reward. DA is produced from L-tyrosine in the brain. The enzyme tyrosine hydroxylase (TH) is responsible for catalyzing the hydroxylation of L-tyrosine into L-DOPA, which is subsequently decarboxylated by the enzyme amino acid decarboxylase (AADC) to form DA (Missale et al. 1998). Once synthesized, DA is stored in presynaptic vesicles by the vesicular monoamine transporter (VMAT) and transported to the synaptic terminal. Upon activation of the dopaminergic neurons, DA is released from the vesicles into the synaptic cleft (Rice, Patel, and Cragg 2011). DA can bind to the metabotropic dopamine receptors, which can be divided into the stimulatory D_1-type (D_1 and D_5) and the inhibitory D_2-type (D_2, D_3 and D_4) receptors (Hurley and Jenner 2006; Missale et al. 1998; Sokoloff and Schwartz 1995). The DA receptor subtypes show a different distribution and expression levels across various brain regions (Beaulieu and Gainetdinov 2011) (Table 1). Locomotion, which is impaired in PD, is mainly mediated by D_1, D_2 and D_3 receptors, but D4 and D5 receptors also seem to be involved, because they are expressed in the motor regions of the brain (Missale et al. 1998; Sibley 1999).

Table 1: Gene expression of dopamine receptor subtypes in different regions of the mammalian brain (animal studies). Adapted from Beaulieu and Gainetdinov 2011.



Levodopa is the most used medication to control symptoms of PD, due to its ability to cross the blood brain barrier and to be converted to DA (Parkinson's Foundation 2020), therefore, increasing DA availability. Other treatments involve dopamine agonists because these drugs mimic the effects of dopamine and are used sometimes to minimize the on-off effects of levodopa. Other important components in dopaminergic transmission are the dopamine transporters (DAT), which are located on the plasma membrane of dopaminergic neurons and are responsible for the rapid reuptake of DA in the presynaptic neuron (Haenisch and Bönisch 2011). Several clinical studies with DAT inhibitors were performed in PD patients, but currently no specific treatment targeting these transporters is available in the clinic (Huot, Fox, and Brotchie 2016). However, DAT activity can be used as a biomarker for the diagnosis of early stages of PD (Romero et al. 2019). The degradation of DA occurs through the action of the enzymes monoamine oxidase (MAO) and catecholamine O-methyl transferase (COMT) (Huotari et al. 2002; Youdim, Edmondson, and Tipton 2006). MAO or COMT inhibitors are clinically used in the treatment of PD patients, the first can be used as a monotherapy in the early stages of the disease and the second is used as an adjuvant to increase the availability of levodopa in the brain (Parkinson's Foundation 2020).

The adenosinergic system

The importance of the dopaminergic system in PD is unquestionable, but the interaction of the dopaminergic system with other neurotransmitter systems suggests that these systems could be involved in PD as well. The adenosine system, for example, closely interacts with the dopaminergic system. Dopamine D_2 receptors form heterodimers with adenosine A_{2A} receptors and thus can have allosteric interactions. Adenosine A_{2A} receptor agonists can reduce the affinity of a D_2 receptor agonist (Ferre et al. 1991; Fuxe et al. 2003). Based on these findings, researchers suggested that adenosine A_{2A} receptor antagonists may exhibit potential for the treatment of PD, as they could increase the affinity of D_2 receptor agonist like DA and thus compensate for the degeneration of the dopaminergic system (Casadó-Anguera et al. 2016).

Adenosine is an important neuromodulator that exerts stimulating activity through the adenosine A_{2A} and A_{2B} receptor subtypes and inhibitory activity via the

adenosine A_1 and A_3 receptor subtypes (Ralevic and Burnstock 1998; Zimmermann 2011). In physiological situations, the action of adenosine is mediated only by A_1 and A_{2A} receptors, due to the low basal adenosine levels and the high affinity of adenosine for these receptors. The physiological role of the high affinity A_1 receptors is reinforced by its wide distribution in neural tissue. Adenosine A_{2A} receptors, on the other hand, are mainly expressed in the basal ganglia, blood vessels and immune cells (Chen, Eltzschig, and Fredholm 2013), suggesting a more important role in PD.

The major extracellular source of adenosine is adenosine triphosphate (ATP). E-NTPDases hydrolyze ATP into adenosine monophosphate (AMP) and ecto-5'-nucleotidase subsequently hydrolyzes AMP into adenosine. ATP hydrolyzing enzymes play an important role in controlling the activation of adenosine receptors. The key enzyme for adenosine production, ecto-5'-nucleotidase, plays a crucial role in striatal A_{2A} receptor activation (Augusto et al. 2013). Interestingly, adenosine and ATP can also act as modulators in inflammatory process, oxidative stress, excitotoxicity and cell death (Tóth et al. 2019). Studies in rodents showed that striatal injection of 6-hydroxydopamine (6-OHDA) caused an increase in adenosine production by stimulation of striatal AMP and ADP hydrolysis without altering the expression of the genes of enzymes responsible for the dephosphorylation of the adenosine phosphates (Oses et al. 2011).

Several therapies for PD appear to exert their neuroprotective properties, at least in part, by modulation of adenosine-mediated signaling (Nazario, da Silva, and Bonan 2017). Adenosine A_{2A} receptor antagonists can reduce the off-time and the complications of levodopa (Hauser et al. 2008). These “off” episodes occur when the treatment with levodopa stops working and PD symptoms like tremor and difficulty walking start increasing again (FDA 2019). For this reason, the A_{2A} antagonist, Istradefyline, has been approved as a complementary treatment for PD in Japan since 2013 (Torti, Vacca, and Stocchi 2018). This drug was also approved as an adjunctive treatment to levodopa/cardiodopa in adult patients experiencing “off” episodes in United States of America since 2019. The European Medicines Agency has reviewed the product dossier and recently provided marketing authorization for this drug as well (EMA 2020). Caffeine, one of the most consumed psychostimulants in the world, is an A_{2A} adenosine receptor antagonist as well. Caffeine was found to exert neuroprotective effects (Chiu and Freund 2014) and suggested to slow down the progression of degeneration in PD models (Ferré et al. 1992; Morelli et al. 2012; Sonsalla et al. 2012).

Inflammation

Inflammation is an important physiological reaction that, in most cases, is beneficial for the body. However, chronic and exacerbated inflammatory responses can become detrimental to the body, including the brain. In the brain, the response to the tissue damage, danger signals and pathogens is mediated by microglia, the resident macrophages of the central nervous system (Thameem Dheen, Kaur, and Ling 2007). Excessive and chronic activation of microglia leads to an increase in the release of pro-inflammatory cytokines that could eventually lead to neuronal damage (Zhang et al. 2005). Moreover, peripheral inflammation can induce an inflammatory response in the brain. For example, microbiota changes, or irritable bowel syndrome can lead to the increase of the permeability of the blood-brain barrier through the release of inflammatory mediators and LPS, thus enabling the influx of the peripheral immune cells and immune mediators (Kortekaas et al. 2005).

Neuroinflammation is a common hallmark of neurodegenerative diseases such as PD (Suescun, Chandra, and Schiess 2019). The confirmation that PD is accompanied with neuroinflammation was made through postmortem analysis of specific inflammatory markers in the brain of PD patients. These findings were later confirmed with neuroimaging techniques that could visualize the presence of neuroinflammation *in vivo* (Cebrián, Loike, and Sulzer 2014; Gerhard et al. 2006; Hirsch et al. 2016; Toulorge, Schapira, and Hajj 2016). In addition, increased levels of pro-inflammatory mediators have been found in the blood of PD patients (Kim et al. 2018). After many years of study, however, it is still not clear whether neuroinflammation is the cause or the consequence of neurodegeneration in PD (Tansey and Goldberg 2010). Another key question is if the exacerbated activation microglia originates from the brain or has an extra-cerebral origin.

Gut-Brain Axis

Interactions of the brain and the gut are well described in pathologies like depression, anxiety, inflammatory bowel disease, Alzheimer disease and PD (Gracie, Hamlin, and Ford 2019; Hirschberg et al. 2019; Peirce and Alviña 2019). Although it is known that the gut and the brain interact in PD, it is not entirely clear yet what happens first: degeneration in the brain or gut modifications (Borghammer and Van Den Berge 2019).

A problem for the research on PD is that the diagnosis can only be made at a late stage of the disease, which makes it difficult to discover the origin of the disease. Environmental, genetic and lifestyle factors can possibly induce pathological processes in the gut and brain that can aggravate each other.

Patients with PD are more likely to suffer gastrointestinal problems, such as constipation, and these symptoms can appear ten years before the diagnose (Poewe et al. 2017). H. Braak and colleagues (2003) performed pathological analysis of the gut of PD patients and observed that there is α-synuclein deposition in the gut (H Braak et al. 2003). A systematic review and meta-analyses performed in 2019 demonstrated a correlation between the α-synuclein deposition in the gut and PD. The authors concluded that the α-synuclein deposition in the gut may be used as an adjuvant for the diagnosis (Bu et al. 2019). The deposition of α-synuclein is highly correlated with neuroinflammation (Gelders, Baekelandt, and Perren 2018), which may be due to a direct immune response that increases the expression of inflammatory mediators (Gelders et al. 2018) or could be related to changes in microbiota composition (Fitzgerald, Murphy, and Martinson 2019). Animal studies revealed that mice receiving microbiota transplant from PD patients show enhanced motor impairment and neuroinflammation, as compared to a control group (Sampson et al. 2016). Several studies show that gut composition of patients with PD have an increased relative abundance of bacteria from the genera *Akkermansia*, *Lactobacillus*, and *Bifidobacterium* and decreased abundances of *Prevotella*, *Faecalibacterium*, and *Blautia* (Bedarf et al. 2017; Keshavarzian et al. 2015; Scheperjans et al. 2015; Unger et al. 2016).

It is interesting to mention that alterations in the dopaminergic system have been associated with constipation, one of the first symptoms of PD patients (Colucci et al. 2012; Garrido-Gil et al. 2018; Jiménez et al. 2014). Colucci and colleagues (2012) found a reduced expression of D_2 receptors in the proximal (-66.8%) and distal (-54.5%) colon, as well as reduced peristalsis efficiency in rats with 6-OHDA induced lesions in striatum. This observation was confirmed by Garrido-Gil and colleagues (2018), who showed in rodents that nigrostriatal dopaminergic lesions cause a decrease in colonic D_1 and D_2 receptor expression (Garrido-Gil et al. 2018). Vice versa, colonic inflammation can increase nigrostriatal dopaminergic cell death in PD model, which is accompanied by an increase in neuroinflammatory markers (Gil-Martínez et al. 2019). These observations support the hypothesis that the gut-brain axis is involved

in the pathology of PD, and could help explain why constipation is one of the first occurring symptoms.

Diet

Evidences suggests that life style is an important factor that may prevent, reduce or accelerate the progression of PD. The influence of lifestyle before PD diagnosis has been researched in epidemiological studies (Morris et al. 2010; Paul et al. 2019; Xu et al. 2011), showing that factors like caffeine and alcohol (moderated consumption) intake, and exercise may have a protective effect against PD, whereas smoking and alcoholism were associated with an increased risk (Paul et al. 2019). Other risk factors like a bad diet, diabetes, high cholesterol, traumatic brain injury, cancer and exposure to pesticides were also reported in the literature (Ascherio and Schwarzschild 2016).

One of the life style factors that can affect the onset and progression of PD is the diet (Mischley, Lau, and Bennett 2017). For example, Western diet (high intake of fat, sugar and red meat) was found to increase the risk to develop PD, whereas a Mediterranean diet (high intake of fibers) decreases it (Maraki et al. 2019; Mischley et al. 2017). The mechanisms responsible for the effects of diet are not elucidated yet, but can be a direct effect of components in diet or an indirect effect through diet-induced changes in the composition of the gut microbiota (Figure 4) (Heiman and Greenway 2016; Perez-Pardo et al. 2017).

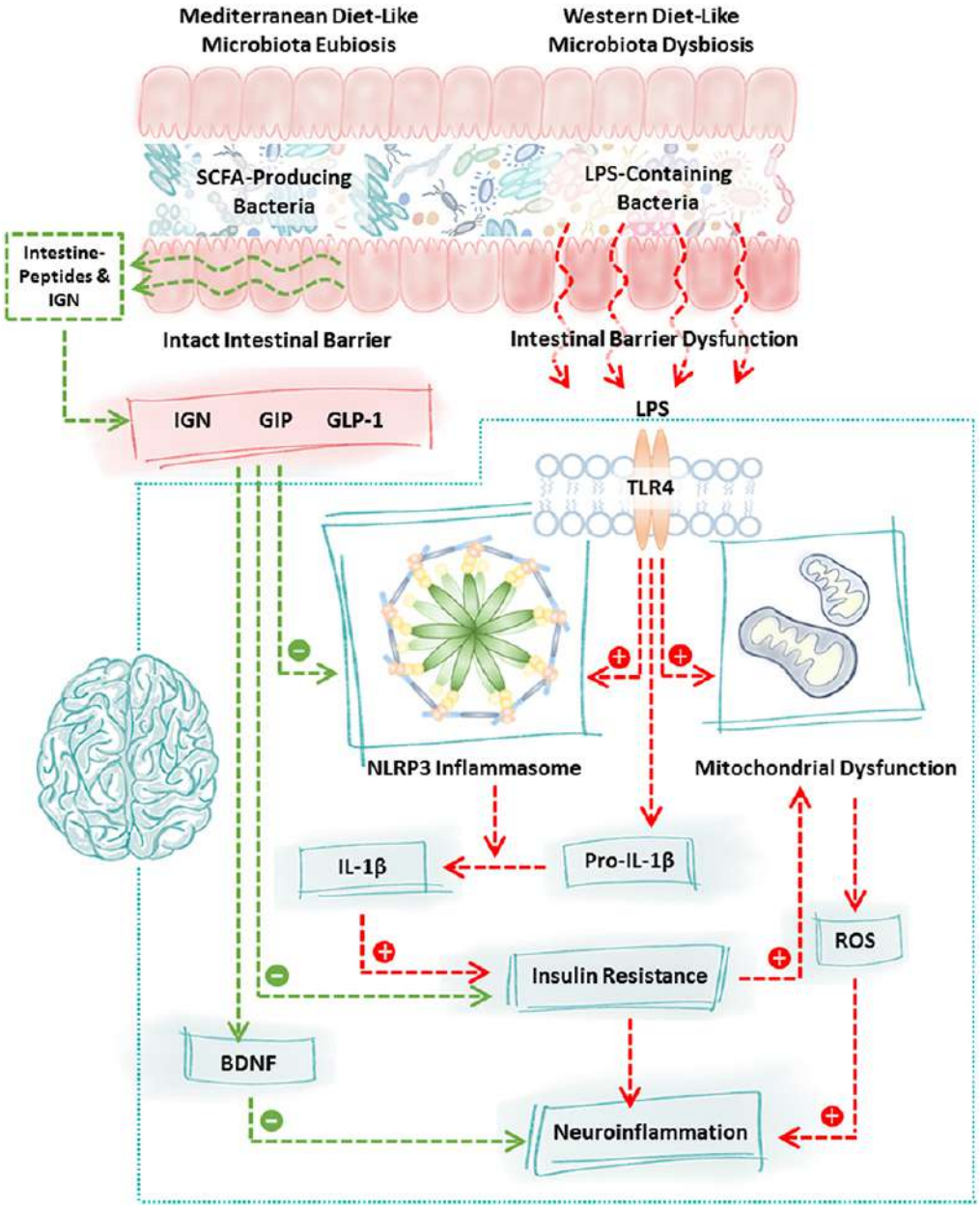


Figure 4: Communication between the intestinal microbiota and the brain. (from Jackson et al. 2019)

Diet can affect intestinal microbiota. For example, Western diet can stimulate the expansion of lipopolysaccharide (LPS)-releasing bacteria and reduce the abundance of short chain fatty acids (SCFA)-producing bacteria, whereas a Mediterranean diet promotes the opposite. LPS-releasing bacteria are known as pro-inflammatory and are capable of inducing a disruption of the intestinal barrier integrity. LPS binds to toll-like receptor 4 (TLR4) and thus can stimulate a cascade of events, summarized in Figure 4, that can contribute to neuroinflammation and neurodegeneration in the brain. In contrast, the ingestion of a Mediterranean diet

increases the production of SCFA and as a consequence fortifies the intestinal barrier, stimulates the intestinal production of glucagon like peptide 1 (GLP-1) and gastrointestinal peptide (GIP), which inhibits NLRP3 inflammasome activation and regulates insulin resistance. SCFA and GLP-1/GIP can activate the intestinal glucogenesis (IGN) of epithelial cells and thus stimulate the *vagus* nerve and the production of brain-derived neurotrophic factor (BDNF). BDNF has several positive effects on the brain, improves neuronal insulin resistance and promotes neural health (Figure 4) (Jackson et al. 2019). Characteristic features of PD patients' microbiome are similar to those observed following consumption of a Western diet (low SCFA-producing bacteria, high LPS-secreting bacteria). This suggests that dietary interventions, such as a Mediterranean diet (or components of the Mediterranean diet), may be a viable approach to blunt neuroinflammation and improve neuronal function in PD patients (Oliveira de Carvalho et al. 2018). Such a lifestyle intervention may also help preventing the onset of the disease.

Another risk factor for PD related with the diet is the development of diabetes type 2. Prevalence of type 2 diabetes mellitus (T2DM) increased over the years, with 465 million people being diagnosed with this condition in 2019, according to data from the international diabetes federation (WHO 2016). People with T2DM have a higher incidence of cognitive decline and an increased risk of developing all types of dementia, which corroborates the idea that diabetes could be an accelerator for the development of neurodegenerative diseases (Umegaki 2012; Yang and Song 2013). The association between PD and T2DM was first reported in 1960 and it was suggested that diabetes accelerated the evolution of both motor and cognitive Parkinsonian symptoms (Schwab 1960). A meta-analysis confirmed that T2DM increases the risk of developing PD by approximately 38% (Yue et al. 2016).

Morris et al. (2010) demonstrated that a high fat diet leads to insulin resistance and accelerates the progression of neurodegeneration in a PD model. In animals on a high-fat diet, dopamine depletion in substance nigra and striatum after 6-OHDA injection in the medial forebrain bundle was correlated with adiposity and insulin resistance (Morris et al. 2010). Likewise, many patients with PD demonstrated abnormal glucose tolerance and hyperglycemia (Lipman, Boykin, and Flora 1974; Marques et al. 2018). The association between T2DM and PD could be explained by the increase in methylglyoxal (MGO) levels in response of the increased glucose levels in the body. MGO is a potent glycation agent that plays an important role in the

exacerbated formation of α-synuclein aggregates and is also involved in insulin resistance (A. Shamsaldeen et al. 2016; Vicente Miranda, El-Agnaf, and Outeiro 2016). In addition, the glycated species produced by MGO activate RAGE (multi-ligand receptor for advanced glycation end products), thereby triggering an inflammatory response. Thus, glycation can contribute to neurodegeneration and neuroinflammation in PD and may constitute the missing link between diabetes and this neurodegenerative disease (Vicente Miranda et al. 2016).

Santiago and Potashkin (2013b, 2013a) provided evidence that PD and T2DM are also strongly linked at the molecular level. They identified 478 genes implicated in both diseases. The genes that are most correlated between PD and T2DM are: *cd63, cdk1, ushbp1, raf1, pkn1, mapk1 and 3, rhoa, crebbp, copb1, akt1, arf1 and 3, braf, ralgds, app, pourf1, rock 1* and *2, and prkca.* Most of these genes are involved in cell survival and metabolism, mitochondrial function, autophagy, inflammatory response, and insulin resistance (Santiago and Potashkin 2013a).

Animal models

Animal models that try to mimic specific features of diseases in general do not have all the characteristics of face, prediction and construct validity (Belzung and Lemoine 2011). This is not different for models of PD: the models cannot reproduce all motor and non-motor symptoms, and show different dynamics of dopaminergic neural loss with increasing age than patients. Despite these shortcomings, the use of animal models is important for unraveling the basic mechanisms underlying the disease and can help in the development of new drugs (reviewed by Tieu 2011). Different species are used as animal models for PD, including invertebrates, such as *Drosophila*, *Caenorhabditis elegans* and snail, and vertebrates like zebrafish, mouse, rat, and monkey (Zeng et al. 2018). These animals have different characteristics that can direct their use as animal model for a specific purpose. For example, monkeys are the closest to humans, but expensive and used less frequently because of ethical reasons; rodents also have physiological and morphological aspects similar to humans, but are cheaper; and zebrafish are the cheapest animal model, but smaller and only share some characteristics similar to humans.

Genetic and pharmacological interventions are used to create models that mimic specific aspects of PD (Meredith, Sonsalla, and Chesselet 2008). The most used

genetic animal models are the ones with mutations in the autosomal dominant *LRRK2* or *SCNA* genes, or mutations in the autosomal recessive *parkin*, *DJ1* or *PINK1* gene (Dawson, Ko, and Dawson 2010). Different pharmacological approaches are also used to produce PD models. In the most frequently used animal model, nigrostriatal damage is induced by intracerebral injection of 6-OHDA. Other neurotoxins are used to induce neurodegeneration in animals as well. The well-known herbicide, Paraquat is capable of inducing PD symptoms after prolonged exposure (Berry, La Vecchia, and Nicotera 2010). Other models include intraperitoneal injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinen (MPTP), a lipophilic molecule that can cross the blood-brain barrier (Toomey et al. 2012) or injection of Rotenone, an organic pesticide that can cause dopaminergic toxicity by interference with the mitochondrial complex I (Glinka, Tipton, and Youdim 1998; Richardson et al. 2007; Schapira et al. 1989; Tanner et al. 2011).

6-OHDA is the most used neurotoxin to model dopaminergic neurodegeneration *in vivo* and *in vitro*. Since this neurotoxin cannot cross the blood-brain barrier, stereotactic injection is required (Schober 2004). The mechanism behind the toxicity of 6-OHDA is largely due to the induction of oxidative stress, respiratory inhibition and formation of reactive oxygen species (Rodriguez-Pallares et al. 2007; Sachs and Jonsson 1975). The stereotaxic injection of 6-OHDA in the striatum leads to a progressive, retrograde degeneration of the nigrostriatal neurons, with up to 70% of the dopaminergic neurons being destroyed in 4 weeks (Shimohama et al. 2003). This makes it an interesting model for a longitudinal study. Normally only a unilateral lesion is generated, resulting in a “hemiparkinson’s model” (Perese et al. 1989), in which the contralateral hemisphere can serve as internal control. Furthermore, bilaterally affected animals require intensive care (Cenci, Whishaw, and Schallert 2002). The damage induced to the nigrostriatal system in the hemiparkinson’s model causes asymmetric motor symptoms. However, it does not mimic all pathological features of PD, like tremor and rigidity. Other options to damage the dopaminergic system are to inject 6-OHDA into substantia nigra or the medial forebrain bundle, but both methods cause rapid cell death (Przedbroski et al. 1995).

Some studies investigate the mechanisms involved in PD using the zebrafish as an animal model. The dopaminergic pathway in zebrafish shows large similarities with that in rats (Figure 5) (Parker et al. 2013). The dorsal nucleus of the ventral telencephalon of zebrafish is suggested to be homologous to the mammalian striatum, and receives projections of dopaminergic neurons from the ventral diencephalon,

resembling the nigrostriatal pathway of mammals (Rink and Wullimann 2002). MPTP induces a decrease in dopamine levels in adult zebrafish (Lam, Korzh, and Strahle 2005). Rotenone induces cataleptic behavior and neuronal loss in zebrafish comparable to that seen in rodents (Makhija and Jagtap 2014). Paraquat has been used to mimic the parkinsonian phenotype in zebrafish promoting the typical behavioral, biochemical and molecular changes (Bortolotto et al. 2014). Exposure of zebrafish larvae to 6-OHDA leads to reduced general expression of tyrosine hydroxylase, increased expression of proinflammatory cytokines, and behavioral and locomotor changes (Feng et al. 2014; Parng et al. 2007). Unilateral diencephalic injections of 6-OHDA in adult zebrafish can ablate more than 85% of the dopaminergic neurons (Vijayanathan et al. 2017).

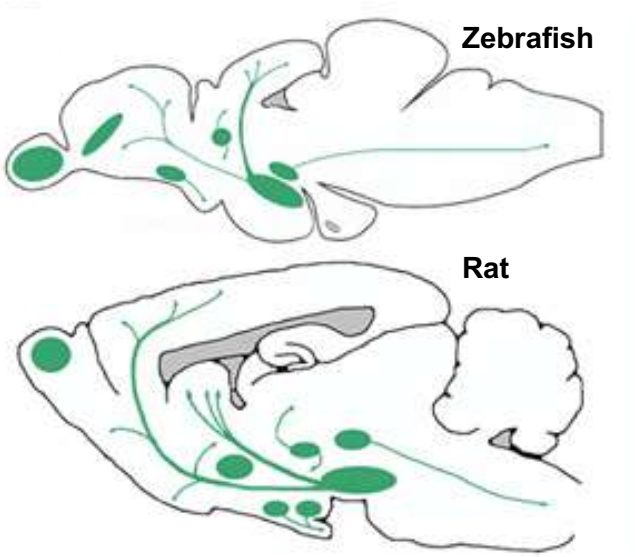


Figure 5: Schematic drawing illustrating the dopaminergic projections in adult zebrafish and rat brain. Adapted from Parker et al. 2013.

PET imaging

To study *in vivo* disease characteristics in animal models and PD patients, functional imaging techniques can be applied. Positron Emission Tomography (PET) is a non-invasive molecular imaging technique that uses tracers labeled with specific positron emitting isotopes to visualize biochemical and physiological processes *in vivo*.

The most frequently used positron emitting radioactive isotopes for the labeling of PET tracers are carbon-11 and fluor-18, with a half-life of 20 and 110 minutes, respectively. These isotopes are used to label biologically active molecules that can be applied as tracers to assess parameters like metabolism, perfusion, receptor binding and transporter availability. The radiotracer is usually injected intravenously before the scan and allowed to distribute throughout the body. The unstable isotope of the tracer will decay and the emitted positron will collide with an electron in the tissue, causing an annihilation event. In this annihilation event, the mass of the positron and electron is converted into two gamma rays with an energy of 511 keV, which will travel in opposite direction from each other (usually with a small deviation in the angle). These gamma rays can be detected by scintillators located in a ring around the patient or the animal. The detection of the two photons is registered if the two photons are recorded by two opposite scintillators at the same time (true coincidence). After true coincidences have been measured and several corrections have been applied, a 3D representation of the tracer distribution can be generated. PET provides quantitative information about the tracer distribution (and thus about the underlying processes). Usually tracer concentration in the tissue of interest is normalized to the injected dose and bodyweight and, therefore, is expressed as the standardized uptake value (SUV) in order to enable comparison between subjects and conditions.

PET can be used in preclinical and clinical researches and has the advantage that findings in animal models can be confirmed in a noninvasive way in patients using the same methodology. PET can capture processes that are difficult to visualize with other modalities, like metabolism, enzymatic activity, protein accumulation and receptor binding (Slough et al. 2016). These characteristics may contribute, in the future, to a faster diagnosis of the disease and more accurate monitoring of therapy efficiency (Eisenmenger et al. 2016).

Different radiotracers have been used to study various aspects of PD in clinical studies, such as: glucose metabolism (^{18}F-FDG), neuroinflammation ([^{11}C]PBR28; [^{11}C]PK11195), the GABAergic ([^{11}C]flumazenil), dopaminergic ([^{11}C]raclopride; 6-[^{18}F]Fluoro-L-DOPA) and adenosinergic ([^{11}C]Preladenant) system (Gerhard et al. 2006; Ibrahim et al. 2016; Sun et al. 2019; Varnäs et al. 2019; Volonté et al. 2001). PET imaging methods for PD have recently been reviewed (Abbasi Gharibkandi and Hosseinimehr 2019).

THESIS OUTLINE

The number of patients with PD increases each year and no curative treatment is available yet. At present, the best treatment option is slowing down the progression of the disease and reducing the symptoms. Early diagnosis and preventive treatment are important factors that can help minimizing disease burden. This requires a better understanding of the basic mechanisms involved in the onset and early progression of the disease. Such information could help the development of new interventions to prevent, slow down or stop the disease at an early stage. The main goal of this thesis is to investigate basic mechanisms underlying PD in different animal models. Since lifestyle is an important risk factor for PD, the influence of diet is investigated as well. This thesis starts with the assessment of basic mechanisms of PD in zebrafish; then the feasibility of PET imaging in zebrafish is explored; and finally, the effect of high-fat diet on the dopaminergic signaling pathway is studied in healthy rats and a rat model of PD.

In **Chapter 2,** the interaction of adenosine receptors with dopaminergic receptors and the potential role of adenosine receptor ligands in the treatment of PD are reviewed. In this chapter, the potential relation of adenosine with lifestyle and diabetes is discussed as well.

In **Chapter 3,** a PD model in zebrafish is described. In this PD model, purinergic and dopaminergic receptors and behavioral parameters were measured at different time points after induction.

In **Chapter 4,** the feasibility of *in vivo* PET imaging in zebrafish is explored. Animal preparation and imaging procedures have been optimized to be applied in different research sites.

Chapter 5 describes a study investigating the effect of a cafeteria diet on the dopaminergic reward system in rats. PET was used to measure dopamine D_2 receptor availability in rats on a cafeteria diet (high caloric diet) and control animals after a challenge with highly palatable food.

Chapter 6 employed ^{11}C-Raclopride PET to investigate the effect of a high-fat diet on D_2 receptor availability, before and after injection of the neurotoxin 6-OHDA as a rat model for PD.

Chapter 7 investigated the effect of high-fat diet on the neuroinflammation in the 6-OHDA rat model of PD, using PET with the tracer ^{11}C-PBR28. In addition, the effect on the gut microbiota composition and cytokines in the blood was evaluated.

References

A. Shamsaldeen, Yousif, Louise S. Mackenzie, Lisa A. Lione, and Christopher D. Benham. 2016. “Methylglyoxal, A Metabolite Increased in Diabetes Is Associated with Insulin Resistance, Vascular Dysfunction and Neuropathies.” *Current Drug Metabolism* 17(4):359–67.

Abbasi Gharibkandi, Nasrin and Seyed Jalal Hosseinimehr. 2019. “Radiotracers for Imaging of Parkinson’s Disease.” *European Journal of Medicinal Chemistry* 166:75–89.

Ascherio, Alberto and Michael A. Schwarzschild. 2016. “The Epidemiology of Parkinson’s Disease: Risk Factors and Prevention.” *The Lancet Neurology* 15(12):1257–72.

Augusto, Elisabete, Marco Matos, Jean Sévigny, Ali El-Tayeb, Margaret S. Bynoe, Christa E. Müller, Rodrigo A. Cunha, and Jiang Fan Chen. 2013. “Ecto-5′- Nucleotidase (CD73)-Mediated Formation of Adenosine Is Critical for the Striatal Adenosine A2A Receptor Functions.” *Journal of Neuroscience* 33(28):11390–99.

Beaulieu, Jean Martin and Raul R. Gainetdinov. 2011. “The Physiology, Signaling, and Pharmacology of Dopamine Receptors.” *Pharmacological Reviews* 63(1):182–217.

Bedarf, J. R., F. Hildebrand, L. P. Coelho, S. Sunagawa, M. Bahram, F. Goeser, P. Bork, and U. Wüllner. 2017. “Functional Implications of Microbial and Viral Gut Metagenome Changes in Early Stage L-DOPA-Naïve Parkinson’s Disease Patients.” *Genome Medicine* 9(1):39.

Belzung, Catherine and Maël Lemoine. 2011. “Criteria of Validity for Animal Models of Psychiatric Disorders: Focus on Anxiety Disorders and Depression.” *Biology of Mood & Anxiety Disorders* 1(1):9.

Berry, C., C. La Vecchia, and P. Nicotera. 2010. “Paraquat and Parkinson’s Disease.” *Cell Death and Differentiation* 17(7):1115–25.

Borghammer, Per and Nathalie Van Den Berge. 2019. “Brain-First versus Gut-First Parkinson’s Disease: A Hypothesis.” *Journal of Parkinson’s Disease* 9(s2):S281–95.

Bortolotto, Josiane W., Giana P. Cognato, Raissa R. Christoff, Laura N. Roesler, Carlos E. Leite, Luiza W. Kist, Mauricio R. Bogo, Monica R. Vianna, and Carla D. Bonan. 2014. “Long-Term Exposure to Paraquat Alters Behavioral Parameters and Dopamine Levels in Adult Zebrafish (Danio Rerio).” *Zebrafish* 11(2):142–53.

Braak, H, U. Rüb, W. P. Gai, and K. Del Tredici. 2003. “Idiopathic Parkinson’s Disease: Possible Routes by Which Vulnerable Neuronal Types May Be Subject to Neuroinvasion by an Unknown Pathogen.” *J Neural Transm* 110:517–36.

Braak, Heiko, Kelly Del Tredici, Udo Rüb, Rob A. I. De Vos, Ernst N. H. Jansen Steur, and Eva Braak. 2003a. “Staging of Brain Pathology Related to Sporadic Parkinson’s Disease.” *Neurobiology of Aging* 24(2):197–211.

Braak, Heiko, Kelly Del Tredici, Udo Rüb, Rob A. I. De Vos, Ernst N. H. Jansen Steur, and Eva Braak. 2003b. “Staging of Brain Pathology Related to Sporadic Parkinson’s Disease.” *Neurobiology of Aging* 24(2):197–211.

Bu, Jin, Jinsong Liu, Kun Liu, and Zhaohui Wang. 2019. “Diagnostic Utility of Gut α-Synuclein in Parkinson’s Disease: A Systematic Review and Meta-Analysis.” *Behavioural Brain Research* 364:340–47.

Casadó-Anguera, Verònica, Jordi Bonaventura, Estefanía Moreno, Gemma Navarro, Antoni Cortés, Sergi Ferré, and Vicent Casadó. 2016. “Evidence for the Heterotetrameric Structure of the Adenosine A 2A -Dopamine D 2 Receptor Complex.” *Biochemical Society Transactions* 44(2):595–600.

Cebrián, Carolina, John D. Loike, and David Sulzer. 2014. “Neuroinflammation in Parkinson’s Disease Animal Models: A Cell Stress Response or a Step in Neurodegeneration?” *Current Topics in Behavioral Neurosciences* 22:237–70.

Cenci, M. Angela, Ian Q. Whishaw, and Timothy Schallert. 2002. “Animal Models of Neurological Deficits: How Relevant Is the Rat?” *Nature Reviews Neuroscience* 3(7):574–79.

Chen, Jiang Fan, Holger K. Eltzschig, and Bertil B. Fredholm. 2013. “Adenosine Receptors as Drug Targets-What Are the Challenges?” *Nature Reviews Drug Discovery* 12(4):265–86.

Chiu, Gabriel S. and Gregory G. Freund. 2014. “Modulation of Neuroimmunity by Adenosine and Its Receptors: Metabolism to Mental Illness.” *Metabolism: Clinical and Experimental* 63(12):1491–98.

Colucci, Mario, Marila Cervio, Marisa Faniglione, Stefano De Angelis, Massimo Pajoro, Giovanna Levandis, Cristina Tassorelli, Fabio Blandini, Fausto Feletti, Roberto De Giorgio, Antonio Dellabianca, Stefano Tonini, and Marcello Tonini. 2012. “Intestinal Dysmotility and Enteric Neurochemical Changes in a Parkinson’s Disease Rat Model.” *Autonomic Neuroscience: Basic and Clinical* 169(2):77–86.

Corti, Olga, Suzanne Lesage, and Alexis Brice. 2011. “WHAT GENETICS TELLS US ABOUT THE CAUSES AND MECHANISMS OF PARKINSON’S DISEASE L.” *Physiol Rev* 91:1161–1218.

Dawson, Ted M., Han Seok Ko, and Valina L. Dawson. 2010. “Genetic Animal Models of Parkinson’s Disease.” *Neuron* 66(5):646–61.

Dorsey, E. R., R. Constantinescu, J. P. Thompson, K. M. Biglan, R. G. Holloway, K. Kieburtz, F. J. Marshall, B. M. Ravina, G. Schifitto, A. Siderowf, and C. M. Tanner. 2007. “Projected Number of People with Parkinson Disease in the Most Populous Nations, 2005 through 2030.” *Neurology* 68(5):384–86.

Doty, Richard L. 2012. “Olfactory Dysfunction in Parkinson Disease.” *Nature Reviews Neurology* 8(6):329–39.

Driver, Jane A., Ashley Smith, Julie E. Buring, J. Michael Gaziano, Tobias Kurth, and Giancarlo Logroscino. 2008. “Prospective Cohort Study of Type 2 Diabetes and the Risk of Parkinson’s Disease.” *Diabetes Care* 31(10):2003–5.

Eisenmenger, Laura B., Eugene J. Huo, John M. Hoffman, Satoshi Minoshima, Manuela C. Matesan, David H. Lewis, Brian J. Lopresti, Chester A. Mathis, David O. Okonkwo, and James M. Mountz. 2016. “Advances in PET Imaging of Degenerative, Cerebrovascular, and Traumatic Causes of Dementia.” *Seminars in Nuclear Medicine* 46(1):57–87.

EMA. 2020. “European Medicines Agency.” in *Definitions*.

FDA. 2019. “FDA Approves New Add-on Drug to Treat off Episodes in Adults with Parkinson’s Disease.” *Case Medical Research*.

Feng, Chien Wei, Zhi Hong Wen, Shi Ying Huang, Han Chun Hung, Chun Hong Chen, San Nan Yang, Nan Fu Chen, Hui Min Wang, Chung Der Hsiao, and Wu Fu Chen. 2014. “Effects of 6-Hydroxydopamine Exposure on Motor Activity and Biochemical Expression in Zebrafish (Danio Rerio) Larvae.” *Zebrafish* 11(3):227–39.

Ferré, S., K. Fuxe, G. von Euler, B. Johansson, and B. B. Fredholm. 1992. “Adenosine-Dopamine Interactions in the Brain.” *Neuroscience* 51(3):501–12.

Ferre, Sergi, Gabriel Von Euler, Björn Johansson, Bertil B. Fredholm, and Kjell Fuxe. 1991. “Stimulation of High-Affinity Adenosine A2 Receptors Decreases the Affinity of Dopamine D2 Receptors in Rat Striatal Membranes.” *Proceedings of the National Academy of Sciences of the United States of America* 88(16):7238–41.

Fitzgerald, Emily, Sarah Murphy, and Holly A. Martinson. 2019. “Alpha-Synuclein Pathology and the Role of the Microbiota in Parkinson’s Disease.” *Frontiers in Neuroscience* 13(APR).

Fuxe, Kjell, L. F. Agnati, K. Jacobsen, J. Hillion, M. Canals, M. Torvinen, B. Tinner-Staines, W. Staines, D. Rosin, A. Terasmaa, P. Popoli, G. Leo, V. Vergoni, C. Lluis, F. Ciruela, R. Franco, and S. Ferré. 2003. “Receptor Heteromerization in Adenosine A2A Receptor Signaling: Relevance for Striatal Function and Parkinson’s Disease.” *Neurology* 61(11 SUPPL. 6).

Garrido-Gil, Pablo, Ana I. Rodriguez-Perez, Antonio Dominguez-Meijide, Maria J. Guerra, and Jose L. Labandeira-Garcia. 2018. “Bidirectional Neural Interaction Between Central Dopaminergic and Gut Lesions in Parkinson’s Disease Models.” *Molecular Neurobiology* 55(9):7297–7316.

Gasser, Thomas. 2001. *ENS TEACHING REVIEW*. Vol. 248.

Gelders, Géraldine, Veerle Baekelandt, and Anke Van der Perren. 2018. “Linking Neuroinflammation and Neurodegeneration in Parkinson’s Disease.” *Journal of Immunology Research* 2018.

Gerhard, Alexander, Nicola Pavese, Gary Hotton, Federico Turkheimer, Meltem Es, Alexander Hammers, Karla Eggert, Wolfgang Oertel, Richard B. Banati, and David J. Brooks. 2006. "In Vivo Imaging of Microglial Activation with [11C](R)-PK11195 PET in Idiopathic Parkinson's Disease." *Neurobiology of Disease* 21(2):404–12.

Gil-Martínez, Ana Luisa, Cristina Estrada, Lorena Cuenca, Juan Antonio Cano, Manuel Valiente, Carlos Manuel Martínez-Cáceres, Emiliano Fernández-Villalba, and María Trinidad Herrero. 2019. "Local Gastrointestinal Injury Exacerbates Inflammation and Dopaminergic Cell Death in Parkinsonian Mice." *Neurotoxicity Research* 35(4):918–30.

Glinka, Yelena, Keith F. Tipton, and Moussa B. H. Youdim. 1998. "Mechanism of Inhibition of Mitochondrial Respiratory Complex I by 6- Hydroxydopamine and Its Prevention by Desferrioxamine." *European Journal of Pharmacology* 351(1):121–29.

Gracie, David J., P. John Hamlin, and Alexander C. Ford. 2019. "The Influence of the Brain–Gut Axis in Inflammatory Bowel Disease and Possible Implications for Treatment." *The Lancet Gastroenterology and Hepatology* 4(8):632–42.

Haenisch, Britta and Heinz Bönisch. 2011. "Depression and Antidepressants: Insights from Knockout of Dopamine, Serotonin or Noradrenaline Re-Uptake Transporters." *Pharmacology and Therapeutics* 129(3):352–68.

Hauser, Robert A., Lisa M. Shulman, Joel M. Trugman, John W. Roberts, Akihisa Mori, Rocco Ballerini, and Neil M. Sussman. 2008. "Study of Istradefylline in Patients with Parkinson's Disease on Levodopa with Motor Fluctuations." *Movement Disorders* 23(15):2177–85.

Heiman, Mark L. and Frank L. Greenway. 2016. "A Healthy Gastrointestinal Microbiome Is Dependent on Dietary Diversity." *Molecular Metabolism* 5(5):317–20.

Hirsch, Lauren, Nathalie Jette, Alexandra Frolkis, Thomas Steeves, and Tamara Pringsheim. 2016. "The Incidence of Parkinson's Disease: A Systematic Review and Meta-Analysis." *Neuroepidemiology* 46(4):292–300.

Hirschberg, Sarah, Barbara Gisevius, Alexander Duscha, and Aiden Haghikia. 2019. "Implications of Diet and the Gut Microbiome in Neuroinflammatory and Neurodegenerative Diseases." *International Journal of Molecular Sciences* 20(12).

Hung, Hui Chuan and Eminy H. Y. Lee. 1996. "The Mesolimbic Dopaminergic Pathway Is More Resistant than the Nigrostriatal Dopaminergic Pathway to MPTP and MPP+ Toxicity: Role of BDNF Gene Expression." *Molecular Brain Research* 41(1–2):16–26.

Huot, Philippe, Susan H. Fox, and Jonathan M. Brotchie. 2016. "Dopamine Reuptake Inhibitors in Parkinson's Disease: A Review of Nonhuman Primate Studies and Clinical Trials." *Journal of Pharmacology and Experimental Therapeutics* 357(3):562–69.

Huotari, Marko, Joseph A. Gogos, Maria Karayiorgou, Olli Koponen, Markus Forsberg, Atso Raasmaja, Juha Hyttinen, and Pekka T. Männistö. 2002. "Brain Catecholamine Metabolism in Catechol- *O* -Methyltransferase (COMT)-Deficient Mice." *European Journal of Neuroscience* 15(2):246–56.

Hurley, M. J. and P. Jenner. 2006. "What Has Been Learnt from Study of Dopamine Receptors in Parkinson's Disease?" *Pharmacology and Therapeutics* 111(3):715–28.

Ibrahim, Nevein, Joanna Kusmirek, Aaron F. Struck, John M. Floberg, Scott B. Perlman, Catherine Gallagher, and Lance T. Hall. 2016. "The Sensitivity and Specificity of F-DOPA PET in a Movement Disorder Clinic." *American Journal of Nuclear Medicine and Molecular Imaging* 6(1):102–9.

Jackson, Aeja, Christopher B. Forsyth, Maliha Shaikh, Robin M. Voigt, Phillip A. Engen, Vivian Ramirez, and Ali Keshavarzian. 2019. "Diet in Parkinson's Disease: Critical Role for the Microbiome." *Frontiers in Neurology* 10:1245.

Jiménez, Marcel, Pere Clavé, Anna Accarino, and Diana Gallego. 2014. "Purinergic Neuromuscular Transmission in the Gastrointestinal Tract; Functional Basis for Future Clinical and Pharmacological Studies." *British Journal of Pharmacology* 171(19):4360–75.

Keshavarzian, Ali, Stefan J. Green, Phillip A. Engen, Robin M. Voigt, Ankur Naqib, Christopher B. Forsyth, Ece Mutlu, and Kathleen M. Shannon. 2015. "Colonic Bacterial Composition in Parkinson's Disease." *Movement Disorders* 30(10):1351–60.

Kim, Ryul, Han Joon Kim, Aryun Kim, Mihee Jang, Ahro Kim, Yoon Kim, Dallah Yoo, Jin Hee

Im, Ji Hyun Choi, and Beomseok Jeon. 2018. “Peripheral Blood Inflammatory Markers in Early Parkinson’s Disease.” *Journal of Clinical Neuroscience* 58:30–33.

Kortekaas, Rudie, Klaus L. Leenders, Joost C. H. Van Oostrom, Willem Vaalburg, Joost Bart, Antoon T. M. Willemsen, and N. Harry Hendrikse. 2005. “Blood-Brain Barrier Dysfunction in Parkinsonian Midbrain in Vivo.” *Annals of Neurology* 57(2):176–79.

Lam, Chen Sok, Vladimir Korzh, and Uwe Strahle. 2005. “Zebrafish Embryos Are Susceptible to the Dopaminergic Neurotoxin MPTP.” *European Journal of Neuroscience* 21(6):1758–62.

de Lau, Lonneke ML and Monique MB Breteler. 2006. “Epidemiology of Parkinson’s Disease.” *Lancet Neurology* 5(6):525–35.

Lipman, Ivan J., Michael E. Boykin, and Roger E. Flora. 1974. “Glucose Intolerance in Parkinson’s Disease.” *Journal of Chronic Diseases* 27(11–12):573–79.

Makhija, Dinesh T. and Aarti G. Jagtap. 2014. “Studies on Sensitivity of Zebrafish as a Model Organism for Parkinson′s Disease: Comparison with Rat Model.” *Journal of Pharmacology and Pharmacotherapeutics* 5(1):39–46.

Maraki, Maria I., Mary Yannakoulia, Maria Stamelou, Leonidas Stefanis, Georgia Xiromerisiou, Mary H. Kosmidis, Efthimios Dardiotis, Georgios M. Hadjigeorgiou, Paraskevi Sakka, Costas A. Anastasiou, Eleni Simopoulou, and Nikolaos Scarmeas. 2019. “Mediterranean Diet Adherence Is Related to Reduced Probability of Prodromal Parkinson’s Disease.” *Movement Disorders* 34(1):48–57.

Marques, Ana, Frédéric Dutheil, Elodie Durand, Isabelle Rieu, Aurélien Mulliez, Maria Livia Fantini, Yves Boirie, and Franck Durif. 2018. “Glucose Dysregulation in Parkinson’s Disease: Too Much Glucose or Not Enough Insulin?” *Parkinsonism and Related Disorders* 55:122–27.

Meredith, Gloria E., Patricia K. Sonsalla, and Marie Francoise Chesselet. 2008. “Animal Models of Parkinson’s Disease Progression.” *Acta Neuropathologica* 115(4):385–98.

Michel, Patrick P., Etienne C. Hirsch, and Stéphane Hunot. 2016. “Understanding Dopaminergic Cell Death Pathways in Parkinson Disease.” *Neuron* 90(4):675–91.

Mischley, Laurie K., Richard C. Lau, and Rachel D. Bennett. 2017. “Role of Diet and Nutritional Supplements in Parkinson’s Disease Progression.”

Missale, Cristina, S. Russel Nash, Susan W. Robinson, Mohamed Jaber, and Marc G. Caron. 1998. “Dopamine Receptors: From Structure to Function.” *Physiological Reviews* 78(1):189–225.

Morelli, Micaela, Fabio Blandini, Nicola Simola, and Robert A. Hauser. 2012. “A 2A Receptor Antagonism and Dyskinesia in Parkinson’s Disease.” 2012.

Morris, Jill K., Gregory L. Bomhoff, John A. Stanford, and Paige C. Geiger. 2010. “Neurodegeneration in an Animal Model of Parkinson’s Disease Is Exacerbated by a High-Fat Diet.” *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 299(4):R1082.

Nazario, Luiza R., Rosane S. da Silva, and Carla D. Bonan. 2017. “Targeting Adenosine Signaling in Parkinson’s Disease: From Pharmacological to Non-Pharmacological Approaches.” *Frontiers in Neuroscience* 11(NOV).

Oliveira de Carvalho, Alessandro, Alberto Souza Sá Filho, Eric Murillo-Rodriguez, Nuno Barbosa Rocha, Mauro Giovanni Carta, and Sergio Machado. 2018. “Physical Exercise For Parkinson’s Disease: Clinical And Experimental Evidence.” *Clinical Practice & Epidemiology in Mental Health* 14(1):89–98.

Oses, Jean Pierre, Cristiane Batassini, Daniela Pochmann, Ana Elisa Böhmer, Fernanda Cenci Vuaden, Roberta Bristot Silvestrin, Alcyr Oliveira, Carla Denise Bonan, Maurício Reis Bogo, Diogo Onofre Souza, Luis Valmor Cruz Portela, João José De Freitas Sarkis, and Tadeu Mello E Souza. 2011. “The Hydrolysis of Striatal Adenine- and Guanine-Based Purines in a 6-Hydroxydopamine Rat Model of Parkinson’s Disease.” *Neurochemical Research* 36(2):215–22.

Parker, Matthew O., Alistair J. Brock, Robert T. Walton, and Caroline H. Brennan. 2013. “The Role of Zebrafish (Danio Rerio) in Dissecting the Genetics and Neural Circuits of Executive Function.” *Frontiers in Neural Circuits* 7(MAR).

Parkinson's Foundation. 2020. "Parkinson's Foundation." Retrieved March 4, 2020 (https://www.parkinson.org/Understanding-Parkinsons/Treatment).

Parng, Chuenlei, Nicole Marie Roy, Christopher Ton, Yingxin Lin, and Patricia McGrath. 2007. "Neurotoxicity Assessment Using Zebrafish." *Journal of Pharmacological and Toxicological Methods* 55(1):103–12.

Paul, Kimberly C., Yu Hsuan Chuang, I. Fan Shih, Adrienne Keener, Yvette Bordelon, Jeff M. Bronstein, and Beate Ritz. 2019. "The Association between Lifestyle Factors and Parkinson's Disease Progression and Mortality." *Movement Disorders* 34(1):58–66.

Peirce, Jason M. and Karina Alviña. 2019. "The Role of Inflammation and the Gut Microbiome in Depression and Anxiety." *Journal of Neuroscience Research* 97(10):1223–41.

Perese, D. A., J. Ulman, J. Viola, S. E. Ewing, and K. S. Bankiewicz. 1989. "A 6-Hydroxydopamine-Induced Selective Parkinsonian Rat Model." *Brain Research* 494(2):285–93.

Perez-Pardo, Paula, Tessa Kliest, Hemraj B. Dodiya, Laus M. Broersen, Johan Garssen, Ali Keshavarzian, and Aletta D. Kraneveld. 2017. "The Gut-Brain Axis in Parkinson's Disease: Possibilities for Food-Based Therapies." *European Journal of Pharmacology* 817:86–95.

Pfeiffer, Ronald F. 2016. "Non-Motor Symptoms in Parkinson's Disease." *Parkinsonism and Related Disorders* 22:S119–22.

Poewe, Werner, Klaus Seppi, Caroline M. Tanner, Glenda M. Halliday, Patrik Brundin, Jens Volkmann, Anette Eleonore Schrag, and Anthony E. Lang. 2017. "Parkinson Disease." *Nature Reviews Disease Primers* 3:1–21.

Polymeropoulos, Mihael H., Christian Lavedan, Elisabeth Leroy, Susan E. Ide, Anindya Dehejia, Amalia Dutra, Brian Pike, Holly Root, Jeffrey Rubenstein, Rebecca Boyer, Edward S. Stenroos, Settara Chandrasekharappa, Aglaia Athanassiadou, Theodore Papapetropoulos, William G. Johnson, Alice M. Lazzarini, Roger C. Duvoisin, Giuseppe Di Iorio, Lawrence I. Golbe, and Robert L. Nussbaum. 1997. "Mutation in the α-Synuclein Gene Identified in Families with Parkinson's Disease." *Science* 276(5321):2045–47.

Przedbroski, S., M. Leviver, H. Jiang, M. Ferreira, V. Jackson-Lewis, D. Donaldson, and D. M. Togasaki. 1995. "Dose-Dependent Lesions of the Dopaminergic Nigrostriatal Pathway Induced by Instrastriatal Injection of 6-Hydroxydopamine." *Neuroscience* 67(3):631–47.

Ralevic, Vera and Geoffrey Burnstock. 1998. "Receptors for Purines and Pyrimidines." *Pharmacological Reviews* 50(3):413–92.

Ray Dorsey, E., Alexis Elbaz, Emma Nichols, Foad Abd-Allah, Ahmed Abdelalim, Jose C. Adsuar, Mustafa Geleto Ansha, Carol Brayne, Jee Young J. Choi, Daniel Collado-Mateo, Nabila Dahodwala, Huyen Phuc Do, Dumessa Edessa, Matthias Endres, Seyed Mohammad Fereshtehnejad, Kyle J. Foreman, Fortune Gbetoho Gankpe, Rahul Gupta, Graeme J. Hankey, Simon I. Hay, Mohamed I. Hegazy, Desalegn T. Hibstu, Amir Kasaeian, Yousef Khader, Ibrahim Khalil, Young Ho Khang, Yun Jin Kim, Yoshihiro Kokubo, Giancarlo Logroscino, João Massano, Norlinah Mohamed Ibrahim, Mohammed A. Mohammed, Alireza Mohammadi, Maziar Moradi-Lakeh, Mohsen Naghavi, Binh Thanh Nguyen, Yirga Legesse Nirayo, Felix Akpojene Ogbo, Mayowa Ojo Owolabi, David M. Pereira, Maarten J. Postma, Mostafa Qorbani, Muhammad Aziz Rahman, Kedir T. Roba, Hosein Safari, Saeid Safiri, Maheswar Satpathy, Monika Sawhney, Azadeh Shafieesabet, Mekonnen Sisay Shiferaw, Mari Smith, Cassandra E. I. Szoeke, Rafael Tabarés-Seisdedos, Nu Thi Truong, Kingsley Nnanna Ukwaja, Narayanaswamy Venketasubramanian, Santos Villafaina, Kidu Gidey Weldegwergs, Ronny Westerman, Tissa Wijeratne, Andrea S. Winkler, Bach Tran Xuan, Naohiro Yonemoto, Valery L. Feigin, Theo Vos, and Christopher J. L. Murray. 2018. "Global, Regional, and National Burden of Parkinson's Disease, 1990–2016: A Systematic Analysis for the Global Burden of Disease Study 2016." *The Lancet Neurology* 17(11):939–53.

Rice, M. E., J. C. Patel, and S. J. Cragg. 2011. "Dopamine Release in the Basal Ganglia." *Neuroscience* 198:112–37.

Richardson, Jason R., W. Michael Caudle, Thomas S. Guillot, Jodi L. Watson, Eiko Nakamaru-Ogiso, Byoung Boo Seo, Todd B. Sherer, J. Timothy Greenamyre, Takao Yagi, Akemi

Matsuno-Yagi, and Gary W. Miller. 2007. “Obligatory Role for Complex {I} Inhibition in the Dopaminergic Neurotoxicity Of.” *Toxicological Sciences : An Official Journal of the Society of Toxicology* 95(1):196–204.

de Rijk, M. C., L. J. Launer, K. Berger, M. M. Breteler, J. F. Dartigues, M. Baldereschi, L. Fratiglioni, A. Lobo, J. Martinez-Lage, C. Trenkwalder, and A. Hofman. 2000. “Prevalence of Parkinson’s Disease in Europe: A Collaborative Study of Population-Based Cohorts. Neurologic Diseases in the Elderly Research Group.” *Neurology* 54(11 Suppl 5):S21-3.

Rink, Elke and Mario F. Wullimann. 2002. “Connections of the Ventral Telencephalon and Tyrosine Hydroxylase Distribution in the Zebrafish Brain (Danio Rerio) Lead to Identification of an Ascending Dopaminergic System in a Teleost.” Pp. 385–87 in *Brain Research Bulletin*. Vol. 57. Brain Res Bull.

Rizek, Philippe, Niraj Kumar, and Mandar S. Jog. 2016. “An Update on the Diagnosis and Treatment of Parkinson Disease.” *CMAJ* 188(16):1157–65.

Rodriguez-Pallares, J., J. A. Parga, A. Muñoz, P. Rey, M. J. Guerra, and J. L. Labandeira-Garcia. 2007. “Mechanism of 6-Hydroxydopamine Neurotoxicity: The Role of NADPH Oxidase and Microglial Activation in 6-Hydroxydopamine-Induced Degeneration of Dopaminergic Neurons.” *Journal of Neurochemistry* 103(1):145–56.

Romero, Klaus, Daniela Conrado, Jackson Burton, Timothy Nicholas, Vikram Sinha, Sreeraj Macha, Malidi Ahamadi, Jesse Cedarbaum, John Seibyl, Kenneth Marek, Peter Basseches, Derek Hill, Ed Somer, Jill Gallagher, David T. Dexter, Arthur Roach, and Diane Stephenson. 2019. “Molecular Neuroimaging of the Dopamine Transporter as a Patient Enrichment Biomarker for Clinical Trials for Early Parkinson’s Disease.” *Clinical and Translational Science* 12(3):240–46.

Sachs, Charlotte and Gösta Jonsson. 1975. “Mechanisms of Action of 6-Hydroxydopamine.” *Biochemical Pharmacology* 24(1):1–8.

Sampson, Timothy R., Justine W. Debelius, Taren Thron, Stefan Janssen, Gauri G. Shastri, Zehra Esra Ilhan, Collin Challis, Catherine E. Schretter, Sandra Rocha, Viviana Gradinaru, Marie Francoise Chesselet, Ali Keshavarzian, Kathleen M. Shannon, Rosa Krajmalnik-Brown, Pernilla Wittung-Stafshede, Rob Knight, and Sarkis K. Mazmanian. 2016. “Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson’s Disease.” *Cell* 167(6):1469-1480.e12.

Santiago, Jose A. and Judith A. Potashkin. 2013a. “Integrative Network Analysis Unveils Convergent Molecular Pathways in Parkinson’s Disease and Diabetes” edited by J. D. Barbour. *PLoS ONE* 8(12):e83940.

Santiago, Jose A. and Judith A. Potashkin. 2013b. “Shared Dysregulated Pathways Lead to Parkinson’s Disease and Diabetes.” *Trends in Molecular Medicine* 19(3):176–86.

Schapira, A. H. V., J. G. Llewelyn, N. J. Patel, and P. K. Thomas. 1989. “Polypeptide and Glycoprotein Abnormalities in Dorsal Root Ganglia of Streptozotocin-Diabetic Rats.” *Journal of the Neurological Sciences* 94(1–3):147–61.

Schapira, Anthony H. V., K. Ray Chaudhuri, and Peter Jenner. 2017. “Non-Motor Features of Parkinson Disease.” *Nature Reviews Neuroscience* 18(7):435–50.

Scheperjans, Filip, Velma Aho, Pedro A. B. Pereira, Kaisa Koskinen, Lars Paulin, Eero Pekkonen, Elena Haapaniemi, Seppo Kaakkola, Johanna Eerola-Rautio, Marjatta Pohja, Esko Kinnunen, Kari Murros, and Petri Auvinen. 2015. “Gut Microbiota Are Related to Parkinson’s Disease and Clinical Phenotype.” *Movement Disorders* 30(3):350–58.

Schober, Andreas. 2004. “Classic Toxin-Induced Animal Models of Parkinson’s Disease: 6-OHDA and MPTP.” *Cell and Tissue Research* 318(1):215–24.

Schwab, Robert S. 1960. “Progression and Prognosis in Parkinson’s Disease.” *Journal of Nervous and Mental Disease* 130(6):556–66.

Shimohama, Shun, Hideyuki Sawada, Yoshihisa Kitamura, and Takashi Taniguchi. 2003. “Disease Model: Parkinson’s Disease.” *Trends in Molecular Medicine* 9(8):360–65.

Sibley, David R. 1999. “NEW INSIGHTS INTO DOPAMINERGIC RECEPTOR FUNCTION USING ANTISENSE AND GENETICALLY ALTERED ANIMALS.” *Annual Review of Pharmacology and Toxicology* 39(1):313–41.

Slough, Courtney, Shane C. Masters, Robin A. Hurley, and Katherine H. Taber. 2016. “Clinical

Positron Emission Tomography (PET) Neuroimaging: Advantages and Limitations as a Diagnostic Tool." *Journal of Neuropsychiatry and Clinical Neurosciences* 28(2):66–71.
Sokoloff, Pierre and Jean Charles Schwartz. 1995. "Novel Dopamine Receptors Half a Decade Later." *Trends in Pharmacological Sciences* 16(8):270–75.
Sonsalla, Patricia K., Lai Yoong Wong, Suzan L. Harris, Jason R. Richardson, Ida Khobahy, Wenhao Li, Bharathi S. Gadad, and Dwight C. German. 2012. "Delayed Caffeine Treatment Prevents Nigral Dopamine Neuron Loss in a Progressive Rat Model of Parkinson's Disease." *Experimental Neurology* 234(2):482–87.
Spillantini, Maria Grazia, Marie Luise Schmidt, Virginia M. Y. Lee, John Q. Trojanowski, Ross Jakes, and Michel Goedert. 1997. "α-Synuclein in Lewy Bodies [8]." *Nature* 388(6645):839–40.
Suescun, Jessika, Shivika Chandra, and Mya C. Schiess. 2019. "The Role of Neuroinflammation in Neurodegenerative Disorders." *Translational Inflammation* 241–67.
Sulzer, David. 2007. "Multiple Hit Hypotheses for Dopamine Neuron Loss in Parkinson's Disease." *Trends in Neurosciences* 30(5):244–50.
Sun, Xun, Fang Liu, Qingyao Liu, Yongkang Gai, Weiwei Ruan, Dilani Neranjana Wimalarathne, Fan Hu, Xubo Tan, and Xiaoli Lan. 2019. "Quantitative Research of C-CFT and 18F-FDG PET in Parkinson's Disease: A Pilot Study with NeuroQ Software." *Frontiers in Neuroscience* 13(APR).
Tanner, Caroline M., Freya Kame, G. Webster Ross, Jane A. Hoppin, Samuel M. Goldman, Monica Korell, Connie Marras, Grace S. Bhudhikanok, Meike Kasten, Anabel R. Chade, Kathleen Comyns, Marie Barber Richards, Cheryl Meng, Benjamin Priestley, Hubert H. Fernandez, Franca Cambi, David M. Umbach, Aaron Blair, Dale P. Sandler, and J. William Langston. 2011. "Rotenone, Paraquat, and Parkinson's Disease." *Environmental Health Perspectives* 119(6):866–72.
Tansey, Malú G. and Matthew S. Goldberg. 2010. "Neuroinflammation in Parkinson's Disease: Its Role in Neuronal Death and Implications for Therapeutic Intervention." *Neurobiology of Disease* 37(3):510–18.
Thameem Dheen, S., Charanjit Kaur, and Eng-Ang Ling. 2007. "Microglial Activation and Its Implications in the Brain Diseases." *Current Medicinal Chemistry* 14(11):1189–97.
Tieu, Kim. 2011. "A Guide to Neurotoxic Animal Models of Parkinson's Disease." *Cold Spring Harbor Perspectives in Medicine* 1(1).
Toomey, James S., Shilpa Bhatia, La'Wanda T. Moon, Elysse A. Orchard, Kerrie H. Tainter, Stephen J. Lokitz, Tracee Terry, J. Michael Mathis, and Andrew D. Penman. 2012. "Pet Imaging a MPTP-Induced Mouse Model of Parkinson's Disease Using the Fluoropropyl-Dihydrotetrabenazine Analog [18F]-DTBZ (AV-133)." *PLoS ONE* 7(6).
Torti, Margherita, Laura Vacca, and Fabrizio Stocchi. 2018. "Istradefylline for the Treatment of Parkinson's Disease: Is It a Promising Strategy?" *Expert Opinion on Pharmacotherapy* 19(16):1821–28.
Tóth, Adrián, Zsófia Antal, Dániel Bereczki, and Beáta Sperlágh. 2019. "Purinergic Signalling in Parkinson's Disease: A Multi-Target System to Combat Neurodegeneration." *Neurochemical Research* 44(10):2413–22.
Toulorge, Damien, Anthony H. V. Schapira, and Rodolphe Hajj. 2016. "Molecular Changes in the Postmortem Parkinsonian Brain." *Journal of Neurochemistry* 139 Suppl 1:27–58.
Umegaki, Hiroyuki. 2012. "Neurodegeneration in Diabetes Mellitus." *Advances in Experimental Medicine and Biology* 724:258–65.
Unger, Marcus M., Jörg Spiegel, Klaus Ulrich Dillmann, David Grundmann, Hannah Philippeit, Jan Bürmann, Klaus Faßbender, Andreas Schwiertz, and Karl Herbert Schäfer. 2016. "Short Chain Fatty Acids and Gut Microbiota Differ between Patients with Parkinson's Disease and Age-Matched Controls." *Parkinsonism and Related Disorders* 32:66–72.
Varnäs, Katarina, Zsolt Cselényi, Aurelija Jucaite, Christer Halldin, Per Svenningsson, Lars Farde, and Andrea Varrone. 2019. "PET Imaging of [11C]PBR28 in Parkinson's Disease Patients Does Not Indicate Increased Binding to TSPO despite Reduced Dopamine Transporter Binding." *European Journal of Nuclear Medicine and Molecular Imaging* 46(2):367–75.

Vicente Miranda, Hugo, Omar M. A. El-Agnaf, and Tiago Fleming Outeiro. 2016. “Glycation in Parkinson’s Disease and Alzheimer’s Disease.” *Movement Disorders* 31(6):782–90.

Vijayanathan, Yuganthini, Fei Tieng Lim, Siong Meng Lim, Chiau Ming Long, Maw Pin Tan, Abu Bakar Abdul Majeed, and Kalavathy Ramasamy. 2017. “6-OHDA-Lesioned Adult Zebrafish as a Useful Parkinson’s Disease Model for Dopaminergic Neuroregeneration.” *Neurotoxicity Research* 32(3):496–508.

Volonté, M. A., R. M. Moresco, C. Gobbo, C. Messa, A. Carpinelli, G. Rizzo, G. Comi, and F. Fazio. 2001. “A PET Study with [11-C]Raclopride in Parkinson’s Disease: Preliminary Results on the Effect of Amantadine on the Dopaminergic System.” Pp. 107–8 in *Neurological Sciences*. Vol. 22.

WHO. 2016. “Global Report on Diabetes.” *Isbn* 978:6–86.

Xu, Qun, Yikyung Park, Xuemei Huang, Albert Hollenbeck, Aaron Blair, Arthur Schatzkin, and Honglei Chen. 2011. “Diabetes and Risk of Parkinson’s Disease.” *Diabetes Care* 34(4):910–15.

Yang, Y. and W. Song. 2013. “Molecular Links between Alzheimer’s Disease and Diabetes Mellitus.” *Neuroscience* 250:140–50.

Youdim, Moussa B. H., Dale Edmondson, and Keith F. Tipton. 2006. “The Therapeutic Potential of Monoamine Oxidase Inhibitors.” *Nature Reviews Neuroscience* 7(4):295–309.

Yue, Xuejing, Hehua Li, Haiqing Yan, Ping Zhang, Li Chang, and Tong Li. 2016. “Risk of Parkinson Disease in Diabetes Mellitus: An Updated Meta-Analysis of Population-Based Cohort Studies.” *Medicine (United States)* 95(18):e3549.

Zeng, Xian Si, Wen Shuo Geng, and Jin Jing Jia. 2018. “Neurotoxin-Induced Animal Models of Parkinson Disease: Pathogenic Mechanism and Assessment.” *ASN Neuro* 10.

Zhang, Wei, Tongguang Wang, Zhong Pei, David S. Miller, Xuefei Wu, Michelle L. Block, Belinda Wilson, Wanqin Zhang, Yong Zhou, Jau-Shyong Hong, and Jing Zhang. 2005. “Aggregated α-Synuclein Activates Microglia: A Process Leading to Disease Progression in Parkinson’s Disease.” *The FASEB Journal* 19(6):533–42.

Zimmermann, Herbert. 2011. “Purinergic Signaling in Neural Development.” *Seminars in Cell and Developmental Biology* 22(2):194–204.

CHAPTER 2

Targeting Adenosine Signaling in Parkinson's Disease: From Pharmacological to Non-pharmacological Approaches

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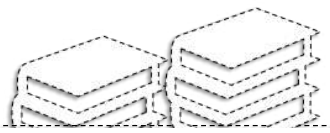

Abstract

Parkinson's disease (PD) is one of the most prevalent neurodegenerative disease displaying negative impacts on both the health and social ability of patients and considerable economical costs. The classical anti-parkinsonian drugs based in dopaminergic replacement are the standard treatment, but several motor side effects emerge during long-term use. This mini-review presents the rationale to several efforts from pre-clinical and clinical studies using adenosine receptor antagonists as a non-dopaminergic therapy. As several studies have indicated that the monotherapy with adenosine receptor antagonists reaches limited efficacy, the usage as a co-adjuvant appeared to be a promising strategy. The formulation of multi-targeted drugs, using adenosine receptor antagonists and other neurotransmitter systems than the dopaminergic one as targets, have been receiving attention since Parkinson's disease presents a complex biological impact. While pharmacological approaches to cure or ameliorate the conditions of PD are the leading strategy in this area, emerging positive aspects have arisen from non-pharmacological approaches and adenosine function inhibition appears to improve both strategies.

Keywords: adenosine, A2AAR, dopaminergic system, neurodegeneration, Parkinson disease

General aspects of parkinson's disease

Parkinson's disease (PD) is the second most prevalent chronic neurodegenerative disease, affecting more than 1% of the elderly population, with diagnostic confirmation occurring when the loss of dopaminergic neurons in the striatum is close to 80% (de Rijk et al., 2000). PD is also diagnosed in people less than 40 years old, named early-onset PD (Crosiers et al., 2011). PD is associated with the formation of Lewy bodies and neurites (Braak et al., 2003), mainly composed of aggregated forms of α-synuclein (Spillantini et al., 1998). The loss of dopaminergic neurons causes a reduction in the release of dopamine, leading to motor symptoms such as bradykinesia, rigidity, imbalance and tremor (Jankovic, 2008). PD presents in sporadic and familial forms. The risk factors involved in the development of PD are both genetic and environmental (Mortimer et al., 2012; Noyce et al., 2012; Van der Mark et al., 2012; Pezzoli and Cereda, 2013). The familial form, with specific genetic targets, represents less than 10% of PD cases (Dawson and Dawson, 2010). The genetic aspects of the disease are linked to mutations in several genes related to a multitude of cellular mechanisms, such as protein aggregation, protein and membrane trafficking, lysosomal autophagy, immune response, synaptic function, endocytosis, inflammation, and metabolic pathways (Redenšek et al., 2017). The genes *SNCA* (PARK1), *UCHL1* (PARK5), *LRRK2* (PARK8), GIGYF2 (PARK11), *OMI/HTRA2* (PARK13), VPS35 (PARK17), and EIF4G1 (PARK18) result in autosomal dominant PD, and PRKN (PARK2), DJ-1 (PARK7), ATP13A2 (PARK9), PLA2G6 (PARK14), FBX07 (PARK15), DNJC6 (PARK19), and SYNJ1 (PARK20) causes autosomal recessive PD (Lautier et al., 2008; Di Fonzo et al., 2009; Klein and Westenberger, 2012; Deng et al., 2015; Bartonikova et al., 2016; Miki et al., 2017; Scott et al., 2017). The gene contribution from other loci (PARK 3, 10, 12, and 16) is under investigation (Dawson and Dawson, 2010). However, a putative causative mutation in the gene that encodes the A_1 adenosine receptor, located in the locus PARK16, has been related to susceptibility to PD (Jaberi et al., 2016). Among the environmental contributors to PD development are occupational exposure of pesticides, such as Rotenone and Paraquat, infection by *Helicobacter* and HCV, low body weight and sedentary lifestyle (McCarthy et al., 2004; Villar-Cheda et al., 2009; Golabi et al., 2017; Sharma and Lewis, 2017; Shen et al., 2017).

The relationship of adenosine and dopamine signalling

Adenosine affects dopaminergic signaling through receptor heteromer formations and shared intracellular pathways. Adenosine is a neuromodulator that acts through the A_1 (A_1AR) and A_3 (A_3AR) inhibitory adenosine receptors and A_{2A} (A_{2A}AR) and A_{2B} (A_{2B}AR) excitatory adenosine receptors (Ralevic and Burnstock, 1998). D1 (D_1DR) and D2 (D_2DR) dopamine receptors are found co-localized with A_{2A}AR and A_1AR, $mGluR_5$ and NMDA (Hillion et al., 2002; Lee et al., 2002; Beggiato et al., 2016). The dopamine-adenosine receptor heteromers are constituted mainly of D_1DR/A_1AR and D_2DR/A_{2A}AR, displaying antagonistic properties. A_1AR agonist decreases the binding potential of dopamine to D_1DR, and reduces the D_1DR-induced cAMP production, while A_1AR antagonists activate D_1DR increasing cAMP levels (Ferré et al., 1998). A_3AR activation appears to have some influence on dopamine release and vesicular transport, while no functional impacts have been registered in dopamine receptors (Gołembiowska and Zylewska, 1998; Björklund et al., 2008; Shen et al., 2011).

The heteromerization of D_2DR/A_{2A}AR is one of the most studied receptors interaction. A_{2A}AR agonists reduce the *in vitro* affinity of the D_2DR agonist through an increase in D_2DR Kd without affecting receptor density (Ferré et al., 1991). *In vivo* studies confirmed these findings since the administration of A_{2A}AR antagonist increased the effects of the D_2DR agonist in the rat striatum and basal ganglia, while the action of A_{2A}AR agonists was opposite (Hillefors-Berglund et al., 1995; Strömberg et al., 2000). This heteromerization was confirmed through co-immunoprecipitation, fluorescence resonance energy, bioluminescence resonance energy transfer and *ex vivo* proximity ligation studies (Hillion et al., 2002; Canals et al., 2003; Trifilieff et al., 2011; Fernández-Dueñas et al., 2015). Studies with PET in the human brain showed the increased binding of a D_2DR antagonist, after the administration of caffeine, a nonselective antagonist of adenosine receptors (Volkow et al., 2015).

The interaction between adenosinergic and dopaminergic receptors has been described as intramembrane, involving direct interaction between receptors, or the modulation of G-proteins and the consequent influence on cAMP-dependent proteins (Fuxe et al., 1998; Ferré et al., 2001; Hillion et al., 2002; Fredholm and Svenningsson, 2003). The administration of D_2DR antagonists can reduce the cAMP production by A_{2A}AR and the D_2 agonist administration induces increase in cAMP

levels by A_{2A}AR (Vortherms and Watts, 2004; Botsakis et al., 2010). A_{2A}AR stimulation, *in vitro*, causes the phosphorylation and activation of DARPP-32, which can be inhibited by D_2DR activation (Nishi et al., 1997). A_{2A}AR antagonists increase D_2DR-dependent regulation of *c-fos*, which is more intense when dopaminergic neurodegeneration is presented (Pollack and Fink, 1995; Svenningsson et al., 1999). Compelling evidence for the impairment of D_2DR/A_{2A}AR oligomers in the striatum of rats was obtained in experimental Parkinsonism induced by 6-hydroxydopamine (6-OHDA) (Fernández-Dueñas et al., 2015). The ventral striopallidal GABA pathway appears to be a target of $mGlu_5$R/D_2DR/A_{2A}AR interactions. The co-administration of A_{2A}AR and $mGlu_5$R agonist enhances GABA release compared with $mGlu_5$R agonist alone, and this effect decreases with the administration of D_2DR agonists (Díaz-Cabiale et al., 2002). In addition, D_2DR/A_{2A}AR controls NMDA-mediated excitation in neurons from the nucleus accumbens through a direct protein–protein interaction (Azdad et al., 2009).

Support for the A_{2A}AR antagonism hypothesis from animal studies

The co-expression of D_2DR/A_{2A}AR receptors and their close functional and structural association in the striatopallidal GABAergic neurons reveals sites for therapeutic intervention and has received attention in the last three decades (Fink et al., 1992; Kase, 2001; Kelsey et al., 2009). The non-specific blockade of adenosine receptors by methylxanthines produces contralateral rotations in animals with dopaminergic lesions induced by 6-OHDA, since contralateral rotations have been related to an indirect stimulation of dopamine receptors in the lesioned area (Watanabe et al., 1981; Herrera-Marschitz et al., 1988).

During the late 1990s and early 2000s, exciting results from animal models of Parkinsonism indicated that A_{2A}AR antagonism improves motor activity by reducing the postsynaptic effects of dopamine depletion. Caffeine neuroprotection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced lesion showed to be especially dependent on A_{2A}AR from the striatal neurons, but not exclusively (Chen et al., 2001; Xu et al., 2016). The A_{2A}AR antagonist KW6002 (Istradefylline) was shown to be powerful enough to increase locomotion activity and potentiate dopaminergic agonist motor effects in MPTP- and 6-OHDA-lesioned animals (Kanda et al., 1998, 2000; Grondin et al., 1999; Koga et al., 2000; Bibbiani et al., 2003). The anti-

parkinsonian effects of KW6002 and similar drugs, such as KW17837, appear to be dose-dependent, effective in the postsynapse and beyond the direct effect on the dopaminergic system, and act on glutamatergic/gabaergic neurotransmission and monoamine oxidase activity (Bibbiani et al., 2003; Petzer et al., 2003; Tanganelli et al., 2004; Orru et al., 2011). MSX-3, a water-soluble precursor of the highly specific A_{2A}AR antagonist MSX-2, which exhibits greater potency for A_{2A}AR than KW6002, appeared to be a candidate of monotherapy since it alleviates the symptomatic parkinsonian locomotor deficiency in a genetic model of dopaminergic degeneration (Yang et al., 2007; Marcellino et al., 2010).

While some studies advocated that A_{2A}AR antagonism, as a monotherapy, could reach a mildly lower or similar efficacy of L-DOPA treatment without inducing dyskinesia (Grondin et al., 1999; Pinna et al., 2007), the promisor effect of these drugs appeared to be when co-administrated with L-DOPA, simultaneously inhibiting A_{2A}AR and activating D_2DR. A_{2A}AR-knockout animals demonstrated weak and transitory rotational sensitization and no sensitized grooming as a response to L-DOPA (Fredduzzi et al., 2002). The blockade of adenosine receptors by caffeine promoted additive or synergistic interactions with L-DOPA (Yu et al., 2006), whereas the co-administration of specific A_{2A}AR antagonists, such as KW6002, ST1535, and L-DOPA, potentiated the anti-parkinsonian effect of L-DOPA without exacerbating dyskinesia (Kanda et al., 2000; Koga et al., 2000; Bibbiani et al., 2003; Matsuya et al., 2007; Tronci et al., 2007). However, some studies using several A_{2A}AR antagonists, such as SCH4123-48, BIIB014 (Vipanedant), KW6002 and caffeine, when administered concomitantly and chronically with L-DOPA, failed to avoid dyskinesia (Jones et al., 2013).

The mechanism behind the effects of A_{2A}AR antagonists alone or as co-adjuvant drugs appears to beyond actions on dopaminergic system (Fuxe et al., 2009; Maggio et al., 2009; Figure Figure1).1). The A_{2A}AR exerts its neuronal activity in the striatum in a manner that is partially independent of D_2Rs (Chen et al., 2001). Actually, KW6002 decreases the neuronal activity of the striatopallidal indirect pathway in the absence of D_2R-mediated signaling (Aoyama et al., 2000). Dopaminergic neurodegeneration induced by transgenic mutant human α-synuclein is prevented in mice lacking the A_{2A}AR reinforcing the potential of shared downstream pathways (Ferraro et al., 2012). However, the adenylate cyclase activity did not differ in a genetic model of PD, suggesting that coupling to G-proteins of dopaminergic and

adenosinergic receptors should be a target (Botsakis et al., 2010). Regional differences appear in the anti-parkinsonian ability of A_{2A}AR antagonism, since caffeine given at or before MPTP exposure blocks the nigral neurodegenerative process without restoring the striatal nerve terminal neurochemical features (Sonsalla et al., 2012). Motor sensitization developed in unilaterally 6-OHDA-lesioned rats submitted to L-DOPA has been associated with an overexpression of the GABA-synthesizing enzyme glutamic acid decarboxylase, dynorphin, and enkephalin mRNAs in the striatal efferent indirect pathway (Fink et al., 1992; Tronci et al., 2007). The impact of A_{2A}AR antagonism over enkephalin content seems to promote motor recovery in D_2DR-knockout animals, but did not promote changes in the preproenkephalin mRNA in a 6-OHDA model (Fink et al., 1992; Aoyama et al., 2000). The functional relation of D_2DR/A_{2A}AR in striatal medium spiny neurons appears to receive contributions of cholinergic signaling with consequences for the anti-tremor benefits of A_{2A}AR antagonists (Simola et al., 2006; Tozzi et al., 2011; Salamone et al., 2013). The existence of A_{2A}AR/$mGlu_5$R heteromers and shared intracellular cascades steps, such as the stimulation of DARPP32 phosphorylation, increase in cAMP levels and elevated *c-fos* expression, provides clues to the possible contribution of glutamatergic and adenosinergic signaling to the beneficial effects of adenosine receptor antagonism (Nash and Brotchie, 2000; Kachroo et al., 2005). Effects resembling akinesia in 6-OHDA-lesioned rats were fully reversed by either a single treatment of an A_{2A}AR antagonist or an $mGlu_5$R antagonist at higher doses, or by a combined treatment with ineffective doses of each compound (Coccurello et al., 2004). Increased A_{2A}AR mRNA levels, decreased DARPP-32 phosphorylation and increased phosphorylation of ERK1/2 appeared in 6-OHDA-lesioned rats that display L-DOPA motor sensitization (Tomiyama et al., 2004; Song et al., 2009). This altered downstream signaling pathway is recovered by CSC (8-(3-chlorostryryl) caffeine), an A_{2A}AR antagonist (Song et al., 2009). Amelioration of motor response by A_{2A}AR antagonism seems to be accompanied by the rescue of dopamine, dopamine metabolites, glutamate, and GABA striatal levels as well as the reversal of astroglial and microglial activation and antioxidant properties with beneficial outcomes on cognition (Aguiar et al., 2008; Gołembiowska et al., 2013; Uchida et al., 2014).

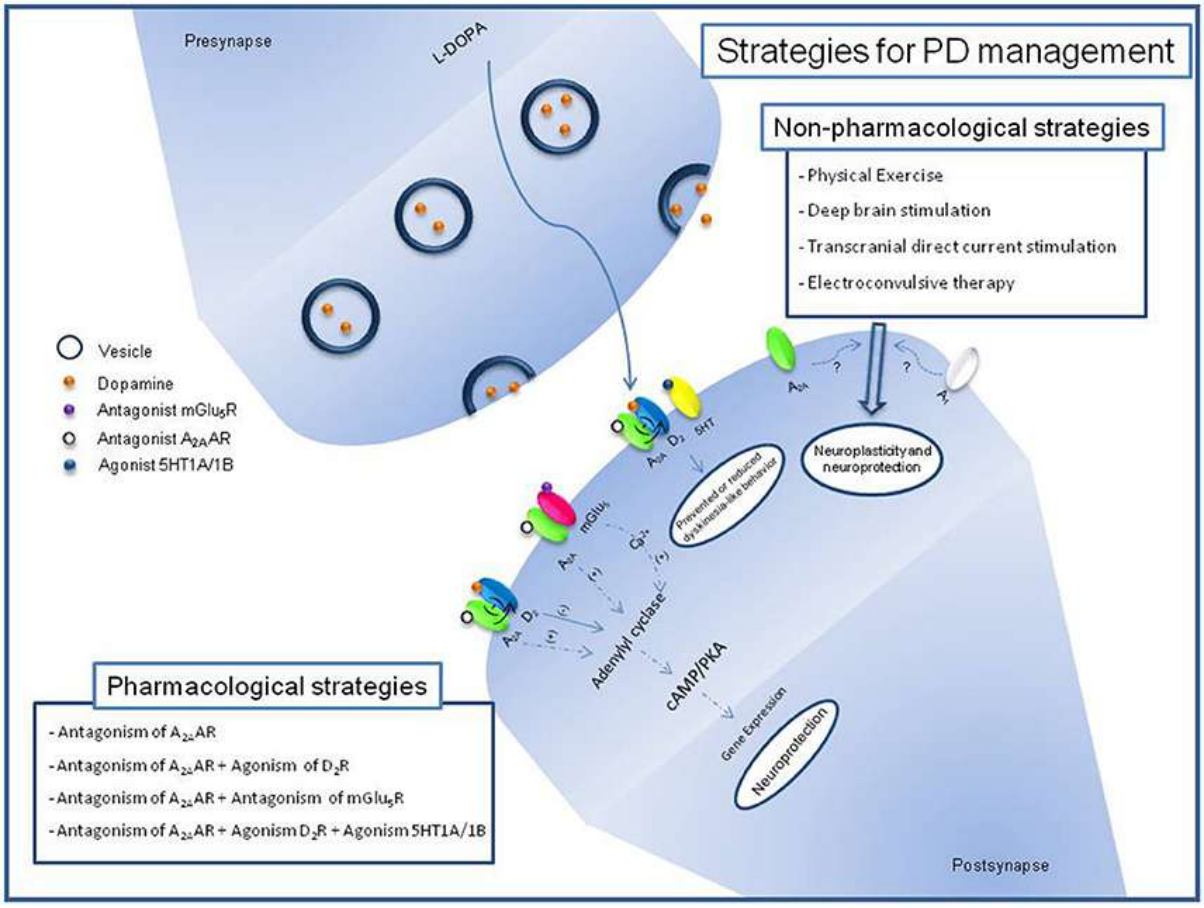

Figure 1: Schematic description of pharmacological and non-pharmacological strategies for PD management and its relation with adenosinergic signaling. Block of $A_{2A}AR$ by antagonist induces reduction of positive effects over Adenylyl cyclase and negative effects over D2R signaling. Block of $mGlu_5R$ reduces its positive effects over Adenilyl cyclase through release of Ca^{2+}. Recent studies with non-phamacological strategies for PD have been related it with adenosine receptors expression.

Prodrugs such as DP-L-A2AANT were designed to conjugate the beneficial effects against dopaminergic degeneration obtained by the combined action of dopamine and $A_{2A}AR$ antagonists in central nervous system (Dalpiaz et al., 2012). In addition to the potential dual action on adenosinergic and dopaminergic systems, the complimentary action on glutamatergic and adenosinergic systems appeared as prospective targets for dual anti-parkinsonian approaches. The combination of $A_{2A}AR$ antagonists and NR2B or $mGlu_5R$ antagonists has demonstrated attractive effects on motor activity with potential in the treatment of PD (Michel et al., 2014, 2015; Beggiato et al., 2016). $A_{2A}AR$–CB_1-D_2DR-receptor-heteromer has been suggested as a

component of motor alterations associated with dyskinesia and a possible target of multi-targeted drugs (Bonaventura et al., 2014; Pinna et al., 2014). The effects of caffeine-derived compounds over A_{2A}AR and that of monoamine oxidase B have revealed that these proteins are targets for synergistic action with benefits on dopaminergic degeneration (Petzer and Petzer, 2015). Sulphanylphthalimides are also presented as a dual-targeted-direct compound acting in A_1AR and monoamine oxidase B (Van der Walt et al., 2015). The association of L-dopa, serotonin 5-HT1A/1B receptor agonist and A_{2A}AR antagonist also demonstrated a promissory strategy in 6-OHDA-lesioned rats exhibiting prevented or reduced dyskinetic-like behavior without impairing motor activity (Pinna et al., 2016).

Support for the A_{2A}AR antagonism hypothesis from clinical tests

The A_{2A}AR biding sites and mRNA levels in PD patients with dyskinesia are increased in striatopallidal pathway neurons in relation to healthy patients (Martinez-Mir et al., 1991; Calon et al., 2004). These data, in association with the experimental benefits of A_{2A}AR antagonists in dopaminergic degenerative diseases increased the enthusiasm regarding non-dopaminergic drug development. Table Table11 updates the clinical trials assigned in the EUA and European Union using adenosine receptor antagonists. Istradefylline had long-term tolerability and safety, including as an adjuvant therapy to levodopa (Hauser et al., 2003; Stacy et al., 2008). In 2008, US Food and Drug Administration issued a non-approvable letter to the use of Istradefylline in humans based in the concern if the efficacy findings support clinical utility of Istradefylline in patients with PD. However, Kyowa Hakko Kirin has received approval for the use of Istradefylline as adjunctive therapy in Japan (Dungo and Deeks, 2013; Mizuno et al., 2013). After the additional data request, a 12-week randomized study to evaluate oral Istradefylline in subjects with moderate to severe PD ended with disappointing results, since Istradefylline did not change the off time per day (NCT01968031). However, a clinical trial is currently open (NCT02610231). Preladenant was evaluated as monotherapy to patients with early PD since it reduced the mean daily off time in a phase II study; however, no evidence has supported its efficacy in phase III studies (Hauser, 2011; Stocchi et al., 2017). BIIB014 and SCH900800 also failed to prove efficacy in clinical trials, while Tozadenant showed a mean daily off time reduction accompanied by adverse events of dyskinesia, nausea,

and dizziness (Hauser et al., 2014). A safety and efficacy study of Tozadenant to treat end of dose wearing off in PD patients using L-DOPA is currently open (NCT02453386). Multiple epidemiological studies indicate that caffeine is able to prevent PD development (Ross et al., 2000; Ascherio et al., 2001). In a pilot study of caffeine for daytime sleepiness in PD, there was evident benefit on the motor manifestations of disease with no adverse effects (Postuma et al., 2012). Recently, a clinical trial has aimed to evaluate the efficacy of caffeine for motor and non-motor aspects of disease (NCT01738178). Nowadays, changing the dose and frequency of daily drug taking had no benefits in the use of adenosine receptor antagonists as a monotherapy or as an adjuvant of current Parkinsonism treatment.

Drug	Sponsor	Identifier number (year)	Parkinson's disease patient condition	Outcome measures (dose tested)	Phase	Status	Results
Istradefylline (KW6002)	Kyowa Hakko Kirin Co., Ltd	NCT02610231* (2015)	Moderate to severe disease	Safety and tolerability (20 or 40 mg oral daily)	III	Active – not recruiting	-
		NCT01968031* (2013) 2013-002254-70** (2014)	Moderate to Severe Disease	Efficacy and safety (20 or 40 mg daily)	III	Completed	No change in the OFF time
		NCT00957203* (2009)	Advanced disease treated with levodopa	Long-term safety and efficacy (20 or 40 mg daily)	III	Completed	
		NCT00955526* (2009)	Levodopa-Treated	Efficacy in reducing the mean total hours of awake time per day spent in the OFF state (20 or 40 mg daily)	III	Completed	Reduction in daily OFF time
		NCT00456794* (2007)	Advanced disease treated with levodopa/carbidopa	Safety and efficacy compared with placebo in subjects with OFF-time (20 and 60 mg daily)	II	Completed	Significant reduction in OFF time, and was well tolerated as adjunctive treatment to levodopa
		NCT00456586* (2007)	Advanced disease treated with levodopa/carbidopa	Safety and efficacy compared with placebo in subjects with OFF phenomena(40 mg daily)	II	Completed	Istradefylline was safe, well toler-ated, and effective at improving end-of-dose wearing
		NCT00455507* (2007)	Advanced disease treated with levodopa.	Efficacy for reducing the mean total hours of awake time per day spent in the OFF state(20 or 40 mg daily)	II	Completed	
		2004-002844-93** (2005)	Motor response complications on levodopa therapy	Long-term tolerability and safety (20 or 40 mg daily)	III	Completed	Istradefylline was well tolerated as adjunctive therapy to levodopa for subjects with Parkinson's disease
		NCT00250393* (2005)	Not specified	Change in Unified Parkinson's Disease Rating Scale (UPDRS) part-III (Motor examination) (40 mg daily)	II	Completed	
		NCT00203957* (2005)	Motor Response Complications on Levodopa	Confirmation of long term tolerability and safety (20 or 40 mg daily)	III	Completed	
		NCT00199420* (2005)	Aadvanced disease treated with levodopa	Percentage of OFF time (10, 20 or 40 mg daily)	III	Completed	

		NCT00199407* (2005)	Advanced disease treated with levodopa	Efficacy for reducing the percentage of OFF time (20 mg daily)	III	Completed	
		NCT00199394* (2005)	Advanced disease treated with levodopa	Percentage of awake time spent in the OFF state (40 mg daily)	III	Completed	
		NCT00199381* (2005)	Patients Who Have Recently Completed One Year of Treatment With Istradefylline	Long-term tolerability and safety (20 or 40 mg daily)	III	Completed	The Sponsor decided to terminate the study early (not for safety reasons)
		NCT00199368* (2005)	Patients With Motor Response Complications on Levodopa Therapy. who have completed prior istradefylline studies	Safety Study (20 or 40 mg daily)	III	Completed	
		NCT00199355* (2005)	Advanced disease treated with levodopa /DCI.	OFF time (20 or 40 mg daily)	II		
	NINDS	NCT00006337* (2000)	Not specified	Effects on symptoms and dyskinesias	II	Completed	
SCH900800	Merck Sharp & Dohme Corp.	NCT01500707* (2011)	Moderate to Severe Disease treated with Levodopa	Pharmacokinetics of SCH 900800 (20 mg daily)	I	Study withdrawn	-
Preladenant (SCH 420814)	Merck Sharp & Dohme Corp.	NCT01294800* (2011)	Moderate to severe disease experiencing motor fluctuations and receiving levodopa	Efficacy on "off" time (2, 5, 10 mg twice/day)	II	Completed	Change From Baseline in Mean “Off” Time
		NCT01227265* (2010)	Moderate to severe disease	Efficacy and safety (2-5 mg twice/day)	III	Completed	Not superior to placebo in reducing off time from baseline
		NCT01155479* (2010)	Early Parkinson's disease	Efficacy and safety (2,5, 10 mg twice/day)	III	Completed	Change From Baseline in motor impairments and disability
		2009-015161-31** (2010)	Moderate to severe disease	Efficacy and Safety (2,5, 10 mg twice/day)	III	Completed	
		2009-015162-57** (2010)	Moderate to severe disease	Extension Study (2,5, 10 mg twice/day)	III	Study withdrawn	Lack of efficacy in the parent studies.
		NCT01155466* (2010)	Moderate to severe disease	Stability in levodopa dose (2, 5, 10 mg twice/day)	III	Completed	No change From Baseline in Mean "Off" Time
		2009-013552-72** (2010)	Early Parkinson’s disease	Dose-Range-Finding Efficacy and Safety (2, 5, or 10 mg twice/day)	III	Completed	No statistically significant or clinically meaningful difference vs. placebo
		NCT01215227* (2010)	Moderate to severe disease	Long-term safety and tolerability from patients of NCT01155466 and NCT01227265 (2, 5, 10 mg twice/day)			Terminated early due to the lack of efficacy in the parent studies NCT1155466 and NCT01227265.
		NCT00845000* (2009)	Levodopa treated	Effects on the dyskinesia and antiparkinsonian actions of a levodopa infusion (10 or 100 mg daily)	I	Completed	
		NCT00537017* (2007)	Moderate to severe disease	Long term safety (5 mg twice daily)	II	Completed	Long-term preladenant treatment (5 mgtwice a day) was well tolerated and provided sustained OFF time reductions

							and ON time increases
		NCT00406029* (2006)	Not specified	Efficacy and safety when used together with a stable dose of L-dopa/dopa decarboxylase (1, 2, 5 and 10 mg twice a day)	II	Completed	Mean daily off time reduced (5 mg and 10 mg)
Tozadenant (SYN115)	Biotie Therapies Inc.	NCT03051607* 2016-003961-25** (2017)	Experiencing end of dose "Wearing-Off"	Safety and Tolerability(120 mg oral twice daily)	III	Recruiting	-
		2014-005630-60 ** (2015)	Levodopa-Treated Experiencing End-of-Dose "Wearing-Off"	Efficacy and Safety as Adjunctive Therapy to Levodopa (60 mg oral daily)	III	Active	-
		2011-005054-59 ** (2013)	Experiencing end of dose "Wearing-Off"	Safety and efficacy as an adjunct to levodopa (60 mg oral daily)	II	Completed	
		NCT01283594* (2011)	Motor fluctuations on levodopa	Safety and efficacy as an adjunct to levodopa(60, 120, 180, 240 mg twice/day)	II/III	Completed	Tozadenant (120 or 180 mg) was generally well tolerated and was effective at reducing off-time.
BIIB014	Oxford BioMedica	NCT00627588* (2008)	Early Parkinson's disease	Safety, Efficacy and Dose Evaluation	I/II	Completed	
Caffeine	McGill University Health Center	NCT01738178* (2012)	Not specified	Motor effects of caffeine persist (or even magnify)helps reduce dose of other PD meds and/or prevents their side effects (200 mg daily)	III	Completed	-
	Ron Postuma	NCT01190735* (2010)	Not specified	Optimal caffeine dose with maximal motor benefit and the least amount of undesirable adverse effects (100-200 mg twice/day)	II	Completed	
		NCT00459420* (2007)	Not specified	Effect on sleepiness and motor symptoms (100-200 mg daily)	II/III	Completed	No significant benefit on excessive daytime sleepiness

Table 1

A_{2A}AR antagonists under clinical investigation for Parkinson's disease.

*ClinicalTrials.gov

***EU Clinical Trials Register*.

Association of A_{2A}AR antagonism and non-pharmacological approaches

Non-pharmacological approaches are strategies to combine, reinforce and complement the pharmacological options for the management and prevention of PD (Figure (Figure1).1). Dance, treadmill and aquatic exercises feasibility to PD management have been evaluated in clinical trials with benefits to life quality, based in cognitive and motor features (Picelli et al., 2016; Carroll et al., 2017; Shanahan et al., 2017). Recently, it was demonstrated that treadmill exercises induce brain

activation in PD (Maidan et al., 2017). These benefits have been reproduced in animal models of PD suggesting that physical exercise prevents the development of L-DOPA-induced dyskinesia and its association with hyperphosphorylation of DARPP-32, c-Fos expression and increased brain-derived neurotrophic factor (BDNF) levels (Gyárfás et al., 2010; Aguiar et al., 2013; Shin et al., 2017). Studies with wheel running rats revealed that A_1AR and A_{2A}AR expression is reduced in the striatum, reinforcing the idea that physical exercise is able to promote neuroplasticity and neuroprotection to brain regions related to motor control, probably through the reduction of antagonistic adenosine effects over dopamine signaling (Clark et al., 2014).

Deep Brain Stimulation (DBS) was approved by the FDA in 2002 as therapy for advanced PD (Suarez-Cedeno et al., 2017). From studies with animals, DBS appeared to have a neuroprotective effect against loss of dopaminergic neurons induced by classical dopaminergic neurotoxins (Maesawa et al., 2004). The use of A_{2A}AR antagonism as an adjuvant of DBS in rodents suggests the potential to enhance the response in the treatment of parkinsonian symptoms, such as tremor (Collins-Praino et al., 2013). While clinical studies using transcranial direct current stimulation (tDCS) in PD suggest possible locomotor benefits, the biological mechanism is still under investigation (Benninger et al., 2011). In rodents, tDCS on the cerebral cortex promotes cognitive effects involving A_1AR, although the adenosinergic participation in tDCS responses of PD has not been evaluated (Márquez-Ruiz et al., 2012). Electroconvulsive therapy (ECT) has been proposed to be efficient for both motor and non-motor symptoms in PD with psychological problems (Nishioka et al., 2014; Calderón-Fajardo et al., 2015). The proposed mechanism for ECT includes the enhancement of dopaminergic transmission in the striatum and an increase in the levels of levodopa by disrupting the blood–brain barrier (Kennedy et al., 2003). The purinergic system appears to be influenced by ECT, since the action, metabolism and release of nucleotide and nucleoside are altered under ECT, but no correlation with PD was identified until now (Gleiter et al., 1989; Busnello et al., 2008; Sadek et al., 2011). A combination of drugs and non-pharmacological therapies could warrant new investigations into the preclinical and clinical studies, with hope for the amelioration and affects in PD prevention, management and treatment.

Conclusions

This review highlights the need to intensify research into adenosine signaling in the development of PD therapies. The interaction between adenosine and dopamine signaling has been extensively studied and contributed to knowledge of the role of non-dopamingergic neurotransmitters in the PD. As cholinergic, glutamatergic, GABAergic, canabinergic and serotoninergic systems appear together with adenosinergic system in the myriad of pathways involved in the PD, appearing together with the possibility of improved results from dual or multi-targeted anti-parkisonism approaches opened a new area of drug development. In addition, the association of pharmacological and non-pharmacological approaches brings new perspectives for a more effective treatment of PD and improved of quality of life for PD patients.

Author contributions

LN, RdS, and CB equally contributed to the definition of the scope and to the writing of the manuscript.

Conflict of interest statement

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

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Glossary

Abbreviations

A_1AR	A_1 adenosine receptor
A_{2A}AR	A_{2A} adenosine receptor
A_{2B}AR	A_{2B} adenosine receptor

A_3AR	A_3 adenosine receptor
BDNF	brain-derived neurotrophic factor
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa
D_1DR	D_1 dopamine receptor
D_2DR	D_2 dopamine receptor
PD	Parkinson's disease
6-OHDA	6-hydroxydopamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

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References

1. Aguiar A. S., Jr., Moreira E. L., Hoeller A. A., Oliveira P. A., Córdova F. M., Glaser V., et al. . (2013). Exercise attenuates levodopa-induced dyskinesia in 6-hydroxydopamine-lesioned mice. Neuroscience 243, 46–53. 10.1016/j.neuroscience.2013.03.039
2. Aguiar L. M., Macêdo D. S., Vasconcelos S. M., Oliveira A. A., de Sousa F. C., Viana G. S. (2008). CSC, an adenosine A_{2A} receptor antagonist and MAO B inhibitor, reverses behavior, monoamine neurotransmission, and amino acid alterations in the 6-OHDA-lesioned rats. Brain Res. 1191, 192–199. 10.1016/j.brainres.2007.11.051
3. Aoyama S., Kase H., Borrelli E. (2000). Rescue of locomotor impairment in dopamine D2 receptor-deficient mice by an adenosine A2A receptor antagonist. J. Neurosci. 20, 5848–5852.
4. Ascherio A., Zhang S. M., Hernán M. A., Kawachi I., Colditz G. A., Speizer F. E., et al. . (2001). Prospective study of caffeine consumption and risk of Parkinson's disease in men and women. Ann. Neurol. 50, 56–63. 10.1002/ana.1052
5. Azdad K., Gall D., Woods A. S., Ledent C., Ferré S., Schiffmann S. N. (2009). Dopamine D_2 and adenosine A_{2A} receptors regulate NMDA-mediated excitation in accumbens neurons through A_{2A}-D_2 receptor heteromerization. Neuropsychopharmacology 34, 972–986. 10.1038/npp.2008.144
6. Bartonikova T., Mensikova K., Mikulicova L., Vodicka R., Vrtel R., Godava M., et al. . (2016). Familial atypical parkinsonism with rare variant in VPS35 and FBXO7 genes: a case report. Medicine (Baltimore) 95:e5398. 10.1097/MD.0000000000005398
7. Beggiato S., Tomasini M. C., Borelli A. C., Borroto-Escuela D. O., Fuxe K., Antonelli T., et al. . (2016). Functional role of striatal A_{2A}, D_2, and mGlu5 receptor interactions in regulating striatopallidal GABA neuronal transmission. J. Neurochem. 138, 254–264. 10.1111/jnc.13652
8. Benninger D. H., Lomarev M., Lopez G., Pal N., Luckenbaugh D. A., Hallett M. (2011). Transcranial direct current stimulation for the treatment of focal hand dystonia. Mov. Disord. 26, 1698–1702. 10.1002/mds.
9. Bibbiani F., Oh J. D., Petzer J. P., Castagnoli N., Jr., Chen J. F., Schwarzschild M. A., et al. . (2003). A_{2A} antagonist prevents dopamine agonist-induced motor complications in animal models of Parkinson's disease. Exp. Neurol. 184, 285–294. 10.1016/S0014-4886(03)00250-4
10. Björklund O., Halldner-Henriksson L., Yang J., Eriksson T. M., Jacobson M. A., Daré E., et al. . (2008). Decreased behavioral activation following caffeine, amphetamine and darkness in A3 adenosine receptor knock-out mice. Physiol. Behav. 95, 668–676. 10.1016/j.physbeh.2008.09.018
11. Bonaventura J., Rico A. J., Moreno E., Sierra S., Sánchez M., Luquin N., et al. (2014). L-DOPA-treatment in primates disrupts the expression of A_{2A} adenosine-CB_1 cannabinoid-D_2 dopamine receptor heteromers in the caudate nucleus. Neuropharm. 79, 90–100. 10.1016/j.neuropharm.2013.10.036
12. Botsakis K., Pavlou O., Poulou P. D., Matsokis N., Angelatou F. (2010). Blockade of adenosine A_{2A} receptors downregulates DARPP-32 but increases ERK1/2 activity in striatum of dopamine deficient “weaver” mouse. Neurochem. Int. 56, 245–249. 10.1016/j.neuint.2009.10.007
13. Braak H., Rüb U., Gai W. P., Del Tredici K. (2003). Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion

by an unknown pathogen. J. Neural Transm. (Vienna) 110, 517–536. 10.1007/s00702-002-0808-2

14. Busnello J. V., Oses J. P., da Silva R. S., Feier G., Barichello T., Quevedo J., et al. . (2008). Peripheral nucleotide hydrolysis in rats submitted to a model of electroconvulsive therapy. Prog. Neuropsychopharmacol. Biol. Psychiatry 32, 1829–1833. 10.1016/j.pnpbp.2008.08.007
15. Calderón-Fajardo H., Cervantes-Arriaga A., Llorens-Arenas R., Ramírez-Bermudez J., Ruiz-Chow Á., Rodríguez-Violante M. (2015). Electroconvulsive therapy in Parkinson's disease. Arq Neuropsiquiatr. 73, 856–860. 10.1590/0004-282X20150131
16. Calon F., Dridi M., Hornykiewicz O., Bédard P. J., Rajput A. H., Di Paolo T. (2004). Increased adenosine A_{2A} receptors in the brain of Parkinson's disease patients with dyskinesias. Brain 127(Pt 5), 1075–1084. 10.1093/brain/awh128
17. Canals M., Marcellino D., Fanelli F., Ciruela F., de Benedetti P., Goldberg S. R., et al. . (2003). Adenosine A_{2A}-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. J. Biol. Chem. 278, 46741–46749. 10.1074/jbc.M306451200
18. Carroll L. M., Volpe D., Morris M. E., Saunders J., Clifford A. M. (2017). Aquatic exercise therapy for people with Parkinson disease: a randomized controlled trial. Arch. Phys. Med. Rehabil. 98, 631–638. 10.1016/j.apmr.2016.12.006
19. Chen J. F., Xu K., Petzer J. P., Staal R., Xu Y. H., Beilstein M., et al. (2001). Neuroprotection by caffeine and A_{2A} adenosine receptor inactivation in a model of Parkinson's disease. J. Neurosci. 21:RC143.
20. Clark P. J., Ghasem P. R., Mika A., Day H. E., Herrera J. J., Greenwood B. N., et al. . (2014). Wheel running alters patterns of uncontrollable stress-induced cfos mRNA expression in rat dorsal striatum direct and indirect pathways: a possible role for plasticity in adenosine receptors. Behav. Brain Res. 272, 252–263. 10.1016/j.bbr.2014.07.006
21. Coccurello R., Breysse N., Amalric M. (2004). Simultaneous blockade of adenosine A_{2A} and metabotropic glutamate mGlu5 receptors increase their efficacy in reversing Parkinsonian deficits in rats. Neuropsychopharmacology 29, 1451–1461. 10.1038/sj.npp.1300444
22. Collins-Praino L. E., Paul N. E., Ledgard F., Podurgiel S. J., Kovner R., Baqi Y., et al. . (2013). Deep brain stimulation of the subthalamic nucleus reverses oral tremor in pharmacological models of parkinsonism: interaction with the effects of adenosine A_{2A} antagonism. Eur. J. Neurosci. 38, 2183–2191. 10.1111/ejn.12212
23. Crosiers D., Theuns J., Cras P., Van Broeckhoven C. (2011). Parkinson disease: insights in clinical, genetic and pathological features of monogenic disease subtypes. J. Chem. Neuroanat. 42, 131–141. 10.1016/j.jchemneu.2011.07.003
24. Dalpiaz A., Cacciari B., Vicentini C. B., Bortolotti F., Spalluto G., Federico S., et al. . (2012). A novel conjugated agent between dopamine and an A_{2A} adenosine receptor antagonist as a potential anti-Parkinson multitarget approach. Mol. Pharm. 9, 591–604. 10.1021/mp200489d
25. Dawson T., Dawson V. L. (2010). The role of Parkin in Familial and Sporadic Parkinson's Disease. Mov. Disord. 25, S32–S39. 10.1002/mds.22798
26. Deng S., Deng X., Yuan L., Song Z., Yang Z., Xiong W., et al. . (2015). Genetic analysis of SNCA coding mutation in Chinese Han patients with Parkinson disease. Acta Neurol. Belg. 115, 267–271. 10.1007/s13760-014-0347-2
27. de Rijk M. C., Launer L. J., Berger K., Breteler M. M., Dartigues J. F., Baldereschi M., et al. . (2000). Prevalence of Parkinson's disease in Europe: a collaborative study

of population-based cohorts. Neurology 54(11 Suppl. 5), S21–S23. 10.1212/WNL.54.11.21A
28. Díaz-Cabiale Z., Vivó M., Del Arco A., O'Connor W. T., Harte M. K., Müller C. E., et al. (2002). Metabotropic glutamate mGlu5 receptor-mediated modulation of the ventral striopallidal GABA pathway in rats. Interactions with adenosine A_{2A} and dopamine D_2 receptors. Neurosci. Lett. 324, 154–158. 10.1016/S0304-3940(02)00179-9
29. Di Fonzo A., Fabrizio E., Thomas A., Fincati E., Marconi R., Tinazzi M., et al. (2009). GIGYF2 mutations are not a frequent cause of familial Parkinson's disease. Parkinsonism Relat. Disord. 15, 703–705. 10.1016/j.parkreldis.2009.05.001
30. Dungo R., Deeks E. D. (2013). Istradefylline: first global approval. Drugs 73, 875–882. 10.1007/s40265-013-0066-7
31. Fernández-Dueñas V., Taura J. J., Cottet M., Gómez-Soler M., López-Cano M., Ledent C., et al. . (2015). Untangling dopamine-adenosine receptor-receptor assembly in experimental parkinsonism in rats. Dis. Model. Mech. 8, 57–63. 10.1242/dmm.018143
32. Ferré S., Popoli P., Giménez-Llort L., Rimondini R., Müller C. E., Strömberg I., et al. . (2001). Adenosine/dopamine interaction: implications for the treatment of Parkinson's disease. Parkinsonism Relat. Disord. 7, 235–241. 10.1016/S1353-8020(00)00063-8
33. Ferré S., Torvinen M., Antoniou K., Irenius E., Civelli O., Arenas E., et al. . (1998). Adenosine A1 receptor-mediated modulation of dopamine D1 receptors in stably cotransfected fibroblast cells. J. Biol. Chem. 273, 4718–4724. 10.1074/jbc.273.8.4718
34. Ferré S., von Euler G., Johansson B., Fredholm B. B., Fuxe K. (1991). Stimulation of high affinity adenosine A-2 receptors decreases the affinity of dopamine D-2 receptors in rat striatal membranes. Proc. Natl. Acad. Sci. U.S.A. 88, 7238–7241 10.1073/pnas.88.16.7238
35. Ferraro L., Beggiato S., Tomasini M. C., Fuxe K., Antonelli T., Tanganelli S. (2012). A_{2A}/D_2 receptor heteromerization in a model of Parkinson's disease. Focus on striatal aminoacidergic signaling. Brain Res. 1476, 96–107. 10.1016/j.brainres.2012.01.032
36. Fink J. S., Weaver D. R., Rivkees S. A., Peterfreund R. A., Pollack A. E., Adler E. M., et al. . (1992). Molecular cloning of the rat A_2 adenosine receptor: selective co-expression with D_2 dopamine receptors in rat striatum. Brain Res. Mol. Brain Res. 14, 186–195. 10.1016/0169-328X(92)90173-9
37. Fredduzzi S., Moratalla R., Monopoli A., Cuellar B., Xu K., Ongini E., et al. . (2002). Persistent behavioral sensitization to chronic L-DOPA requires A_{2A} adenosine receptors. J. Neurosci. 22, 1054–1062.
38. Fredholm B. B., Svenningsson P. (2003). Adenosine-dopamine interactions: development of a concept and some comments on therapeutic possibilities. Neurology 61(11 Suppl. 6), S5–S9. 10.1212/01.WNL.0000095204.89871.FF
39. Fuxe K., Ferré S., Zoli M., Agnati L. F. (1998). Integrated events in central dopamine transmission as analyzed at multiple levels. Evidence for intramembrane adenosine A_{2A}/dopamine D_2 and adenosine A1/dopamine D1 receptor interactions in the basal ganglia. Brain Res. Brain Res. Rev. 26, 258–273. 10.1016/S0165-0173(97)00049-0
40. Fuxe K., Marcellino D., Guidolin D., Woods A. S., Agnati L. (2009). Brain receptor mosaics and their intramembrane receptor-receptor interactions: molecular

integration in transmission and novel targets for drug development. J. Acupunct. Meridian Stud. 2, 1–25. 10.1016/S2005-2901(09)60011-X
41. Gleiter C. H., Deckert J., Nutt D. J., Marangos P. J. (1989). Electroconvulsive shock (ECS) and the adenosine neuromodulatory system: effect of single and repeated ECS on the adenosine A1 and A2 receptors, adenylate cyclase, and the adenosine uptake site. J. Neurochem. 52, 641–646. 10.1111/j.1471-4159.1989.tb09168.x
42. Golabi P., Otgonsuren M., Sayiner M., Arsalla A., Gogoll T., Younossi Z. M. (2017). The Prevalence of Parkinson Disease Among Patients With Hepatitis C Infection. Ann. Hepatol. 16, 342–348. 10.5604/01.3001.0009.8588
43. Gołembiowska K., Wardas J., Noworyta-Sokołowska K., Kaminska K., Górska A. (2013). Effects of adenosine receptor antagonists on the *in vivo* LPS-induced inflammation model of Parkinson's disease. Neurotox. Res. 24, 29–40. 10.1007/s12640-012-9372-1
44. Gołembiowska K., Zylewska A. (1998). N6-2-(4-aminophenyl)ethyladenosine (APNEA), a putative adenosine A3 receptor agonist, enhances methamphetamine-induced dopamine outflow in rat striatum. Pol. J. Pharmacol. 50, 299–305.
45. Grondin R., Bédard P. J., Hadj T. A., Grégoire L., Mori A., Kase H. (1999). Antiparkinsonian effect of a new selective adenosine A_{2A} receptor antagonist in MPTP-treated monkeys. Neurology 52, 1673–1677. 10.1212/WNL.52.8.1673
46. Gyárfás T., Knuuttila J., Lindholm P., Rantamäki T., Castrén E. (2010). Regulation of brain-derived neurotrophic factor (BDNF) and cerebral dopamine neurotrophic factor (CDNF) by anti-parkinsonian drug therapy *in vivo*. Cell. Mol. Neurobiol. 30, 361–368. 10.1007/s10571-009-9458-3
47. Hauser R. A. (2011). Future treatments for Parkinson's disease: surfing the PD pipeline. Int. J. Neurosci. 121, 53–62. 10.3109/00207454.2011.620195
48. Hauser R. A., Hubble J. P., Truong D. D., Istradefylline US-001 Study Group (2003). Randomized trial of the adenosine A_{2A} receptor antagonist istradefylline in advanced PD. Neurology 61, 297–303. 10.1212/01.WNL.0000081227.84197.0B
49. Hauser R. A., Olanow C. W., Kieburtz K. D., Pourcher E., Docu-Axelerad A., Lew M., et al. . (2014). Tozadenant (SYN115) in patients with Parkinson's disease who have motor fluctuations on levodopa: a phase 2b, double-blind, randomised Trial. Lancet Neurol. 13, 767–776. 10.1016/S1474-4422(14)70148-6
50. Herrera-Marschitz M., Casas M., Ungerstedt U. (1988). Caffeine produces contralateral rotation in rats with unilateral dopamine denervation: comparisons with apomorphine-induced responses. Psychopharmacology (Berl) 94, 38–45. 10.1007/BF00735878
51. Hillefors-Berglund M., Liu Y., von Euler G. (1995). Persistent, specific and dose-dependent effects of toluene exposure on dopamine D2 agonist binding in the rat caudate-putamen. Toxicology 100, 185–194. 10.1016/0300-483X(95)03084-S
52. Hillion J., Canals M., Torvinen M., Casado V., Scott R., Terasmaa A., et al. . (2002). Coaggregation, cointernalization, and codesensitization of adenosine A_{2A} receptors and dopamine D2 receptors. J. Biol. Chem. 277, 18091–18097 10.1074/jbc.M107731200
53. Jaberi E., Rohani M., Shahidi G. A., Nafissi S., Arefian E., Soleimani M., et al. . (2016). Mutation in ADORA1 identified as likely cause of early-onset parkinsonism and cognitive dysfunction. Mov. Disord. 31, 1004–1011. 10.1002/mds.26627
54. Jankovic J. (2008). Parkinson's disease: clinical features and diagnosis. J. Neurol. Neurosurg. Psychiatr. 79, 368–376. 10.1136/jnnp.2007.131045

55. Jones N., Bleickardt C., Mullins D., Parker E., Hodgson R. (2013). A_{2A} receptor antagonists do not induce dyskinesias in drug-naive or L-dopa sensitized rats. Brain Res. Bull. 98, 163–169. 10.1016/j.brainresbull.2013.07.001
56. Kachroo A., Orlando L. R., Grandy D. K., Chen J. F., Young A. B., Schwarzschild M. A. (2005). Interactions between metabotropic glutamate 5 and adenosine A_{2A} receptors in normal and parkinsonian mice. J. Neurosci. 25, 10414–10419. 10.1523/JNEUROSCI.3660-05.2005
57. Kanda T., Jackson M. J., Smith L. A., Pearce R. K., Nakamura J., Kase H., et al. (2000). Combined use of the adenosine A_{2A} antagonist KW-6002 with L-DOPA or with selective D_1 or D_2 dopamine agonists increases antiparkinsonian activity but not dyskinesia in MPTP-treated monkeys. Exp. Neurol. 162, 321–327. 10.1006/exnr.2000.7350
58. Kanda T., Tashiro T., Kuwana Y., Jenner P. (1998). Adenosine A_{2A} receptors modify motor function in MPTP-treated common marmosets. Neuroreport. 9, 2857–2860. 10.1097/00001756-199808240-00032
59. Kase H. (2001). New aspects of physiological and pathophysiological functions of adenosine A_{2A} receptor in basal ganglia. Biosci. Biotechnol. Biochem. 65, 1447–1457. 10.1271/bbb.65.1447
60. Kennedy P., Evans M. J., Berry C., Mullin J. (2003). Comparative analysis of goal achievement during rehabilitation for older and younger adults with spinal cord injury. Spinal Cord. 41, 44–52. 10.1038/sj.sc.3101386
61. Kelsey J. E., Langelier N. A., Oriel B. S., Reedy C. (2009). The effects of systemic, intrastriatal, and intrapallidal injections of caffeine and systemic injections of A_{2A} and A1 antagonists on forepaw stepping in the unilateral 6-OHDA-lesioned rat. Psychopharmacology (Berl) 201, 529–539. 10.1007/s00213-008-1319-0
62. Klein C., Westenberger A. (2012). Genetics of Parkinson's disease. Cold Spring Harb. Perspect. Med. 2:a008888. 10.1101/cshperspect.a008888
63. Koga K., Kurokawa M., Ochi M., Nakamura J., Kuwana Y. (2000). Adenosine A_{2A} receptor antagonists KF17837 and KW-6002 potentiate rotation induced by dopaminergic drugs in hemi-Parkinsonian rats. Eur. J. Pharmacol. 408, 249–255. 10.1016/S0014-2999(00)00745-7
64. Lautier C., Goldwurm S., Dürr A., Giovannone B., Tsiaras W. G., Pezzoli G., et al. . (2008). Mutations in the GIGYF2 (TNRC15) gene at the PARK11 locus in familial Parkinson disease. Am. J. Hum. Genet. 82, 822–833. 10.1016/j.ajhg.2008.01.015
65. Lee F. J., Xue S., Pei L., Vukusic B., Chéry N., Wang Y., et al. . (2002). Dual regulation of NMDA receptor functions by direct protein-protein interactions with the dopamine D1 receptor. Cell 111, 219–230. 10.1016/S0092-8674(02)00962-5
66. Maesawa S., Kaneoke Y., Kajita Y., Usui N., Misawa N., Nakayama A., et al. . (2004). Long-term stimulation of the subthalamic nucleus in hemiparkinsonian rats: neuroprotection of dopaminergic neurons. J. Neurosurg. 100, 679–687. 10.3171/jns.2004.100.4.0679
67. Maggio R., Aloisi G., Silvano E., Rossi M., Millan M. J. (2009). Heterodimerization of dopamine receptors: new insights into functional and therapeutic significance. Parkinsonism Relat. Disord. 15, S2–7. 10.1016/S1353-8020(09)70826-0
68. Maidan I., Rosenberg-Katz K., Jacob Y., Giladi N., Hausdorff J. M., Mirelman A. (2017). Disparate effects of training on brain activation in Parkinson disease. Neurology 89, 1804–1810. 10.1212/WNL.0000000000004576
69. Marcellino D., Lindqvist E., Schneider M., Müller C. E., Fuxe K., Olson L., et al. . (2010). Chronic A_{2A} antagonist treatment alleviates parkinsonian locomotor

deficiency in MitoPark mice. Neurobiol. Dis. 40, 460–466. 10.1016/j.nbd.2010.07.008
70. Márquez-Ruiz J., Leal-Campanario R., Sánchez-Campusano R., Molaee-Ardekani B., Wendling F., Miranda P. C., et al. . (2012). Transcranial direct-current stimulation modulates synaptic mechanisms involved in associative learning in behaving rabbits. Proc. Natl. Acad. Sci. U.S.A. 109, 6710–6715. 10.1073/pnas.1121147109
71. Martinez-Mir M. I., Probst A., Palacios J. M., Adenosine A. (1991). Receptors: selective localization in the human basal ganglia and alterations with disease. Neuroscience 42, 697–706. 10.1016/0306-4522(91)90038-P
72. Matsuya T., Takuma K., Sato K., Asai M., Murakami Y., Miyoshi S., et al. . (2007). Synergistic effects of adenosine A_{2A} antagonist and L-DOPA on rotational behaviors in 6-hydroxydopamine-induced hemi-Parkinsonian mouse model. Pharmacol Sci. 103, 329–332. 10.1254/jphs.SCZ070058
73. McCarthy S., Somayajulu M., Sikorska M., Borowy-Borowski H., Pandey S. (2004). Paraquat induces oxidative stress and neuronal cell death; neuroprotection by water-soluble Coenzyme Q10. Toxicol. Appl. Pharmacol. 201, 21–31. 10.1016/j.taap.2004.04.019
74. Michel A., Downey P., Nicolas J. M., Scheller D. (2014). Unprecedented therapeutic potential with a combination of A2A/NR2B receptor antagonists as observed in the 6-OHDA lesioned rat model of Parkinson's disease. PLoS ONE 9:e114086. 10.1371/journal.pone.0114086
75. Michel A., Downey P., Van Damme X., De Wolf C., Schwarting R., Scheller D. (2015). Behavioural Assessment of the A2a/NR2B combination in the unilateral 6-OHDA-lesioned rat model: a new method to examine the therapeutic potential of non-dopaminergic drugs. PLoS ONE 10:e0135949. 10.1371/journal.pone.0135949
76. Miki Y., Tanji K., Mori F., Kakita A., Takahashi H., Wakabayashi K. (2017). PLA2G6 accumulates in Lewy bodies in PARK14 and idiopathic Parkinson's disease. Neurosci. Lett. 645, 40–45. 10.1016/j.neulet.2017.02.027
77. Mizuno Y., Kondo T., Japanese Istradefylline Study Group (2013). Adenosine A_{2A} receptor antagonist istradefylline reduces daily OFF time in Parkinson's disease. Mov. Disord. 28, 1138–1141. 10.1002/mds.25418
78. Mortimer J. A., Borenstein A. R., Nelson L. M. (2012). Associations of welding and manganese exposure with Parkinson disease: review and meta-analysis. Neurology 79, 1174–1180. 10.1212/WNL.0b013e3182698ced
79. Nash J. E., Brotchie J. M. (2000). A common signaling pathway for striatal NMDA and adenosine A2a receptors: implications for the treatment of Parkinson's disease. J. Neurosci. 20, 7782–7789.
80. Nishi A., Snyder G. L., Greengard P. (1997). Bidirectional regulation of DARPP-32 phosphorylation by dopamine. J. Neurosci. 17, 8147–8155.
81. Nishioka K., Tanaka R., Shimura H., Hirano K., Hatano T., Miyakawa K., et al. . (2014). Quantitative evaluation of electroconvulsive therapy for Parkinson's disease with refractory psychiatric symptoms. J. Neural. Transm. (Vienna) 121, 1405–1410. 10.1007/s00702-014-1212-4
82. Noyce A. J., Bestwick J. P., Silveira-Moriyama L., Hawkes C. H., Giovannoni G., Lees A. J., et al. . (2012). Meta-analysis of early nonmotor features and risk factors for Parkinson disease. Ann. Neurol. 72, 893–901. 10.1002/ana.23687
83. Orru M., Bakešová J., Brugarolas M., Quiroz C., Beaumont V., Goldberg S. R., et al. (2011). Striatal pre-and postsynaptic profile of adenosine A_{2A} receptor antagonists. PLoS ONE 6:e16088 10.1371/journal.pone.0016088

84. Petzer J. P., Petzer A. (2015). Caffeine as a lead compound for the design of therapeutic agents for the treatment of Parkinson's disease. Curr. Med. Chem. 22, 975–988. 10.2174/0929867322666141215160015
85. Petzer J. P., Steyn S., Castagnoli K. P., Chen J. F., Schwarzschild M. A., Van der Schyf C. J., et al. . (2003). Inhibition of monoamine oxidase B by selective adenosine A_{2A} receptor antagonists. Bioorg. Med. Chem. 11, 1299–1310. 10.1016/S0968-0896(02)00648-X
86. Pezzoli G., Cereda E. (2013). Exposure to pesticides or solvents and risk of Parkinson disease. Neurology 80, 2035–2041. 10.1212/WNL.0b013e318294b3c8
87. Picelli A., Varalta V., Melotti C., Zatezalo V., Fonte C., Amato S., et al. . (2016). Effects of treadmill training on cognitive and motor features of patients with mild to moderate Parkinson's disease: a pilot, single-blind, randomized controlled trial. Funct. Neurol. 31, 25–31. 10.11138/FNeur/2016.31.1.025
88. Pinna A., Bonaventura J., Farré D., Sánchez M., Simola N., Mallol J., et al. (2014). L-DOPA disrupts adenosine A_{2A}-cannabinoid CB(1)-dopamine D(2) receptor heteromer cross-talk in the striatum of hemiparkinsonian rats: biochemical and behavioral studies. Exp. Neurol. 253, 180–191. 10.1016/j.expneurol.2013.12.021
89. Pinna A., Ko W. K., Costa G., Tronci E., Fidalgo C., Simola N., et al. . (2016). Antidyskinetic effect of A_{2A} and 5HT1A/1B receptor ligands in two animal models of Parkinson's disease. Mov. Disord. 31, 501–511. 10.1002/mds.26475
90. Pinna A., Pontis S., Borsini F., Morelli M. (2007). Adenosine A_{2A} receptor antagonists improve deficits in initiation of movement and sensory motor integration in the unilateral 6-hydroxydopamine rat model of Parkinson's disease. Synapse 61, 606–614. 10.1002/syn.20410
91. Pollack A. E., Fink J. S. (1995). Adenosine antagonists potentiate D2 dopamine-dependent activation of Fos in the striatopallidal pathway. Neuroscience 68, 721–728. 10.1016/0306-4522(95)00168-I
92. Postuma R. B., Lang A. E., Munhoz R. P., Charland K., Pelletier A., Moscovich M., et al. . (2012). Caffeine for treatment of Parkinson disease: a randomized controlled trial. Neurology 79, 651–658. 10.1212/WNL.0b013e318263570d
93. Ralevic V., Burnstock G. (1998). Receptors for purines and pyrimidines. Pharmacol. Rev. 50, 413–492.
94. Redenšek S., Trošt M., DolŽan V. (2017). Genetic determinants of parkinson's disease: can they help to stratify the patients based on the underlying molecular defect? Front. Aging Neurosci. 9:20. 10.3389/fnagi.2017.00020
95. Ross G. W., Abbott R. D., Petrovitch H., Morens D. M., Grandinetti A., Tung K. H., et al. . (2000). Association of coffee and caffeine intake with the risk of Parkinson disease. JAMA 283, 2674–2679. 10.1001/jama.283.20.2674
96. Sadek A. R., Knight G. E., Burnstock G. (2011). Electroconvulsive therapy: a novel hypothesis for the involvement of purinergic signalling. Purinergic Signal. 7, 447–452. 10.1007/s11302-011-9242-y
97. Salamone J. D., Collins-Praino L. E., Pardo M., Podurgiel S. J., Baqi Y., Müller C. E., et al. (2013). Conditional neural knockout of the adenosine A_{2A} receptor and pharmacological A_{2A} antagonism reduce pilocarpine-induced tremulous jaw movements: studies with a mouse model of parkinsonian tremor. Eur. Neuropsychopharmacol. 23, 972–977. 10.1016/j.euroneuro.2012.08.004
98. Scott L., Dawson V. L., Dawson T. M. (2017). Trumping neurodegeneration: targeting common pathways regulated by autosomal recessive Parkinson's disease genes. Exp. Neurol. 298(Pt B), 191–201. 10.1016/j.expneurol.2017.04.008

99. Shanahan J., Morris M. E., Bhriain O. N., Volpe D., Lynch T., Clifford A. M. (2017). Dancing for Parkinson Disease: a randomized trial of irish set dancing compared with usual care. Arch. Phys. Med. Rehabil. 98, 1744–1751. 10.1016/j.apmr.2017.02.017
100. Sharma J. C., Lewis A. (2017). Weight in Parkinson's Disease: phenotypical significance. Int. Rev. Neurobiol. 134, 891–919. 10.1016/bs.irn.2017.04.011
101. Shen H., Luo Y., Yu S. J., Wang Y. (2011). Enhanced neurodegeneration after a high dose of methamphetamine in adenosine A3 receptor null mutant mice. Neuroscience 194, 170–180. 10.1016/j.neuroscience.2011.08.013
102. Shen X., Yang H., Wu Y., Zhang D., Jiang H. (2017). Association of Helicobacter pylori infection with Parkinson's diseases: a meta-analysis. Helicobacter 22:e12398. 10.1111/hel.12398
103. Shin H. K., Lee S. W., Choi B. T. (2017). Modulation of neurogenesis via neurotrophic factors in acupuncture treatments for neurological diseases. Biochem Pharmacol. 141, 132–142. 10.1016/j.bcp.2017.04.029
104. Simola N., Fenu S., Baraldi P. G., Tabrizi M. A., Morelli M. (2006). Dopamine and adenosine receptor interaction as basis for the treatment of Parkinson's disease. J. Neurol. Sci. 248, 48–52. 10.1016/j.jns.2006.05.038
105. Song L., Kong M., Ma Y., Ba M., Liu Z. (2009). Inhibitory effect of 8-(3-chlorostryryl) caffeine on levodopa-induced motor fluctuation is associated with intracellular signaling pathway in 6-OHDA-lesioned rats. Brain Res. 1276, 171–179. 10.1016/j.brainres.2009.04.028
106. Sonsalla P. K., Wong L. Y., Harris S. L., Richardson J. R., Khobahy I., Li W., et al. . (2012). Delayed caffeine treatment prevents nigral dopamine neuron loss in a progressive rat model of Parkinson's disease. Exp. Neurol. 234, 482–487. 10.1016/j.expneurol.2012.01.022
107. Spillantini M. G., Crowther R. A., Jakes R., Hasegawa M., Goedert M. (1998). Alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc. Natl. Acad. Sci. U.S.A. 95, 6469–6473. 10.1073/pnas.95.11.6469 [PMC free article] [PubMed]
108. Stacy M., Silver D., Mendis T., Sutton J., Mori A., Chaikin P., et al. . (2008). A 12-week, placebo-controlled study (6002-US-006) of istradefylline in Parkinson disease. Neurology 70, 2233–2240. 10.1212/01.wnl.0000313834.22171.17
109. Stocchi F., Rascol O., Hauser R. A., Huyck S., Tzontcheva A., Capece R., et al. . (2017). Randomized trial of preladenant, given as monotherapy, in patients with early Parkinson disease. Neurology 88, 2198–2206. 10.1212/WNL.0000000000004003
110. Strömberg I., Popoli P., Müller C. E., Ferré S., Fuxe K. (2000). Electrophysiological and behavioural evidence for an antagonistic modulatory role of adenosine A_{2A} receptors in dopamine D_2 receptor regulation in the rat dopamine-denervated striatum. Eur. J. Neurosci. 12, 4033–4037. 10.1046/j.1460-9568.2000.00288.x
111. Suarez-Cedeno G., Suescun J., Schiess M. C. (2017). Earlier Intervention with Deep Brain Stimulation for Parkinson's Disease. Parkinsons. Dis. 2017:9358153. 10.1155/2017/9358153
112. Svenningsson P., Fourreau L., Bloch B., Fredholm B. B., Gonon F., Le Moine C. (1999). Opposite tonic modulation of dopamine and adenosine on c-fos gene expression in striatopallidal neurons. Neuroscience 89, 827–837. 10.1016/S0306-4522(98)00403-5
113. Tanganelli S., Sandager Nielsen K., Ferraro L., Antonelli T., Scheel-Krüger J. (2004). Striatal plasticity at the network level. Focus on adenosine A_{2A} and

D_2 interactions in models of Parkinson's Disease. Parkinsonism Relat. Disord. 10, 273–280. 10.1016/j.parkreldis.2004.02.015

114. Tomiyama M., Kimura T., Maeda T., Tanaka H., Kannari K., Baba M. (2004). Upregulation of striatal adenosine A_{2A} receptor mRNA in 6-hydroxydopamine-lesioned rats intermittently treated with L-DOPA. Synapse 52, 218–222. 10.1002/syn.20011
115. Tozzi A., de Iure A., Di Filippo M., Tantucci M., Costa C., Borsini F., et al. (2011). The distinct role of medium spiny neurons and cholinergic interneurons in the D2/A2A receptor interaction in the striatum: implications for Parkinson's disease. J. Neurosci. 31, 1850–1862. 10.1523/JNEUROSCI.4082-10.2011
116. Trifilieff P., Rives M. L., Urizar E., Piskorowski R. A., Vishwasrao H. D., Castrillon J., et al. . (2011). Detection of antigen interactions *ex vivo* by proximity ligation assay: endogenous dopamine D2-adenosine A_{2A} receptor complexes in the striatum. Biotechniques 51, 111–118. 10.2144/000113719
117. Tronci E., Simola N., Borsini F., Schintu N., Frau L., Carminati P., et al. . (2007). Characterization of the antiparkinsonian effects of the new adenosine A_{2A} receptor antagonist ST1535: acute and subchronic studies in rats. Eur. J. Pharmacol. 566, 94–102 10.1016/j.ejphar.2007.03.021
118. Uchida S., Kadowaki-Horita T., Kanda T. (2014). Effects of the adenosine A_{2A} receptor antagonist on cognitive dysfunction in Parkinson's disease. Int. Rev. Neurobiol. 119, 169–189. 10.1016/B978-0-12-801022-8.00008-8
119. Van der Mark M., Brouwer M., Kromhout H., Nijssen P., Huss A., Vermeulen R. (2012). Is pesticide use related to Parkinson disease? Some clues to heterogeneity in study results. Environ. Health Perspect. 120, 340–347. 10.1289/ehp.1103881
120. Van der Walt M. M., Terre'Blanche G., Petzer A., Petzer J. P. (2015). The adenosine receptor affinities and monoamine oxidase B inhibitory properties of sulfanylphthalimide analogues. Bioorg. Chem. 59, 117–123. 10.1016/j.bioorg.2015.02.005
121. Villar-Cheda B., Sousa-Ribeiro D., Rodriguez-Pallares J., Rodriguez-Perez A. I., Guerra M. J., Labandeira-Garcia J. L. (2009). Aging and sedentarism decrease vascularization and VEGF levels in the rat substantia nigra. Implications for Parkinson's disease. J. Cereb. Blood Flow Metab. 29, 230–234. 10.1038/jcbfm.2008.127
122. Volkow N. D., Wang G. J., Logan J., Alexoff D., Fowler J. S., Thanos P. K., et al. . (2015). Caffeine increases striatal dopamine D2/D3 receptor availability in the human brain. Transl. Psychiatry 5, e549. 10.1038/tp.2015.46
123. Vortherms T. A., Watts V. J. (2004). Sensitization of neuronal A_{2A} adenosine receptors after persistent D_2 dopamine receptor activation. J. Pharmacol. Exp. Ther. 308, 221–227. 10.1124/jpet.103.057083
124. Watanabe H., Ikeda M., Watanabe K. (1981). Properties of rotational behaviour produced by methylxanthine derivatives in mice with unilateral striatal 6-hydroxydopamine-induced lesions. J. Pharmacobiodyn. 4, 301–307. 10.1248/bpb1978.4.301
125. Xu K., Di Luca D. G., Orrú M., Xu Y., Chen J. F., Schwarzschild M. A. (2016). Neuroprotection by caffeine in the MPTP model of parkinson's disease and its dependence on adenosine A_{2A} receptors. Neuroscience. 322, 129–137. 10.1016/j.neuroscience.2016.02.035
126. Yang M., Soohoo D., Soelaiman S., Kalla R., Zablocki J., Chu N., et al. . (2007). Characterization of the potency, selectivity, and pharmacokinetic profile for

six adenosine A_{2A} receptor antagonists. Naunyn Schmiedebergs. Arch. Pharmacol. 375, 133–144. 10.1007/s00210-007-0135-0

127. Yu L., Schwarzschild M. A., Chen J. F. (2006). Cross-sensitization between caffeine- and L-dopa-induced behaviors in hemiparkinsonian mice. Neurosci. Lett. 393, 31–35. 10.1016/j.neulet.2005.09.036

CHAPTER 3

Evaluation of behavioral and biochemical parameters after an intra-encephalic injection of 6-OHDA in adult zebrafish

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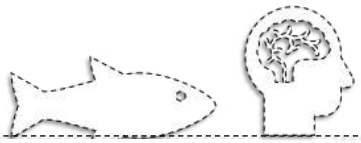

Abstract

The purinergic system strongly interacts with the dopaminergic system, often via allosteric interactions within heteromeric receptor complexes. The most studied heteromer complex consists of the dopamine D_2 and the adenosine A_{2A} receptor. The aim of this study was to investigate the interaction of adenosinergic and dopaminergic signalling during neurodegeneration in a zebrafish model of Parkinson's disease. To this purpose, behavioural parameters, dopamine levels and expression levels of adenosine and dopamine receptors in the brain in of adult zebrafish injected with 6-hydroxydopamine (6-OHDA) were studied. The animals were evaluated 3, 7 and 28 days after injection of 6-OHDA in the right telencephalon (8 μg/μL). The locomotor parameters 'turn angle' and 'time spent in the superior zone' were decreased on day 28 and 7, respectively. Dopamine levels in the whole brain were not altered, but D_2 and A_{2A1} receptor expression was higher on day 3, which was normalized on day 28. In conclusion, the exposure of adult zebrafish to intra-encephalic injection of 6-OHDA, even in a dose that produces mild locomotor effects, is able to transiently affect dopaminergic and adenosinergic receptor expression. This refined locomotor impairment after intra-encephalic injection of 6-OHDA occurred without detectable changes in dopamine levels.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting about 1% of the elderly population (Tysnes & Storstein, 2017). PD is characterized by a progressive degeneration of dopaminergic neurons. When a patient is diagnosed with PD, the loss of striatal dopaminergic neurons is already close to 80% (Fearnley & Lees, 1991). Current treatment is focussed on compensation for the loss of dopaminergic signalling, but treatment efficacy is decreasing when the disease is progressing. The dopaminergic system strongly interacts with the purinergic system, especially because adenosine and dopamine receptors can form heterodimers and thus influence each other's action. Especially the interaction between A_{2A} and D_2 receptors has received much attention (Ferré & Ciruela, 2019). *In vivo* rat studies showed that A_{2A} antagonists can enhance D_2 receptor binding and function (Bortolotto et al., 2014a; Hillefors-Berglund, Hedlund, & von Euler, 1995; Strömberg, Popoli, Müller, Ferré, & Fuxe, 2000). This finding turned the A_{2A} receptor into a potential therapeutic target for PD (Beggiato et al., 2014).

The 6-hydroxydopamine (6-OHDA) model is a frequently used animal model to investigate specific characteristics of PD in rodents (Hernandez-Baltazar, Zavala-Flores, & Villanueva-Olivo, 2017). This model selectively destroys the dopaminergic neurons with little collateral damage in other brain regions or peripheral organs. In this model, 6-OHDA is injected directly in the brain of the animals, since the toxic substance is not capable to cross the blood brain barrier (Simola, Morelli, & Carta, 2007).

Zebrafish is a well-known model in basic research because of the possibility to use different disease models, mutants, CRISPR and avatars. Moreover, the complexity of their structure is already sufficient to investigate physiological and behavioural parameters (Costa et al., 2020; Lam & Peterson, 2019; J. Li et al., 2019; Orger & de Polavieja, 2017). In zebrafish, the use of the 6-OHDA model has recently been introduced, but a better understanding of its characteristics is still necessary (Caldwell et al., 2019; Vijayanathan et al., 2017). Zebrafish have been used to study the mechanisms underlying PD. In studies with zebrafish larvae, dopaminergic degeneration was induced by adding 6-OHDA in the medium. These studies found a decrease in the mobility of the larvae, reduced tyrosine hydrolase levels and a lower dopaminergic cell count (Cronin & Grealy, 2017; M. Li, Zhou, Xu, Song, & Lu, 2018).

However, PD only occurs in adults and elderly people and therefore the use of adult zebrafish is more appropriate for this research.

The aim of this study was to investigate whether adult zebrafish injected with 6-OHDA in the brain can be used to study the interaction of adenosinergic and dopaminergic signalling during neurodegeneration. To this purpose, we investigated behavioural parameters, dopamine levels and adenosine and dopamine receptor expression levels.

Material and Methods

Animals

All animals were obtained from the local breeding facility of Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. Adult zebrafish (*Danio rerio*) (6–12 months; AB background; 3–5 cm) of both sexes were used. Animals were housed in groups of 20 (5 animals/L) in thermostatically controlled (28 ± 2 °C) tanks. The water was from reverse osmosis quality reconstituted with marine salt (Cristalsea™, Marinemix) at 0.4 parts per thousand. The water was kept under constant chemical and mechanical filtration and aeration (7.20 mg $O_2.L^{-1}$) in a recirculating system. Fish were maintained under a 14–10 h day/night photoperiod cycle, fed three times a day with commercial flakes (TetraMin®, NC, USA), supplemented with live brine shrimp twice a day. Adult zebrafish were cryoeuthanized for gene expression and dopamine analyses. All protocols were performed according to Brazilian legislation and approved by the Institutional Animal Care Committee (7563 CEUA-PUCRS). The use and maintenance of zebrafish were according to the ‘‘Guide for the Care and Use of Laboratory Animals’’ published by the United States National Institutes of Health.

Intra-encephalic 6-OHDA injections

The zebrafish was anesthesed using a tricaine solution of 0.1 g/L (MS-222) and placed on a humidified sponge (with the same solution) in a dorsal position. An insulin needle was used to make a small incision to open the skull. A capillary glass needle (0.58 mm inside diameter) was made in a puller (Pull-1000, WPI, São Paulo, Brazil) and a volume

of 8 nL was injected directly in the right telencephalon region of the animal. The injected solution contained 0.02% of acid ascorbic and 8 µg/µL of 6-OHDA in 0.9% saline. The control group was injected with saline. The procedures were executed in the dark and the capillary needle was wrapped in aluminium foil to avoid light-catalysed oxidation of 6-OHDA. Figure 1 represents the experimental design of the study. Different doses of 6-OHDA (2, 4, 8 and 16 µg/µL) were tested to determine the optimal concentration (data not shown). The dose 8 µg/µL was chosen, because it was the lowest dose that had an effect on the parameter 'turn angle', an indicator of locomotor coordination.

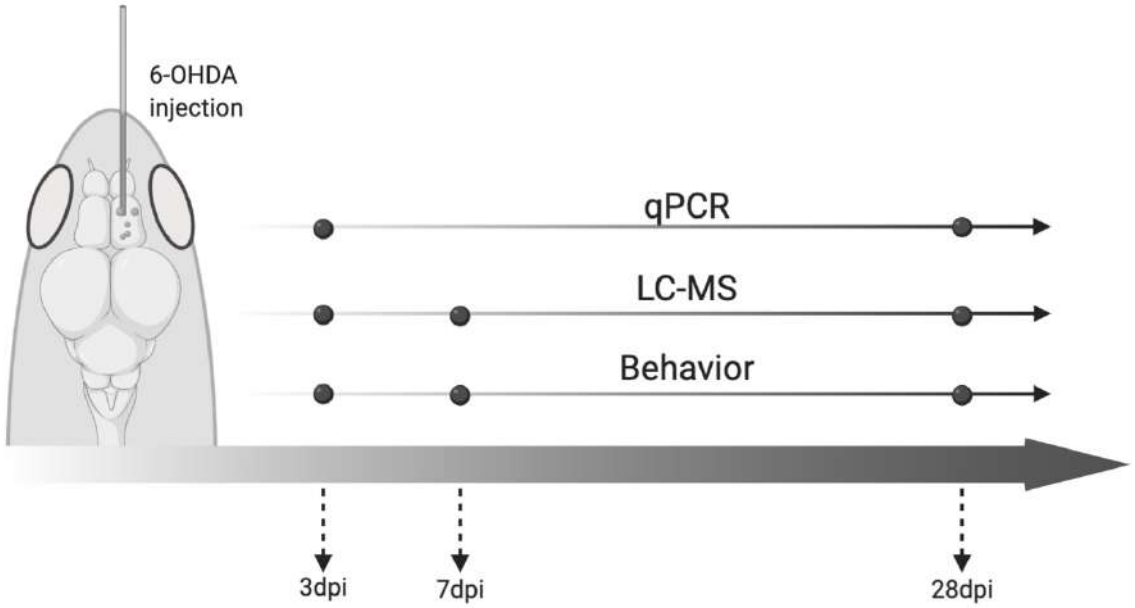

Figure 1: Experimental design of the study. Following the injection of 6-OHDA (8 µg/µL), behavioural tests and LC-MS experiments were performed on day 3, 7 and 28 post injection (dpi). qPCR was performed on day 3 and 28. (Made in ©BioRender - biorender.com)

Dopamine Quantification

Encephala were collected and the encephala of 3 zebrafish were combined and stored in 2 mL microtubes with 300 µL of formic acid (0.1 M). The experiments were performed in quadruplicate. The samples were homogenised with Ultra-Turrax (T10 basic IKA®) and centrifuged at 15.000 RPM for 25 minutes (4ºC). The samples were maintained

on ice at all time. Samples were fractionated in two aliquots, one (5µL) to quantify the amount of protein according to Bradford's method (Bradford, 1976), and another to quantify dopamine by liquid chromatography tandem mass spectrometry (LC-MS/MS). The LC-MS/MS apparatus consisted of an Agilent 1290 Infinity liquid chromatograph (Agilent, Paolo Alto, USA) coupled to an Agilent 6460 Triple Quad mass spectrometer (Agilent, Paolo Alto, USA). The chromatography was performed on an Agilent Zorbax Eclipse PLUS C18 RRHD column (5 x 2.1 mm, 1.8 micra, Agilent, Paolo Alto, USA) in gradient mode. The mobile phase for gradient elution consisted of (A) 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The gradient elution started with a mobile phase composition of 2% B, and was linearly changed to 95% B after 4.5 min. This composition was maintained for 1.3 min before returning to the starting conditions. The total time of the chromatographic run was 7.5 min, the mobile phase flow rate was 0.2 mL/min, and the injected sample volume was 10 µL. Dopamine was ionized by an electrospray source operated in positive mode, using a capillary voltage of 4.5 kV, a nitrogen gas flow of 10 L/min at 300 °C, and a nebulizer pressure of 35 psi. The spectrometer was operated in Multiple reaction monitoring mode, monitoring the transitions (m/z) of 154>137.1, and 154>90.1 for dopamine quantification and qualification, respectively. A calibration curve was prepared for concentrations ranging from 1.0 to 20.0 ng/mL of dopamine dissolved in 0.1% formic acid. Quantification was performed by external calibration and the results were expressed as ng of dopamine/mg of protein.

Gene expression analysis by real-time PCR

Encephala from 3 animals were combined to investigate *d2, a2a1* and *a2a2* gene expression. Four independent experiments were performed for each group. Total RNA was isolated with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA was quantified by spectrophotometry, and the cDNA was synthesized from 1 µg of total RNA using the High-capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's instructions. Quantitative real-rime PCR was performed using Powerup™ SYBR™ Green Master Mix (Applied Biosystems) to detect double-stranded cDNA. Reactions were carried out in a final volume of 20 µL using 1 µL of diluted cDNA (1:2), 10 µL of power up reagent (Brand), 0.6 µL of reverse and forward primers (final concentration of 300 nM) (Table

1) (Boehmier et al., 2004; Capiotti et al., 2016; Mccurley & Callard, 2008), completing the final volume with 8.4 µL of water. The PCR cycling conditions included an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 51°C (*ef1a* and *b-actin*), 60°C (*a2a1* and *a2a2*) or 58°C (*drd2a*) for annealing and 15 s at 72 °C for extension. At the end of cycling protocol, a melting-curve analysis was included, and the fluorescence was measured between 60 and 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems, California, USA). The efficiency *per* sample was calculated using LinRegPCR 11.0 software (http://LinRegPCR.nl) and the stability of the reference genes *ef1α* and *b-actina* (M-value), and the optimal number of reference genes according to the pairwise variation (V) were analysed by using GeNorm 3.5 software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method (Nery et al., 2014).

Table 1: Primer sequences for RT-qPCR experiments included in this study.

	Sequence	Accession	Reference
***β-actin* - F**	CGAGCAGGAGATGGGAACC	AF057040	(Mccurley & Callard, 2008)
***β-actin* - R**	CAACGGAAACGCTCATTGC		
***ef1α* - F**	CTTCTCAGGCTGACTGTGC	AY422992	(Mccurley & Callard, 2008)
***ef1α* - R**	CCGCTAGCATTACCCTCC		
***a2a1* - F**	GCGAACTGTACGCCGAGCAGAG	AY945800	(Capiotti et al., 2016)
***a2a1* - R**	TTATTCCCAGTGAGCGGCGACTC		
***a2a2* - F**	GGATTGGGTCATGTACCTGGCCATC	AY945801	(Capiotti et al., 2016)
***a2a2* - R**	GCTGTTTCCAATGGCCAGCCTG		
***drd2a* - F**	GACGGAACTCACTTCAATGGAG	AY183456	(Boehmier et al., 2004)
***drd2a* - R**	GCCATTGCTTGAAGTTGTACAG		

Locomotion

Animals were individually placed into the experimental tank (30 × 15 × 10 cm, length × height × width) and habituated to the tank for 60 s, as previously described (Gerlai, Lahav, Guo, & Rosenthal, 2000). There was no drug exposure during behavioral experiments. The locomotor activity was video recorded for 5 min after the habituation period and analysed using the Ethovision® XT8 software package (Noldus, Netherlands) (Menezes et al., 2015). The distance travelled and the absolute turn angle was measured. The absolute turn angle represents the sum of all vector angles of movements created from one position to the next.

Statistical Analyses

Data were expressed as mean difference ± standard error of the difference and 95% confidence intervals (CI) per treatment and checked for normality using the Shapiro-Wilk Test. All data were analysed by one-way ANOVA or two-way ANOVA depending on the group distribution. Differences between groups were considered statistically significant when the probability (p) was <0.05. The statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Locomotion

The locomotor parameters distance traveled, velocity (total distance divided by mobile time), turn angle and time spent in the superior zone were measured 3, 7 and 28 days after injection of 6-OHDA. The distance traveled showed no significant main effects of time and treatment, and no significant interactions between these factors. In the velocity measurements, we found a significant main effect of time ($F(1.659, 58.06) = 17.38$, $p < 0.0001$), but neither a main effect of treatment nor any significant interaction was observed. Turn angle demonstrated a significant main effect of time ($F(1.177, 77.13) = 10.14$, $p=0.0012$). No main effect of treatment was found, but the interaction between time and treatment showed a significant decrease in turn angle in 6-OHDA treated animals at 28 days post injection, as compared to controls (mean diff = 5.5, 95% CI of diff. = 1.8 - 9.3, $p= 0.0017$). Time in the superior zone demonstrated a main effect of time ($F(1.462, 92.84) = 14.39$, $p<0.0001$), but not of the treatment. The interaction between time and treatment revealed a significant difference between treatment groups on day 7 (mean diff= 35.4, 95% CI of diff. = 5.4 to 65.3, $p=0.016$).

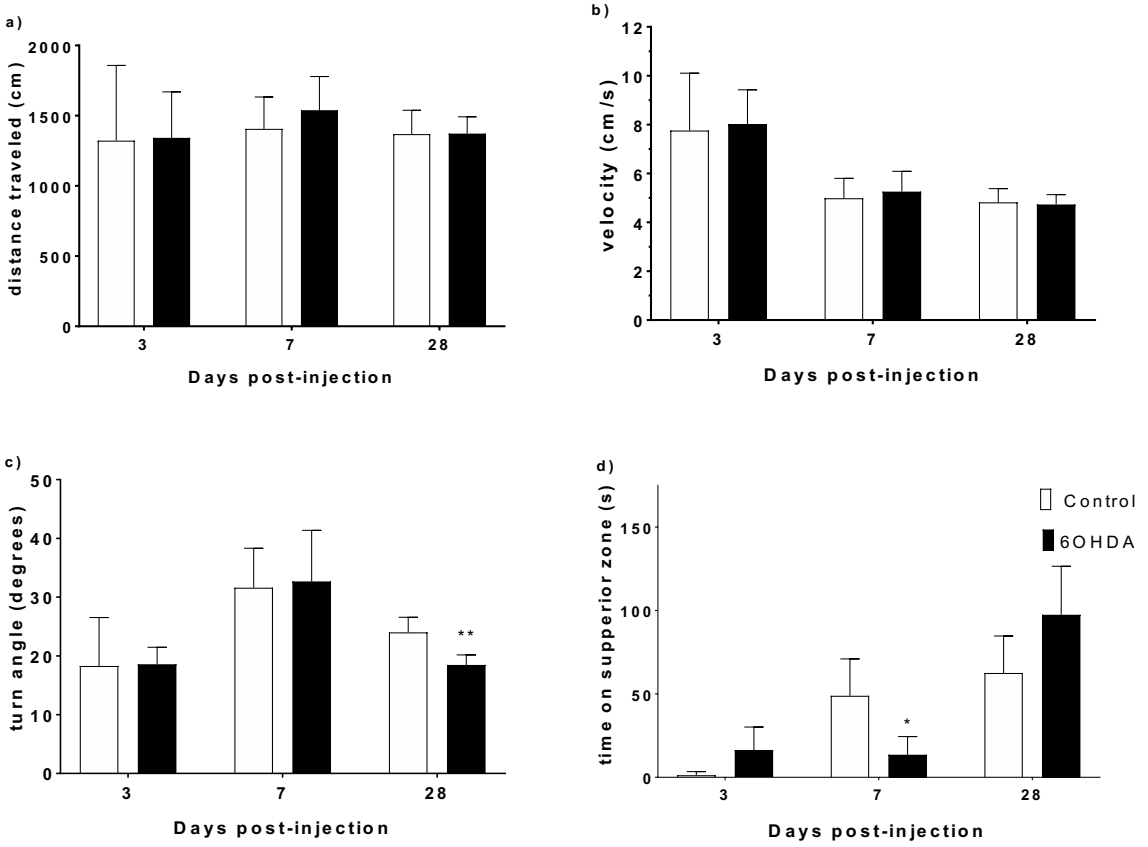

Figure 3: Locomotion of zebrafish on day 3, 7, and 28 after the injection of 6-OHDA (n = 8-10): distance traveled (cm) (a), velocity (cm/s) (b), turn angle (degrees) (c) and time in the superior zone (d). Two-way ANOVA, followed by the Sidak's multiple comparison test were used to analyze differences between groups and time points. Statistically significant differences between groups are indicated by: *p<0.05, **p<0.01

Dopamine Quantification

We analyzed the levels of dopamine in zebrafish brains with LC-MS 3, 7 and 28 after injection of an 8 µg/µL dose of 6-OHDA. We found a main effect of time ($F(1.126, 6.191) = 7.021$, $p = 0.035$), but no significant main effect of treatment. The interaction between time and group also did not show any significant differences ($p>0.05$).

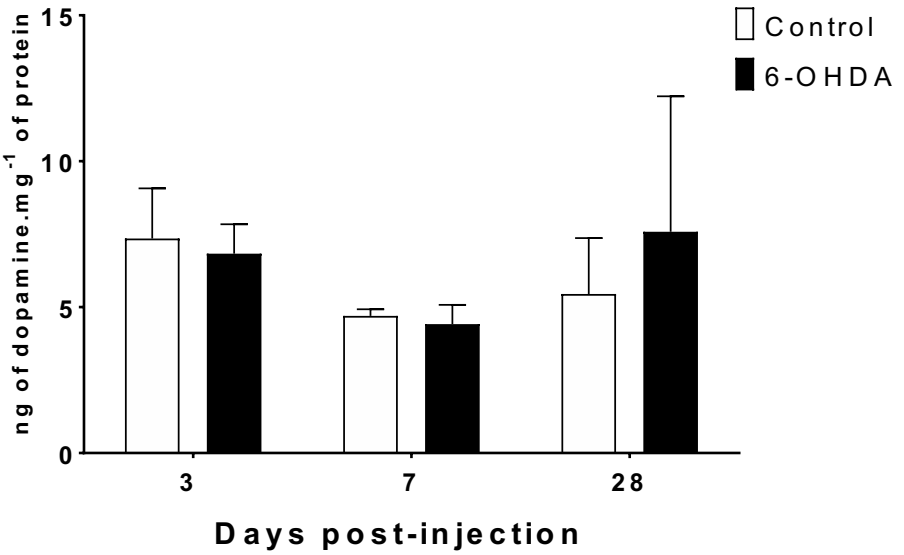

Figure 4: Dopamine levels (ng of dopamine/mg of protein) 3, 7, and 28 days after 6-OHDA injection (n = 4). Two-way ANOVA was used to compare groups and time points, followed by the Sidak's multiple comparison test.

qPCR

Adenosine A_{2A1} and A_{2A2} receptor and dopamine D_2 receptor gene expression in the whole brain was assessed prior to the appearance of locomotor effects of 6-OHDA (day 3) and in a period with locomotor effects (day 28). The number of D_2 receptor transcripts in 6-OHDA-treated animals on day 3 was increased when compared to the saline group ($p<0.05$). This effect of 6-OHDA on D_2 receptor expression had disappeared on day 28 (Figure 5a). The injection of 6-OHDA also increased A_{2A1} adenosine receptor gene expression on day 3, but this effect was not statistically significant yet. However, the number of A_{2A1} adenosine receptor transcripts significantly decreased between day 3 and 28, when was normalized to the level of controls ($p<0.05$) (Figure 5b). The expression of a2a2 RNA was not affected by the 6-OHDA injection, neither on day 3 nor on day 28.

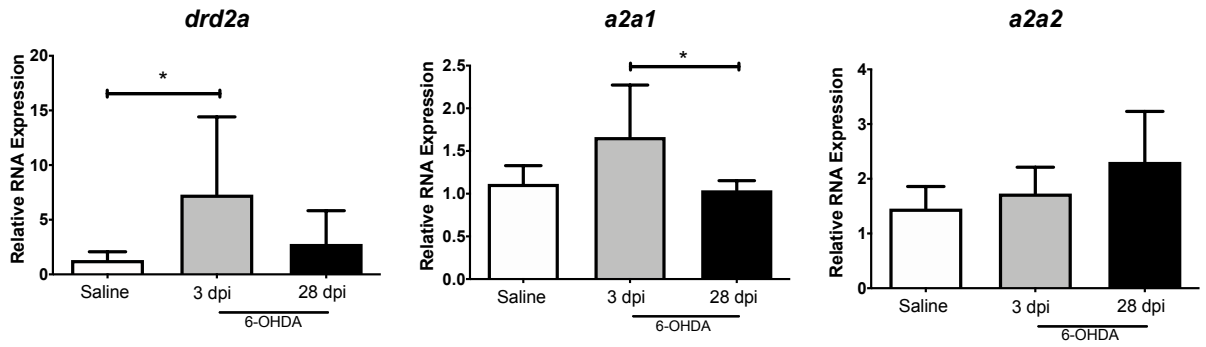

Figure 5: Relative RNA expression of the *d2* (a), *a2a1* (b) and *a2a2(*c) gene on day 3 and 28 post 6-OHDA injection (n=4). *b-actin* and *elf1a* were used as constitutive controls. One-way ANOVA was used to compare groups, followed by Dunn's multiple comparisons test. *p<0.05.

Discussion

In this study, we evaluated behavioural, biochemical and gene expression parameters in adult zebrafish after intra-encephalic injection of 6-OHDA, as a new animal model for PD. The 6-OHDA injection promoted changes in the locomotor parameter 'turn angle' and the anxiety parameter 'time spent in the superior zone'. No alterations in whole brain dopamine levels as a result of 6-OHDA injection were detected. A transient increase in the expression of D_2 receptors accompanied by a trend towards an increase in A_{2A1} expression 3 days after intra-encephalic 6-OHDA injection was observed, but these effects on receptor expression were no longer present anymore 28 days after 6-OHDA administration.

Intra-encephalic injection of 6-OHDA in zebrafish had a significant effect on turn angle measures. This measure corresponds to the locomotor capacity of a highly coordinated movement (Blazina, Vianna, & Lara, 2013) and is affected by dopaminergic loss (Soares et al., 2017). The turn angle reflects the movement relative to a center point, measuring also the direction of the movement, resulting in a measure of motor coordination and swimming directions (Blazina et al., 2013). In our study, this parameter was decreased on day 28 and therefore reflects the long-term effect of the 6-OHDA injection. The sensitivity of this parameter to dopaminergic loss was also confirmed in other studies, showing a dose-dependent response after paraquat exposure, in particular an increase between day 1 and 6 after 6-OHDA injections followed by a normalization after 9 days (Anichtchik, Kaslin, Peitsaro, Scheinin, &

Panula, 2003; Bortolotto et al., 2014b). The time spent in the superior zone is considered a measure of anxiety in zebrafish, with more time in the upper zone indicating less anxiety. In our study, the animals spent less time in the superior zone on day 7, as compared to the control group. Borttoloto and colleagues (2014) did not observe any change in the time spent in the superior zone of aquarium for animals treated with paraquat (Bortolotto et al., 2014b). The effects seen in our work are likely not related to basal locomotor impairment, since the total distanced traveled and the velocity were not affected. This lack of locomotor impairment was also found in a similar model with an intra-diencephalic injection of 6-OHDA in adult zebrafish (Vijayanathan et al., 2017).

Compensatory mechanism could explain the increase in D_2 receptor expression in 6-OHDA-treated animals on day 3. These results are in accordance with other studies that investigate the temporal effect of 6-OHDA injection in the brain. Genfen and colleagues (1990) showed that after the 6-OHDA injection in rat striatum, the D_2 expression increased as a compensation for the decrease in dopaminergic neurotransmission (Gerfen et al., 1990). This increase in D_2 expression could be reversed with a D_2 agonist. Other studies with zebrafish and models of PD have already confirmed such a temporary decrease in dopamine and full recovery 30 days after the induction of the model (Anichtchik et al., 2003; Vijayanathan et al., 2017).

While some locomotor parameters and D_2 receptor expression were altered in our study, the brain dopamine levels were not affected by the injection of 6-OHDA. Maybe the fact that we evaluated the dopamine contents of the whole brain could have masked local effects of 6-OHDA on dopamine levels in the right telencephalon region.

As a result of gene duplication of the teleost group, zebrafish have two copies of the A_{2A} receptor (Boehmler et al., 2009). Here, we showed that the A_{2A1} receptor showed a tendency towards increased expression on day 3, followed by a significant decrease to baseline levels on day 28. The similarity in the temporal profile of A_{2A1} and D_2 expression suggests a possible interaction between these two receptors in zebrafish. The importance of the A_{2A} receptor in PD has already been reported and it is known that patients with PD have an increased expression of A_{2A} in putamen (Varani et al., 2010). However, clinical trials with A_{2A} antagonists like preladenant have been suspended because of lack of response to the drug (Hauser et al., 2011, 2015). Yet, Istradefyline, an A_{2A} antagonist, has recently been approved by United States and is under review in Europe. The drug has already been used in Japan as an adjuvant in

the treatment with cardiodopa/levodopa since 2013 (EMA, 2020; FDA, 2019). For this reason, better understanding of the role of the purinergic system in Parkinson's disease is important. Studies in the zebrafish model described in this manuscript could facilitate this research. Animal models are important tools to help increase our understanding of the basic mechanism of PD and contribute to improve current treatment management. Further studies involving pharmacological and genetic manipulation of adult zebrafish could contribute to the investigation of relationship between purinergic and dopaminergic neurotransmission.

Conclusion

Zebrafish present refined locomotor impairment after intra-encephalic injection of 6-OHDA without detectable changes in dopamine levels. The exposure of adult zebrafish to intraencephalic injection of 6-OHDA, even in a dose that produces only mild locomotor effects, affected dopaminergic and adenosinergic receptor expression. These results may help to clarify the early events before neurodegeneration take place in this model.

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References

Anichtchik, O. V., Kaslin, J., Peitsaro, N., Scheinin, M., & Panula, P. (2003). Neurochemical and behavioural changes in zebrafish Danio rerio after systemic administration of 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Journal of Neurochemistry*, *88*(2), 443–453. https://doi.org/10.1111/j.1471-4159.2004.02190.x

Beggiato, S., Antonelli, T., Tomasini, M., Borelli, A., Agnati, L., Tanganelli, S., … Ferraro, L. (2014). Adenosine A2A-D2 Receptor-Receptor Interactions in Putative Heteromers in the Regulation of the Striato-Pallidal GABA Pathway: Possible Relevance for Parkinson's Disease and its Treatment. *Current Protein & Peptide Science*, *15*(7), 673–680. https://doi.org/10.2174/1389203715666140901103205

Blazina, A. R., Vianna, M. R., & Lara, D. R. (2013). The spinning task: A new protocol to easily assess motor coordination and resistance in zebrafish. *Zebrafish*, *10*(4), 480–485. https://doi.org/10.1089/zeb.2012.0860

Boehmier, W., Obrecht-Pflumio, S., Canfield, V., Thisse, C., Thisse, B., & Levenson, R. (2004). Evolution and expression of D2 and D3 dopamine receptor genes in zebrafish. *Developmental Dynamics*, *230*(3), 481–493. https://doi.org/10.1002/dvdy.20075

Boehmler, W., Petko, J., Woll, M., Frey, C., Thisse, B., Thisse, C., … Levenson, R. (2009). Identification of zebrafish A2 adenosine receptors and expression in developing embryos. *Gene Expression Patterns*, *9*(3), 144–151. https://doi.org/10.1016/j.gep.2008.11.006

Bortolotto, J. W., Cognato, G. P., Christoff, R. R., Roesler, L. N., Leite, C. E., Kist, L. W., … Bonan, C. D. (2014a). Long-Term Exposure to Paraquat Alters Behavioral Parameters and Dopamine Levels in Adult Zebrafish (*Danio Rerio*). *Zebrafish*, *11*(2), 142–153. https://doi.org/10.1089/zeb.2013.0923

Bortolotto, J. W., Cognato, G. P., Christoff, R. R., Roesler, L. N., Leite, C. E., Kist, L. W., … Bonan, C. D. (2014b). Long-term exposure to paraquat alters behavioral parameters and dopamine levels in adult zebrafish (Danio Rerio). *Zebrafish*, *11*(2), 142–153. https://doi.org/10.1089/zeb.2013.0923

Bradford, M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1006/abio.1976.9999

Caldwell, L. J., Davies, N. O., Cavone, L., Mysiak, K. S., Semenova, S. A., Panula, P., … Becker, T. (2019). Regeneration of dopaminergic neurons in adult zebrafish depends on immune system activation and differs for distinct populations. *Journal of Neuroscience*, *39*(24), 4694–4713. https://doi.org/10.1523/JNEUROSCI.2706-18.2019

Capiotti, K. M., Siebel, A. M., Kist, L. W., Bogo, M. R., Bonan, C. D., & Da Silva, R. S. (2016). Hyperglycemia alters E-NTPDases, ecto-5′-nucleotidase, and ectosolic and cytosolic adenosine deaminase activities and expression from encephala of adult zebrafish (Danio rerio). *Purinergic Signalling*, *12*(2), 211–220. https://doi.org/10.1007/s11302-015-9494-z

Costa, B., Ferreira, S., Póvoa, V., Cardoso, M. J., Vieira, S., Stroom, J., … Fior, R. (2020). Developments in zebrafish avatars as radiotherapy sensitivity reporters — towards personalized medicine. *EBioMedicine*, *51*. https://doi.org/10.1016/j.ebiom.2019.11.039

Cronin, A., & Grealy, M. (2017). Neuroprotective and Neuro-restorative Effects of Minocycline and Rasagiline in a Zebrafish 6-Hydroxydopamine Model of Parkinson's Disease. *Neuroscience*, *367*, 34–46. https://doi.org/10.1016/j.neuroscience.2017.10.018

EMA. (2020). European Medicines Agency. In *Definitions*. https://doi.org/10.32388/yokp53

FDA. (2019). FDA approves new add-on drug to treat off episodes in adults with Parkinson's disease. *Case Medical Research*. https://doi.org/10.31525/cmr-1b44443

Fearnley, J. M., & Lees, A. J. (1991). Ageing and parkinson's disease: Substantia nigra regional selectivity. *Brain*, *114*(5), 2283–2301. https://doi.org/10.1093/brain/114.5.2283

Ferré, S., & Ciruela, F. (2019). Functional and Neuroprotective Role of Striatal Adenosine A 2A Receptor Heterotetramers . *Journal of Caffeine and Adenosine Research*, *9*(3), 89–97. https://doi.org/10.1089/caff.2019.0008

Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z., Chase, T. N., Monsma, F. J., & Sibley,

D. R. (1990). D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science*, *250*(4986), 1429–1432. https://doi.org/10.1126/science.2147780

Gerlai, R., Lahav, M., Guo, S., & Rosenthal, A. (2000). Drinks like a fish: Zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. *Pharmacology Biochemistry and Behavior*, *67*(4), 773–782. https://doi.org/10.1016/S0091-3057(00)00422-6

Hauser, R. A., Cantillon, M., Pourcher, E., Micheli, F., Mok, V., Onofrj, M., … Wolski, K. (2011). Preladenant in patients with Parkinson's disease and motor fluctuations: A phase 2, double-blind, randomised trial. *The Lancet Neurology*, *10*(3), 221–229. https://doi.org/10.1016/S1474-4422(11)70012-6

Hauser, R. A., Stocchi, F., Rascol, O., Huyck, S. B., Capece, R., Ho, T. W., … Hewitt, D. (2015). Preladenant as an adjunctive therapy with levodopa in Parkinson disease: Two randomized clinical trials and lessons learned. *JAMA Neurology*, *72*(12), 1491–1500. https://doi.org/10.1001/jamaneurol.2015.2268

Hernandez-Baltazar, D., Zavala-Flores, L. M., & Villanueva-Olivo, A. (2017). The 6-hydroxydopamine model and parkinsonian pathophysiology: Novel findings in an older model. *Neurología (English Edition)*, *32*(8), 533–539. https://doi.org/10.1016/j.nrleng.2015.06.019

Hillefors-Berglund, M., Hedlund, P. B., & von Euler, G. (1995). Effects of CGS 21680 in vivo on dopamine D2 agonist binding in the rat brain. *Brain Research*, *690*(1), 34–40. https://doi.org/10.1016/0006-8993(95)00581-a

Lam, P. Y., & Peterson, R. T. (2019, June 1). Developing zebrafish disease models for in vivo small molecule screens. *Current Opinion in Chemical Biology*, Vol. 50, pp. 37–44. https://doi.org/10.1016/j.cbpa.2019.02.005

Li, J., Li, H., Gu, S., Zi, H.-X., Jiang, L., & Du, J. (2019). One-step Generation of Zebrafish Carrying a Conditional Knockout-Knockin Visible Switch via CRISPR/Cas9-Mediated Intron Targeting. *BioRxiv*, 827352. https://doi.org/10.1101/827352

Li, M., Zhou, F., Xu, T., Song, H., & Lu, B. (2018). Acteoside protects against 6-OHDA-induced dopaminergic neuron damage via Nrf2-ARE signaling pathway. *Food and Chemical Toxicology*, *119*, 6–13. https://doi.org/10.1016/j.fct.2018.06.018

Mccurley, A. T., & Callard, G. V. (2008). *Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment*. https://doi.org/10.1186/1471-2199-9-102

Nery, L. R., Eltz, N. S., Martins, L., Guerim, L. D., Pereira, T. C., Bogo, M. R., & Vianna, M. R. M. (2014). Sustained behavioral effects of lithium exposure during early development in zebrafish: Involvement of the Wnt-β-catenin signaling pathway. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *55*, 101–108. https://doi.org/10.1016/j.pnpbp.2014.04.011

Orger, M. B., & de Polavieja, G. G. (2017). Zebrafish Behavior: Opportunities and Challenges. *Annual Review of Neuroscience*, *40*(1), 125–147. https://doi.org/10.1146/annurev-neuro-071714-033857

Simola, N., Morelli, M., & Carta, A. R. (2007). The 6-hydroxydopamine model of Parkinson's disease. *Neurotoxicity Research*, Vol. 11, pp. 151–167. https://doi.org/10.1007/BF03033565

Soares, J. C., Pereira, T. C. B., Costa, K. M., Maraschin, T., Basso, N. R., & Bogo, M. R. (2017). Developmental neurotoxic effects of graphene oxide exposure in zebrafish larvae (Danio rerio). *Colloids and Surfaces B: Biointerfaces*, *157*, 335–346. https://doi.org/10.1016/j.colsurfb.2017.05.078

Strömberg, I., Popoli, P., Müller, C. E., Ferré, S., & Fuxe, K. (2000). Electrophysiological and behavioural evidence for an antagonistic modulatory role of adenosine A(2A) receptors in dopamine D2 receptor regulation in the rat dopamine-denervated striatum. *European Journal of Neuroscience*, *12*(11), 4033–4037. https://doi.org/10.1046/j.1460-9568.2000.00288.x

Tysnes, O. B., & Storstein, A. (2017, August 1). Epidemiology of Parkinson's disease. *Journal of Neural Transmission*, Vol. 124, pp. 901–905. https://doi.org/10.1007/s00702-017-

1686-y
Varani, K., Vincenzi, F., Tosi, A., Gessi, S., Casetta, I., Granieri, G., … Borea, P. A. (2010). A 2A adenosine receptor overexpression and functionality, as well as TNF-α levels, correlate with motor symptoms in Parkinson's disease . *The FASEB Journal*, *24*(2), 587–598. https://doi.org/10.1096/fj.09-141044
Vijayanathan, Y., Lim, F. T., Lim, S. M., Long, C. M., Tan, M. P., Majeed, A. B. A., & Ramasamy, K. (2017). 6-OHDA-Lesioned Adult Zebrafish as a Useful Parkinson's Disease Model for Dopaminergic Neuroregeneration. *Neurotoxicity Research*, *32*(3), 496–508. https://doi.org/10.1007/s12640-017-9778-x

CHAPTER 4

In vivo PET imaging of zebrafish

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Abstract

The number of studies using imaging tools in zebrafish is growing. Zebrafish are frequently used as a disease model, having a number of attributes, including several physiological characteristics of vertebrates in a small-sized body, which are attractive for basic research. In the present study, we tested the feasibility of *in vivo* PET imaging in healthy adult zebrafish without the necessity to terminate the animal. To optimize imaging conditions, a dose-response curve of the anesthetic tricaine was constructed and subsequently changes in ^{18}F-FDG and ^{18}F-NaF distribution over time in control zebrafish were determined. A concentration of 0.1 g/L of tricaine and a distribution time of 30 min for ^{18}F-FDG and 150 min for ^{18}F-NaF proved to be the optimal conditions. After the standardization, whole-body PET imaging with ^{18}F-FDG could detect significant differences in tracer uptake in the brain area between control and LPS-injected animals. In summary, this study demonstrated that non-invasive PET imaging in zebrafish as a model is feasible.

Key-word: ^{18}F-NaF, ^{18}F-FDG, Inflammation, radiotracers

Introduction

Zebrafish (*Danio rerio*) have been used successfully in several areas of science, due to the rapid external development, optical transparency of the fertilized embryo, tractable genetics, and physiological and behavioral complexity (Phillips & Westerfield, 2014; Schlegel & Gut, 2015). One of the most attractive features of zebrafish as an animal model in research is the low cost of maintenance, representing about ¼ of the costs for rodents (Weintraub, 2017). The zebrafish is already an established model for drug screening (Cully, 2019), but recently zebrafish were also introduced in other fields like in radiation science as an avatar for dose optimization of radiotherapy (Costa et al., 2020).

Positron Emission Tomography (PET) is an imaging tool that allows studying functional processes in the body. With the development of dedicated PET scanners for small animals, which have a higher resolution (1 mm vs 4 mm) than the clinical PET scanners, it became possible to use PET in preclinical research. The main advantage of PET is that it is non-invasive and does not require termination of the animal, hence allowing investigation of e.g. disease progression and treatment efficacy in a single animal over time (Hutchins, Miller, Soon, & Receveur, 2008). Imaging techniques like computed tomography (CT) and magnetic resonance imaging (MRI) have been used in zebrafish. PET imaging has also been performed in zebrafish, but without the recovery of the animals. Quantitative PET tracer uptake was only determined after termination of the zebrafish by means of *ex vivo* biodistribution (Dorsemans et al., 2017) or without the recovery of the animals (Henderson et al., 2019).

The aim of this study was to determine the feasibility of PET imaging in living zebrafish using well-known tracers. In addition, we investigated whether a pathophysiological change can be detected with PET in this model. In this study, the radiotracers ^{18}F-FDG and ^{18}F-NaF were used to visualize glucose metabolism and bone structure, respectively. ^{18}F-FDG is a radiolabeled glucose analogue and a marker of cellular glucose consumption and thus cell viability (Wu, Ma, et al., 2013). ^{18}F-NaF is a well-known radiotracer that is taken up in newly formed bone by osteocytes and is used to investigate bone turnover in several benign and malignant disorders (Segall et al., 2010).

The possibility to study an animal model like zebrafish *in vivo* using PET technology can contribute for the evaluation of therapeutic and diagnostic agents and enables longitudinal studies in disease models. The costs of maintenance and purchase, faster acquisition of results because of the shorter scan time and possibility to scan multiple animals in once, and the availability of genetically modified animals, amongst others, make the zebrafish an interesting animal model to be used in this field. This manuscript is a collaboration between institutes from Brazil, the Netherlands and Belgium, aiming to show that implementation of this new technique at multiple sites is feasible.

Materials and Methods

Animals

Wild-type zebrafish (*Danio rerio*) of 6-12 months were used. They were housed in groups of 20 animals in 15-L thermostated (28 ± 2 °C) tanks (30 cm length: 18 cm depth: 13.5 cm width), kept under constant chemical and mechanical water filtration and aeration (7.20 mg O_2/L). Fish were maintained under a 14:10 h light/dark photoperiod cycle and fed three times a day with commercial flakes (TetraMin®, NC, USA) supplemented with live brine shrimps twice a day. The euthanasia of animals was performed with an overdose of tricaine (0.5 g/L) (Menezes et al., 2014). All protocols were approved by the Institutional Animal Care Committee from Pontifical Catholic University of Rio Grande do Sul (14/00417–CEUA PUCRS) and Ghent University (LA 1400553). The use and maintenance of zebrafish were according to the ‘‘Guide for the Care and Use of Laboratory Animals’’ (National Research Council, 2011).

Immobilization of animals

For the PET image acquisition, the animal needs to be immobile. To assure immobility three different concentrations of buffered (pH=7) tricaine (MS-222) (0.1, 0.12 and 0.15 g/L) were used (n=8 animals per dose). The immobilization latency time (seconds), duration of immobilization (seconds) and the time for recovery (seconds) were determined. The animals were transferred to a recipient

tank with anesthetic dissolved in water. The animals were considered immobilized when they were resting on the bottom of the aquarium. The duration of immobilization was considered the time between the beginning of immobilization and the stop of gill movements, considering 10 minutes as a cut-off time. The animals were placed in another tank with water without anesthetic for the recovery. The time for recovery was defined as the period of time for the fish to go from still gills to moving gills and recovery of swimming movements.

Immobilization device

The immobilization device used for PET acquisition of the anesthetized animals consisted of a closed polypropylene transparent tube of 15 mL (Falcon™, Fisher Scientific, Pittsburgh, PA, USA) filled with a tricaine solution (0.1 g/L). A water recirculation system was not used since all animals survived the time (5 min) required for the PET acquisition. A preliminary test showed that recirculation of water affected PET acquisition, as it induced motion of the animal (data not shown). The general protocol to acquire images is depicted in Figure 1. To maintain the animal in the same position some cotton was added in front of and behind the animals, inside the plastic tube.

PET imaging in healthy animals

Anesthetized (tricaine 0.1 g/L) zebrafish (n=3/group) were intraperitoneally injected with 10 µL of ^{18}F-FDG or ^{18}F-NaF (2-3 MBq). To determine the tracer's distribution in the whole body, PET-CT imaging (β- and X-CUBE, MOLECUBES) was performed 30 and 60 min after ^{18}F-FDG injection and 15, 30, 60 and 150 min after ^{18}F-NaF injection. After tracer distribution in awake, freely moving fish, the zebrafish was put in a 15 mL tube filled with tricaine solution and placed in the PET scanner (5 min scan of 3 animals simultaneously). CT images were obtained for anatomical reference during 2 min (Figure 1). After PET acquisition, the zebrafish were allowed to wake up in a tank with regular water for maintenance. All PET data were iteratively reconstructed using the OSEM algorithm (voxel size: 400 µm), using 30 (^{18}F-FDG) or 50 iterations (^{18}F-NaF). PMOD software (version 3.808) was used to determine tracer uptake

(SUV) in different regions. Tissue density was assumed to be 1 g/mL. In addition, relative uptake was calculated by dividing the tracer uptake (kBq/cc) in a specific region of interest by the tracer uptake (kBq/cc) in a region of interest containing the whole fish. The regions of interest were delineated as shown in Figure 2. Regions of interest were drawn for the head, upper torso, bottom torso, tail, heart (FDG only) and fin (NaF only). These regions were defined using an isocontour tool set at 70% of the maximum uptake within a manually drawn square region.

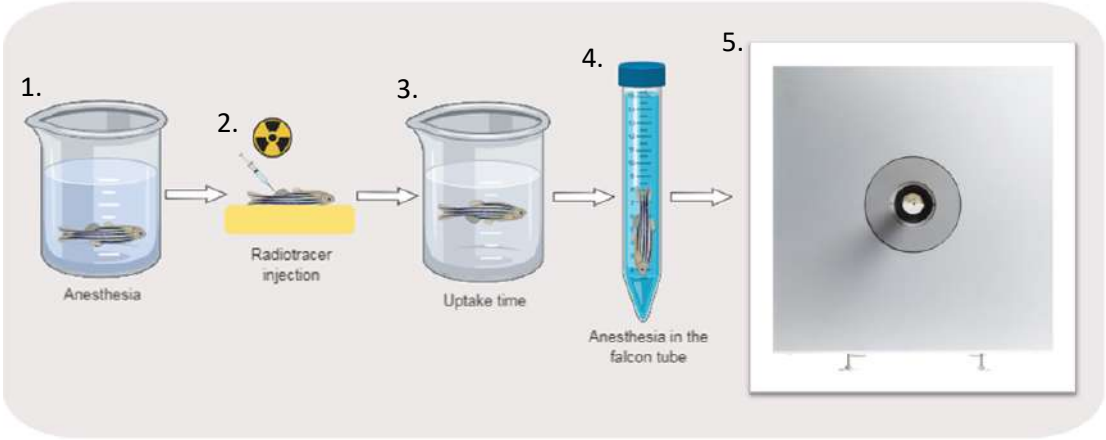

Figure 1: Experimental design for PET image acquisition in zebrafish. 1: Anesthesia of the animal in a tricaine solution (0.1 g/L). 2. Injection of the radiopharmaceutical into the peritoneum. 3. Time to recover from anesthesia and distribution of the radiopharmaceutical. 4. Immobilization of the animal in an immobilization device containing a tricaine solution (0.1 g/L) for image acquisition (the animal needs to lose its posture before the start of the scan). 5. PET scan and CT. After the PET scans the animals were recovered from anesthesia. (Made in ©BioRender - biorender.com)

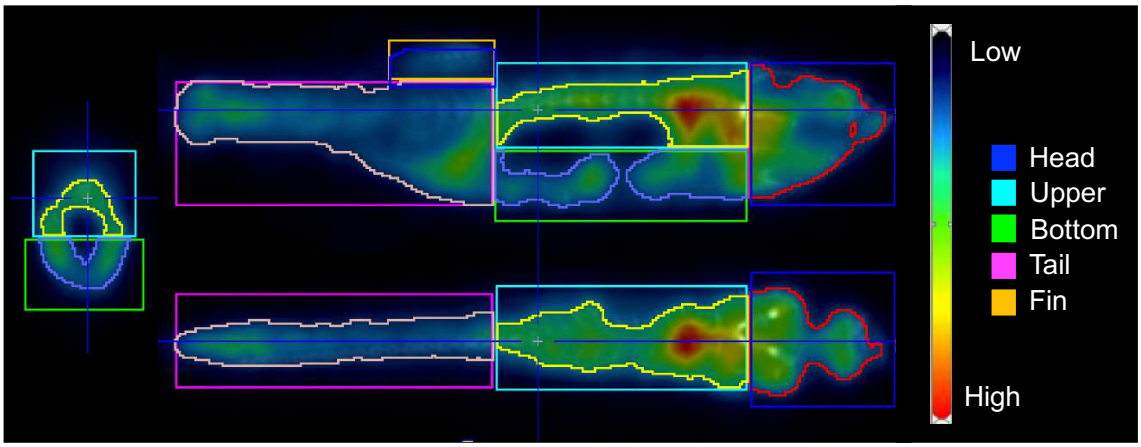

Figure 2: Fused PET-CT images, showing the delineation of the regions of interest for different parts of the animal that were applied for image analysis.

Inflammation model

To investigate if PET is sensitive enough to detect inflamed regions in zebrafish, inflammation was induced by injection of lipopolysaccharide (LPS) as described by Gonçalves et al. (2012). Before intraperitoneal injection of LPS, the animals were anesthetized in a 0.1 g/L tricaine solution until they lost their posture. The fish (n= 5) were intraperitoneally injected with 10 µL of a LPS solution in saline at the dose of 20 µg/g of body weight and placed in a tank with 5 animals per liter water. Control (CTRL, n=5) animals received 10 µL of saline. The animals were injected with ^{18}F-FDG 90 min after LPS injection (20µg/g). After 30 minutes of ^{18}F-FDG distribution in conscious animals, the zebrafish were anesthetized and scanned in a PET camera (Triumph® II microPET LabPET-4®, TriFoil Imaging) (Figure 2). A 5-min static emission scan was performed. All data were reconstructed using a 3D ordered subsets expectation-maximization (MLEM-3D) algorithm with 20 iterations and no attenuation correction. PET data quantification was performed using PMOD version 3.808 (PMOD software/PFUS). Volumes of interest (VOIs) were drawn for the head, upper torso, lower torso and tail region, as described above. ^{18}F-FDG uptake was corrected for injected dose and body weight and expressed as standardized uptake value (SUV) and as relative uptake, as described above.

Statistical analysis

All continuous data are expressed as the mean ± 95% confidence interval (95% CI). Statistical analysis was performed with GraphPad Prism 8. The effect of the anesthetic and differences in tracer uptake over time were analyzed by one or two-way ANOVA and corrected for multiple comparisons by a Tukey's posthoc test. Tracer uptake in inflamed animals was analyzed with an independent samples *T Test*. Differences between groups were considered statistically significant when $p<0.05$.

Results

Anesthetic dose–response curve

The use of anesthetic is important to maintain the animals immobile during the PET scans. To ensure that the animals could recover from anesthesia, different concentrations of anesthetic were tested. The tricaine dose of 0.1 g/L led to a somewhat longer latency time for immobilization and a shorter recovery time than the 0.12 g/L dose. Both doses did not affect gill movement. At the highest dose of 0.15 g/L, it took less time for the animals to immobilize (p=0.0056 compared to 0.1 g/L), but their gill movement stopped quickly (time of immobilization), increasing the chance of death during the experiment (Figure 3). Therefore, the 0.1 g/L dose was selected for the PET experiments.

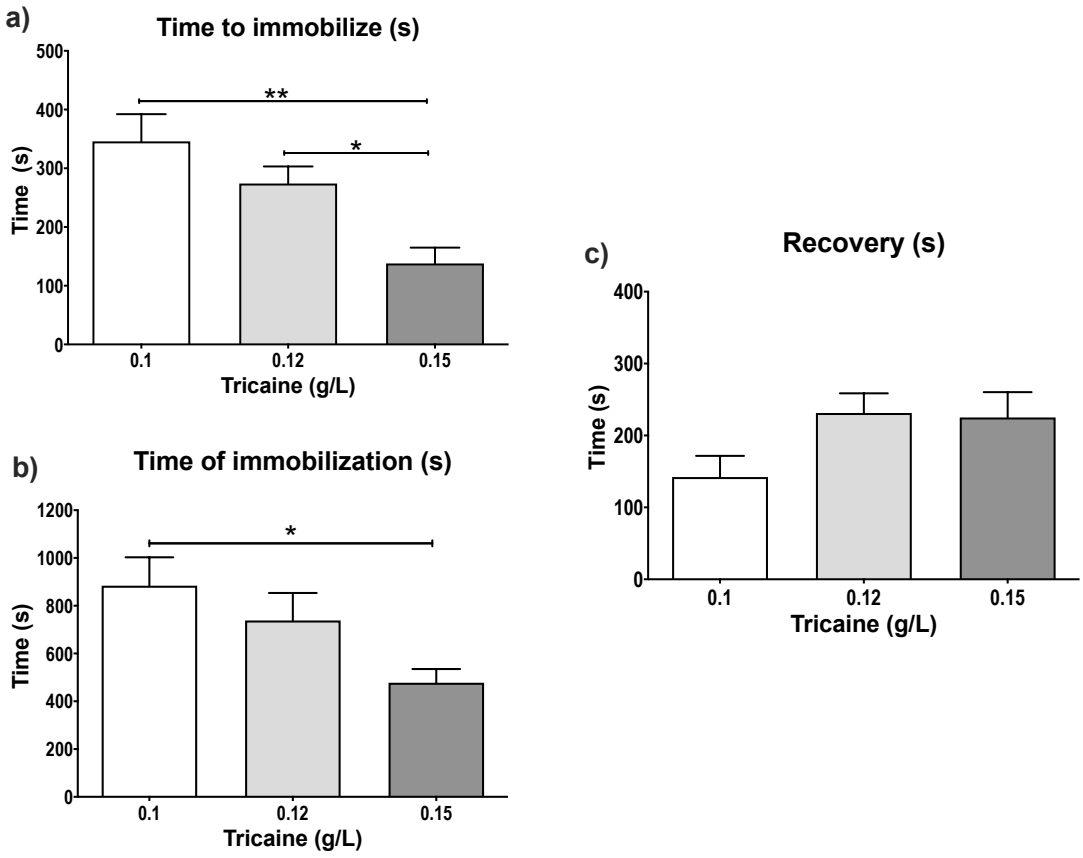

Figure 3: Dose-dependency of tricaine on: a) latency to immobilization; b) time of immobilization; and c) recovery time of adult zebrafish after immobilization. The time is expressed in seconds (s). Data were analyzed by One-way ANOVA.

p<0.05 was considered as statistically significant (n=8). The asterisks represent a significant difference from the 0.1 g/L concentration (**p*<0.05. **p<0.01).

^{18}F-FDG PET in healthy zebrafish

In a first set of pilot experiments, the distribution of ^{18}F-FDG after 3 different uptake times (1, 10, and 20 minutes) was evaluated in 1 animal for each time point. At 20 minutes after the injection, the tracer uptake was already visualized in the brain (data not shown). Distribution times of 30 and 60 minutes after the tracer injection were chosen to further analyze ^{18}F-FDG uptake in various parts of the zebrafish (3 animals per time point).

As can be seen in Figure 3, the highest uptake is observed in the head, heart and the end of the intestine (arrows in the figure) and lowest uptake is found in the tail. Quantification of various body parts is possible, but the variance in SUV_{mean} and SUV_{max} is high. When using the relative uptake instead, this variance decreases, especially at the 60 min time point (Table 1). A main effect of time on SUV_{max} (p=0.02) was found, but not on SUV_{mean} or relative uptake. Quantification of tracer uptake as the relative uptake showed a main effect for the different body parts (p=0.004), which was not observed for SUV_{mean} or SUV_{max}. Significant differences between upper torso and tail at 30 min (p=0.03) and 60 min (p=0.019) and between lower torso and tail at 30 min (p=0.0064) and 60 min (p=0.0023) were found, as well as between tail and heart (p<0.0001).

Table 1: Quantitative ^{18}F-FDG uptake expressed as SUV_{mean}, SUV_{max} and relative uptake in various part of the zebrafish 30 and 60 min after tracer injection. Results are presented as mean±standard deviation and the coefficient of variance (COV).

MEAN±SD (COV)	**SUV_{MEAN}**		**SUV_{MAX}**		**RELATIVE UPTAKE**	
	30 min	60 min	30 min	60 min	30 min	60 min
HEAD	0.56±0.24 (0.44)	0.57±0.32 (0.56)	1.04±0.43 (0.41)	1.12±0.71 (0.63)	0.8±0.24 (0.3)	0.63±0.043 (0.07)
UPPER TORSO	0.62±0.17 (0.26)	0.67±0.11 (0.16)	1.18±0.47 (0.40)	1.51±0.23 (0.15)	0.93±0.27 (0.29)	0.86±0.32 (0.37)
LOWER TORSO	0.78±0.37 (0.48)	0.93±0.52 (0.56)	1.49±0.68 (0.45)	1.85±0.86 (0.46)	1.07±0.01 (0.005)	1.03±0.06 (0.06)
TAIL	0.26±0.06 (0.22)	0.22±0.12 (0.56)	0.50±0.05 (0.10)	0.56±0.30 (0.53)	0.36±0.22 (0.61)	0.24±0.04 (0.16)
HEART	0.72±0.48 (0.67)	0.69±0.35 (0.50)	1.16±0.84 (0.72)	1.14±0.67 (0.59)	0.90±0.44 (0.49)	0.78±0.04 (0.05)

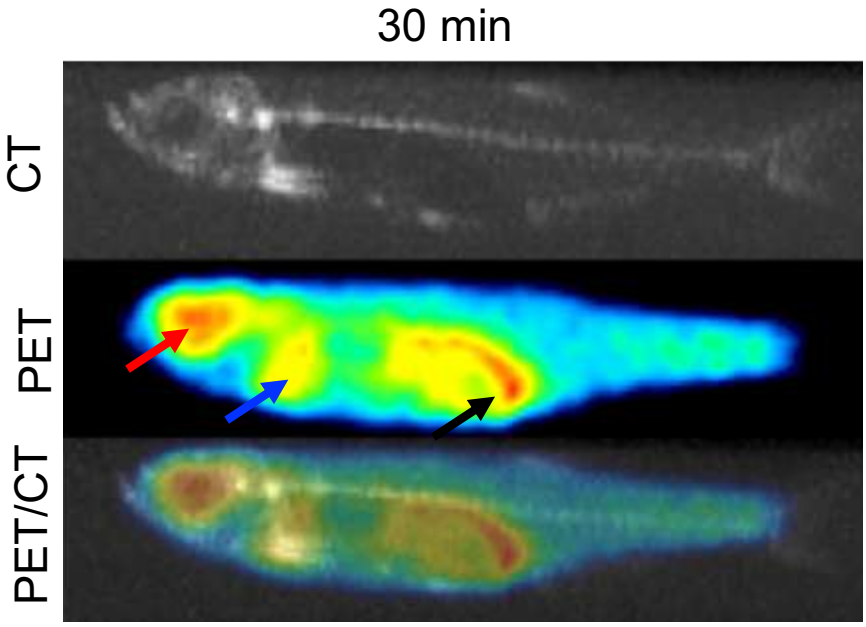

Figure 3: Distribution of ^{18}F-FDG in adult zebrafish at 30 min as measured with a PET/CT system. ^{18}F-FDG was injected 30 minutes before the start of a 5-min PET and 2-min CT acquisition. CT (top), PET (middle) and fused PET-CT images (bottom) acquired 30 min after tracer injection (red arrow indicates the brain; blue arrow the heart and black arrow the end of the intestine).

^{18}F-NaF PET in healthy zebrafish

The distribution of the tracer ^{18}F-NaF was investigated at four time points: 15, 30, 60 and 150 min after tracer injection. PET images of the distribution of the tracer over time are presented in Figure 4. After 15 min the tracer is still mainly localized at the injection site (intraperitoneal), but from 30 min onward tracer uptake can be seen in the bones and is increasing over time. Tracer uptake in the head, upper torso, lower torso, tail and fin was quantified as SUV_{mean}, SUV_{max} or relative uptake. The results show a high variability between animals when the SUV_{mean} and SUV_{max} are used as the quantification methods, whereas using the relative uptake as outcome parameter slightly reduced the variation (Table 2). When ^{18}F-NaF uptake was expressed as SUV_{mean}, a main effect of region ($p<0.0001$) and time ($p=0.002$) was found. The interaction analysis showed a significant difference in tracer uptake in the lower torso between 15 min and 30 min ($p=0.0008$), 60 min ($p=0.0017$) and 150 min $p<0.0001$). At the 15 min time point

a significant difference in tracer uptake between and lower torso and head ($p<0.0001$), upper torso ($p<0.0001$), tail ($p<0.0001$) and fin ($p<0.0001$) was observed, as the tracer was mainly still located at the injection site.

When expressed as SUV_{max}, a main effect of time ($p=0.0016$) and region ($p<0.0001$) on tracer uptake was also found. Again, a significant difference in tracer uptake in the lower torso between the 15 min time point and the 30 min ($p=0.001$), 60 min ($p=0.0038$) and 150 min ($p=0.0002$) time points was observed. Likewise, at the 15 min time point, ^{18}F-NaF uptake in the lower torso was significantly higher than in the head ($p<0.0001$), upper torso ($p<0.0001$), tail ($p<0.0001$) and fin ($p<0.0001$). At 60 min a difference was found between lower torso and fin ($p=0.04$).

When ^{18}F-NaF uptake was expressed as relative uptake, a main effect of time ($p=0.005$) and region ($p<0.0001$) was also found. At 15 min, tracer uptake in the lower torso was significantly higher than in the head ($p=0.0022$), upper torso ($p=0.0028$), tail ($p=0.0005$) and fin ($p=0.0003$). At 30 min, the differences between lower torso and the head ($p=0.02$), upper torso ($p=0.009$), tail ($p=0.002$) and fin ($p=0.0001$) were still statistically significant. At 60 min, ^{18}F-NaF uptake in the lower torso only significantly deferred from uptake in the fin ($p=0.007$), and at 150 min tracer uptake in the lower torso was still significantly higher than uptake in the tail ($p=0.01$) and fin ($p=0.0012$). These data suggest slow migration of intraperitoneally administered ^{18}F-NaF from the injection site.

Table 2: Quantitative ^{18}F-NaF uptake expressed as SUV_{mean}, SUV_{max} and relative uptake in various part of the zebrafish 15, 30, 60 and 150 min after tracer injection. Results are presented as mean±standard deviation and the coefficient of variance (COV).

MEAN±SD (COV)	SUV_{MEAN}				SUV_{MAX}			
	15 min	30 min	60 min	150 min	15 min	30 min	60 min	150 min
HEAD	0.35±0.16 (0.46)	0.34±0.24 (0.70)	0.48±0.44 (0.91)	0.38±0.26 (0.68)	0.71±0.34 (0.48)	0.67±0.48 (0.71)	1.01±0.98 (0.97)	0.76±0.46 (0.60)
UPPER TORSO	0.37±0.11 (0.29)	0.29±0.20 (0.69)	0.52±0.43 (0.82)	0.36±0.25 (0.69)	0.79±0.23 (0.29)	0.64±0.46 (0.72)	1.19±0.98 (0.82)	0.76±0.48 (0.63)
LOWER TORSO	1.68±0.62 (0.37)	0.70±0.22 (0.31)	0.75±0.32 (0.43)	0.56±0.19 (0.34)	3.39±0.90 (0.26)	1.47±0.40 (0.27)	1.68±0.60 (0.36)	1.16±0.36 (0.31)
TAIL	0.16±0.04 (0.25)	0.23±0.11 (0.48)	0.28±0.20 (0.71)	0.23±0.14 (0.61)	0.33±0.07 (0.21)	0.49±0.34 (0.69)	0.66±0.49 (0.74)	0.58±0.41 (0.71)
FIN	0.09±0.08 (0.88)	0.12±0.10 (0.83)	0.19±0.15 (0.79)	0.15±0.09 (0.60)	0.18±0.16 (0.88)	0.23±0.19 (0.82)	0.37±0.29 (0.78)	0.28±0.15 (0.53)

MEAN±SD (COV)	RELATIVE UPTAKE			
	15 min	30 min	60 min	150 min
HEAD	0.21±0.08 (0.38)	0.53±0.37 (0.69)	0.63±0.42 (0.66)	0.71±0.32 (0.45)
UPPER TORSO	0.23±0.03 (0.13)	0.46±0.32 (0.69)	0.74±0.49 (0.66)	0.66±0.29 (0.44)
LOWER TORSO	0.99±0.01 (0.01)	1.15±0.21 (0.18)	0.96±0.10 (0.10)	1.11±0.17 (0.15)
TAIL	0.11±0.07 (0.63)	0.37±0.14 (0.38)	0.42±0.27 (0.64)	0.44±0.17 (0.38)
FIN	0.08±0.09 (1.12)	0.19±0.14 (0.73)	0.26±0.17 (0.65)	0.28±0.09 (0.32)

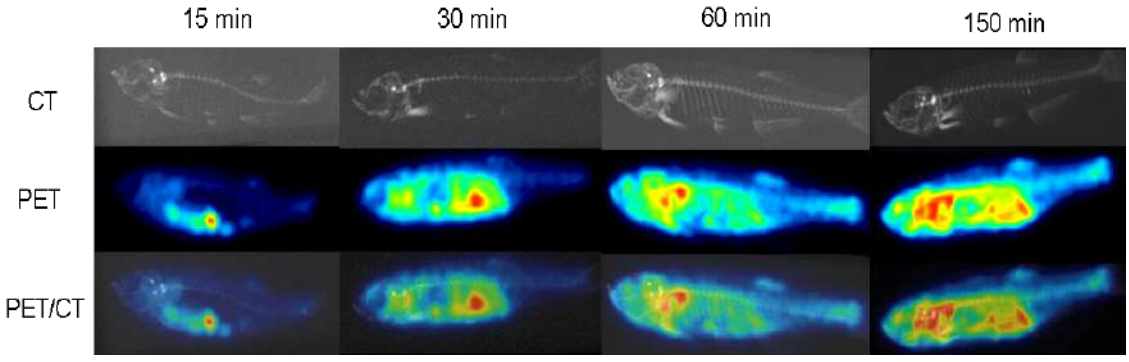

Figure 4: Distribution of ^{18}F-NaF in adult zebrafish over time as measured with PET/CT. ^{18}F-NaF was injected 15, 30, 60 and 150 minutes before the start of a 5-min PET and 2-min CT image acquisition. CT (top), PET (middle) and PET/CT images (bottom) at 15, 30, 60 and 150 min after tracer injection.

^{18}F-FDG PET in LPS-stimulated zebrafish

To investigate if the effect of an intervention could be detected with ^{18}F-FDG PET in zebrafish, zebrafish were injected with LPS to provoke an inflammatory response. When ^{18}F-FDG uptake 30 min after tracer injection was expressed as SUV_{mean}, no significant differences between groups were observed (Figure 5a), likely due to the high within-group variability (Table 3). When tracer uptake was expressed as SUV_{max}, interaction analysis showed a significant increase in radiotracer uptake in the head of LPS-treated animals, as compared to controls (mean difference= -0.42; 95%CI = -0.81 to -0.008; p= 0.04; Figure 5b), but not in any other region. Also, for SUV_{max} the within-group variance was high (Table 3).

When tracer uptake was expressed as relative uptake, interaction analysis also showed a significant increase in radiotracer uptake in the head of LPS-treated animals, as compared to controls (mean difference= -0.31; 95%CI -0.58 to -0.056; p=0.01) (Figure 5c). No significant between-group differences were observed in any other bodypart, despite the within-group variability for the relative uptake being substantially lower than for SUV_{mean} and SUV_{max} (Table 3).

Table 3: Quantitative ^{18}F-FDG uptake expressed as SUV_{mean}, SUV_{max} and relative uptake 30 uptake after tracer injection in control (CTRL) and inflamed animals (LPS). Results are presented as mean±standard deviation and the coefficient of variance (COV).

MEAN±SD (COV)	**SUV_{MEAN}**		**SUV_{MAX}**		**RELATIVE UPTAKE**	
	CTRL	LPS	CTRL	LPS	CTRL	LPS
HEAD	0.37±0.18 (0.48)	0.57±0.26 (0.45)	0.69±0.32 (0.46)	1.10±0.51 (0.46)	0.76±0.22 (0.29)	1.08±0.08 (0.07)
UPPER TORSO	0.39±0.17 (0.43)	0.37±0.19 (0.51)	0.81±0.42 (0.51)	0.79±0.43 (0.54)	0.71±0.07 (0.09)	0.69±0.03 (0.04)
LOWER TORSO	0.58±0.21 (0.36)	0.52±0.32 (0.61)	1.10±0.53 (0.48)	1.01±0.59 (0.58)	1.09±0.04 (0.03)	0.94±0.12 (0.12)
TAIL	0.17±0.06 (0.35)	0.15±0.07 (0.46)	0.34±0.18 (0.53)	0.34±0.16 (0.47)	0.32±0.08 (0.25)	0.28±0.06 (0.21)
HEART	0.48±0.15 (0.31)	0.61±0.36 (0.59)	0.78±0.34 (0.43)	1.01±0.59 (0.58)	0.98±0.30 (0.30)	1.11±0.14 (0.12)

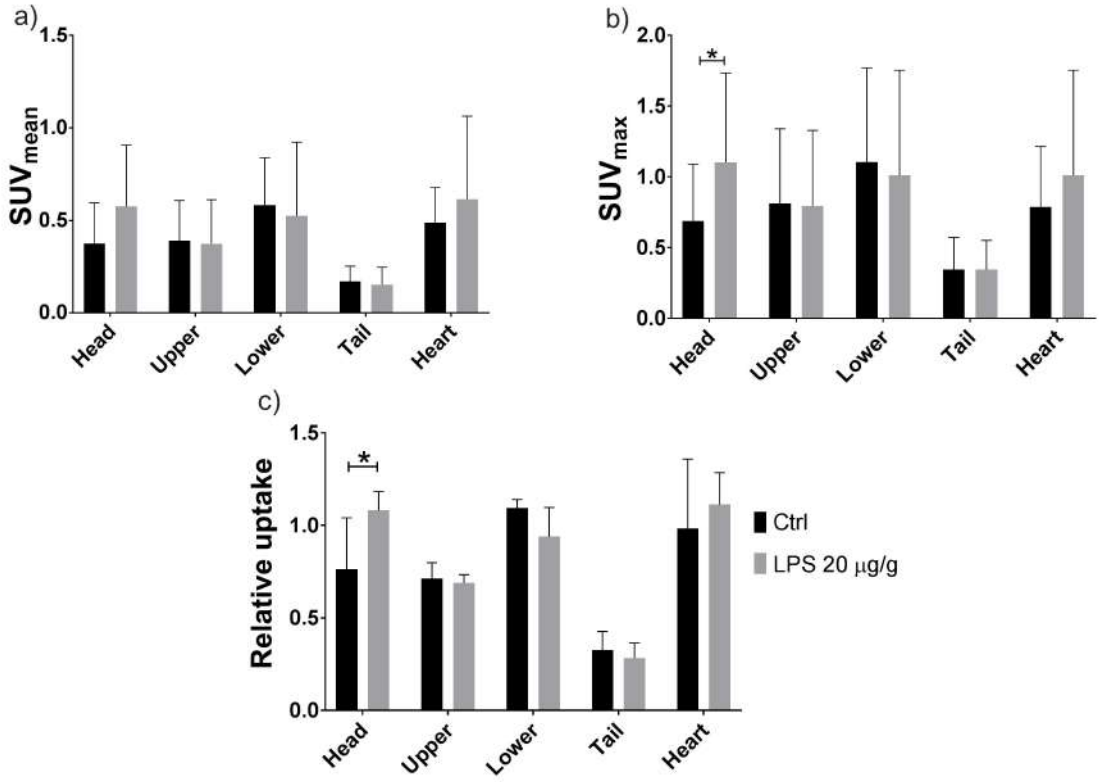

Figure 5: ^{18}F-FDG uptake in different regions (head, upper torso, lower torso and tail), expressed as SUV_{mean} (a), SUV_{max} (b) or relative uptake (c), derived from PET images of control or LPS-treated zebrafish. The bars and error bars represent the mean and 95% confidence intervals, respectively. $p<0.05$ was considered as statistically significant (n=5) and presented with an asterisk in the graphs (*$p<0.05$).

Discussion

In the present study, we demonstrated the feasibility to perform PET imaging in living adult zebrafish with recovery of the animal after the scan. We demonstrated that ^{18}F-FDG PET can detect the inflammatory effect of LPS in this animal model.

For imaging of living zebrafish, it is important that the zebrafish remain immobile during the PET image acquisition. To achieve this a proper immobilization device and anesthesia procedure are required. We selected a Falcon tube (15mL) as immobilization device, because it has adequate dimensions, it is cheap and has a chemical composition (polypropylene) that does not interfere with image acquisition. Kabli et al. (2006) already used a similar

device to keep zebrafish immobile during MRI experiments (Kabli, Alia, Spaink, Verbeek, & De Groot, 2006). However, in that study the authors kept the animals under water recirculation. In this study, the water recirculation made the animals move during the experiment, resulting in a blurring of the image (data not shown). We observed, however, that water circulation is not required if anesthesia is maintained for only a short period of time. The anesthetic used was tricaine at a dose of 0.1 g/L, which is easier to use in zebrafish in comparison to other types of anesthesia, like hypothermia (Collymore, Tolwani, Lieggi, & Rasmussen, 2014). The combination of a closed immobilization device and the optimal anesthetic concentration guaranteed the best conditions to acquire images without affecting the welfare of animals. Is important to keep the image acquisition below 10 min if recovery of the animals is necessary; after this time the gills can stop to move as observed in the tests made with tricaine and the mortality can increase.

Browning et al. (2013) showed that the relative ^{18}F-FDG distribution is not different between fish (zebrafish not included) and human, when considering heart, brain, liver, muscle and kidney (Browning, Wilkes, MacKenzie, Patterson, & Lenox, 2013). Our study demonstrates that the ^{18}F-FDG distribution in zebrafish in a non-pathological state can be visualized by PET. In particular, PET could easily show that the tracer accumulates in the brain, proving that it can cross the blood brain barrier, as observed in humans (Hasselbalch et al., 1996). This demonstrates that it should be possible to test the brain penetration of other PET tracers in zebrafish as well. Another organ in zebrafish that was readily visualized by ^{18}F-FDG PET is the heart, suggesting that cardiac research is feasible as well.

Zebrafish is a teleost and thus has a bone structure. Therefore, the tracer ^{18}F-NaF was chosen to explore the feasibility of bone imaging with PET in zebrafish. In contrast to humans, ^{18}F-NaF was still mostly localized in the abdominal region (site of injection) 15 min after injection. After 30 min, the ^{18}F-NaF uptake in the bones in the head region, vertebral column and fin started to increase until clear bone uptake was observed after 150 min. Apparently, bone uptake of ^{18}F-NaF takes longer in zebrafish than in humans. The differences in ^{18}F-NaF distribution time between zebrafish and humans could be due the fact that humans use bones for support, which requires a different bone composition

and more frequent bone renewal than fish (Wootton & Dore, 1986). Another explanation could be that the tracer is injected intravenously in humans and intraperitoneally in zebrafish. With the latter administration route, the tracer enters the circulation much slower.

Our results show that PET imaging in living heathy adult zebrafish is feasible; it is possible to image multiple animals at the same time and imaging can be done with the recovery of the animals (with a scan duration below 10 min). Zebrafish PET imaging limitations are related to the size of the fish and the resolution of the camera (partial volume effects), as well as the tracer injection, which cannot be done intravenously. Another intrinsic limitation of PET is the lack of anatomical information. To better define the uptake in different organs the use of techniques like MRI in combination with PET could provide a better delineation of different organs.

In this manuscript, three quantification methods, SUV_{mean}, SUV_{max} and the relative uptake, were used. For the calculation of the SUV_{mean} and SUV_{max}, the bodyweight and injected dose are used to correct tracer uptake values. Since bodyweight and injected dose measurements may not be very accurate in small animals, this can increase the variability of the results. When using the relative uptake to quantify uptake, the variance was reduced, because the uptake in the whole fish can be acquired from the same PET image as the uptake in the target region. Remarkably, the reduction in variability by using relative uptake is particularly seen in the ^{18}F-FDG PET data, but is not as apparent in results of ^{18}F-NaF PET. To determine if this effect is truly tracer-specific, other tracers should be tested and the experiment should be repeated using a larger sample size.

Several imaging techniques have been used for detection of inflammation in small animals (Pirko et al., 2003). Here, we showed that PET could detect enhanced ^{18}F-FDG uptake in the head of LPS-treated zebrafish (20 µg/g) as compared to the control group. This result shows that LPS administration has consequences for cellular metabolism in the encephalic area, suggesting that intraperitoneal injection of LPS causes an inflammatory response in the brain of zebrafish. Such an inflammatory response using this dose of LPS has also been observed by ex-vivo methods (Gonçalves et al., 2012). ^{18}F-FDG is extensively used in the preclinical and clinical studies of inflammatory diseases (Irmler et al., 2010; Mäki-Petäjä et al., 2012). The increased ^{18}F-FDG uptake in the brain could

result from the activation of immune cells in the brain, or an increase of neural activity in response to the inflammatory trigger (Jeong, Yoon, & Kang, 2017). Nonetheless, in non-pathological conditions, accumulation of ^{18}F-FDG in the brain is already high, making it difficult to identify the inflammatory response (Wu, Li, Niu, & Chen, 2013). For this reason, other tracers like ^{11}C-PBR28 and ^{11}C-PK11195 could be used to more specifically detect neuroinflammation (Werry et al., 2019). Furthermore, the detection of inflammation in the brain, and beyond, could be improved by adjustment of the dose and time of LPS exposure, which could lead to a stronger inflammatory response, simplifying the differentiation from basal conditions. Also, the use of specific radiotracers that target immune cells could be more effective. Since the use of PET in zebrafish is new, however, more studies need to be done to determine the feasibility of PET with other radiotracers in this species.

Since zebrafish offer a number of well-characterized disease models, this protocol can also be used to studied other types of pathologies like cancer, Parkinson's disease, or Alzheimer's disease, just by changing the radiotracer and the model of disease (Idilli, Precazzini, Mione, & Anelli, 2017; Meshalkina, Kysil, Warnick, Demin, & Kalueff, 2017). This approach could also be very useful in the development of new radiopharmaceuticals, for example to assess blood-brain barrier crossing, for the screening of potential drug treatments and the validation of targets.

Conclusion

This study demonstrates for the first time that *in vivo* PET imaging in living zebrafish is feasible and that PET could detect the effect of an intervention in this species. However, more studies are needed to determine the scope and limitations of this technique.

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References

Browning, Z. S., Wilkes, A. A., MacKenzie, D. S., Patterson, R. M., & Lenox, M. W. (2013). Using PET/CT imaging to characterize 18 F-fluorodeoxyglucose utilization in fish. *Journal of Fish Diseases*, *36*(11), n/a-n/a. https://doi.org/10.1111/jfd.12081

Collymore, C., Tolwani, A., Lieggi, C., & Rasmussen, S. (2014). Efficacy and safety of 5 anesthetics in adult zebrafish (Danio rerio). *Journal of the American Association for Laboratory Animal Science*, *53*(2), 198–203.

Costa, B., Ferreira, S., Póvoa, V., Cardoso, M. J., Vieira, S., Stroom, J., … Fior, R. (2020). Developments in zebrafish avatars as radiotherapy sensitivity reporters — towards personalized medicine. *EBioMedicine*, *51*. https://doi.org/10.1016/j.ebiom.2019.11.039

Cully, M. (2019, October 1). Zebrafish earn their drug discovery stripes. *Nature Reviews. Drug Discovery*, Vol. 18, pp. 811–813. https://doi.org/10.1038/d41573-019-00165-x

Dorsemans, A. C., Lefebvre d'hellencourt, C., Ait-Arsa, I., Jestin, E., Meilhac, O., & Diotel, N. (2017). Acute and chronic models of hyperglycemia in zebrafish: A method to assess the impact of hyperglycemia on neurogenesis and the biodistribution of radiolabeled molecules. *Journal of Visualized Experiments*, *2017*(124), 4–11. https://doi.org/10.3791/55203

Gonçalves, A. F., Páscoa, I., Neves, J. V., Coimbra, J., Vijayan, M. M., Rodrigues, P., & Wilson, J. M. (2012). The inhibitory effect of environmental ammonia on Danio rerio LPS induced acute phase response. *Developmental and Comparative Immunology*, *36*(2), 27–288. https://doi.org/10.1016/j.dci.2011.04.008

Hasselbalch, S. G., Knudsen, G. M., Holm, S., Hageman, L. P., Capaldo, B., & Paulson, O. B. (1996). Transport of D-glucose and 2-fluorodeoxyglucose across the blood-brain barrier in humans. *Journal of Cerebral Blood Flow and Metabolism*, *16*(4), 659–666. https://doi.org/10.1097/00004647-199607000-00017

Henderson, F., Johnston, H. R., Badrock, A. P., Jones, E. A., Forster, D., Nagaraju, R. T., … Hurlstone, A. (2019). Enhanced fatty acid scavenging and glycerophospholipid metabolism accompany melanocyte neoplasia progression in Zebrafish. *Cancer Research*, *79*(9), 2136–2151. https://doi.org/10.1158/0008-5472.CAN-18-2409

Hutchins, G. D., Miller, M. A., Soon, V. C., & Receveur, T. (2008). Small animal PET imaging. *ILAR Journal*, Vol. 49, pp. 54–65. https://doi.org/10.1093/ilar.49.1.54

Idilli, A. I., Precazzini, F., Mione, M. C., & Anelli, V. (2017, September 20). Zebrafish in translational cancer research: Insight into leukemia, melanoma, Glioma and endocrine tumor biology. *Genes*, Vol. 8. https://doi.org/10.3390/genes8090236

Irmler, I. M., Opfermann, T., Gebhardt, P., Gajda, M., Bräuer, R., Saluz, H. P., & Kamradt, T. (2010). In vivo molecular imaging of experimental joint inflammation by combined 18F-FDG positron emission tomography and computed tomography. *Arthritis Research and Therapy*, *12*(6), R203. https://doi.org/10.1186/ar3176

Jeong, Y. J., Yoon, H. J., & Kang, D. Y. (2017). Assessment of change in glucose metabolism in white matter of amyloid-positive patients with Alzheimer disease using F-18 FDG PET. *Medicine (United States)*, *96*(48). https://doi.org/10.1097/MD.0000000000009042

Kabli, S., Alia, A., Spaink, H. P., Verbeek, F. J., & De Groot, H. J. M. (2006). Magnetic resonance microscopy of the adult zebrafish. *Zebrafish*, *3*(4), 431–439. https://doi.org/10.1089/zeb.2006.3.431

Mäki-Petäjä, K. M., Elkhawad, M., Cheriyan, J., Joshi, F. R., Östör, A. J. K., Hall, F. C., … Wilkinson, I. B. (2012). Anti-tumor necrosis factor-α therapy reduces aortic inflammation and stiffness in patients with rheumatoid arthritis. *Circulation*, *126*(21), 2473–2480. https://doi.org/10.1161/CIRCULATIONAHA.112.120410

Meshalkina, D. A., Kysil, E. V., Warnick, J. E., Demin, K. A., & Kalueff, A. V. (2017,

October 6). Adult zebrafish in CNS disease modeling: A tank that's half-full, not half-empty, and still filling. *Lab Animal*, Vol. 46, pp. 378–387. https://doi.org/10.1038/laban.1345

Phillips, J. B., & Westerfield, M. (2014). Zebrafish models in translational research: Tipping the scales toward advancements in human health. *DMM Disease Models and Mechanisms*, *7*(7), 739–743. https://doi.org/10.1242/dmm.015545

Pirko, I., Ciric, B., Johnson, A. J., Gamez, J., Rodriguez, M., & Macura, S. (2003). Magnetic resonance imaging of immune cells in inflammation of central nervous system. *Croatian Medical Journal*, *44*(4), 463–468. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12950151

Schlegel, A., & Gut, P. (2015). Metabolic insights from zebrafish genetics, physiology, and chemical biology. *Cellular and Molecular Life Sciences*, *72*(12), 2249–2260. https://doi.org/10.1007/s00018-014-1816-8

Segall, G., Delbeke, D., Stabin, M. G., Even-Sapir, E., Fair, J., Sajdak, R., & Smith, G. T. (2010). SNM practice guideline for sodium18F-fluoride PET/CT bone scans 1.0. *Journal of Nuclear Medicine*, Vol. 51, pp. 1813–1820. https://doi.org/10.2967/jnumed.110.082263

Weintraub, A. (2017). All eyes on zebrafish. *Lab Animal*, *46*(8), 323–326. https://doi.org/10.1038/laban.1321

Werry, E. L., Bright, F. M., Piguet, O., Ittner, L. M., Halliday, G. M., Hodges, J. R., … Kassiou, M. (2019, July 1). Recent developments in TSPO PET imaging as a biomarker of neuroinflammation in neurodegenerative disorders. *International Journal of Molecular Sciences*, Vol. 20. https://doi.org/10.3390/ijms20133161

Wootton, R., & Dore, C. (1986). The single-passage extraction of 18F in rabbit bone. *Clinical Physics and Physiological Measurement*, *7*(4), 333. https://doi.org/10.1088/0143-0815/7/4/003

Wu, C., Li, F., Niu, G., & Chen, X. (2013). PET imaging of inflammation biomarkers. *Theranostics*, Vol. 3, pp. 448–466. https://doi.org/10.7150/thno.6592

Wu, C., Ma, G., Li, J., Zheng, K., Dang, Y., Shi, X., … Zhu, Z. (2013, January). In vivo cell tracking via 18F-fluorodeoxyglucose labeling: A review of the preclinical and clinical applications in cell-based diagnosis and therapy. *Clinical Imaging*, Vol. 37, pp. 28–36. https://doi.org/10.1016/j.clinimag.2012.02.023

CHAPTER 5

The effect of a cafeteria diet on D_2 receptor availability: a PET imaging study in rats.

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Abstract

Dopamine D_2 receptors are involved in the reward system. Consumption of a diet high in fat and sugar could lead to a decrease in D_2 receptor expression and reduced dopamine turnover. The compulsive eating behavior that is often seen in obese patients might therefore be related to a decreased activation of the reward system or a low number of D_2 receptors. The main objective of this study was to investigate if consumption of a palatable, so-called cafeteria diet would lead to a decreased availability of D_2 receptors in rats. **Methods:** Male Wistar rats were divided in two groups: control diet (chow pellet, 14% fat) or cafeteria diet (high-fat pellet, 45% fat; chocolate, cheese, sugar water). Food consumption and bodyweight were measured daily. Each animal underwent three dynamic [^{11}C]-raclopride PET scans to assess the dopamine D_2 receptor availability: at baseline, after 4 weeks of diet and after consuming palatable condensed sweetened milk expected to lead to dopamine release (challenge). Binding of [^{11}C]-raclopride was assessed using a reference tissue model (SRTM) and expressed as non-displaceable binding potential (BP_{nd}). **Results:** The body weight gain and caloric intake were significantly higher ($p<0.05$) in the cafeteria diet group than in the control diet group. At baseline no significant difference in [^{11}C]-raclopride uptake (BP_{nd}) in the striatum was found between the cafeteria diet and the control group ($p=0.54$). After 4-weeks of the experimental diet, the cafeteria diet group exhibited significantly lower [^{11}C]-raclopride binding in the striatum, when compared with the control group ($p=0.018$, -14.3%). The challenge with highly palatable condensed sweetened milk did not affect striatal tracer uptake. **Conclusion:** Our study demonstrates that the dopamine D_2 receptor availability is affected by consumption of a high caloric diet, suggesting a role of the receptor in obesity. The challenge with highly palatable condensed milk did not affect the dopamine D_2 receptor availability in rats on either diet, so that we cannot conclude if dopamine release by palatable foot is blunted after a high caloric diet.

Introduction

The prevalence of obesity worldwide tripled since 1975, with 40% of the world population being overweight and 13% of this number being obese (WHO, 2016). Being overweight or obese induces a higher risk of developing other health problems, such as cardiovascular disease, musculoskeletal disorders and some cancers (WHO, 2016). Obesity is generally caused by excess energy intake and physical inactivity. However, it has also been suggested that addictive behavior plays a role, as there is an overlap between the neurobiological systems that are involved in both drug addiction and the development of obesity (Barry, Clarke, & Petry, 2009), in particular the reward system. (Kenny, 2011), Activation of the reward system can be considered a neurobiological response to events that stimulate consuming behavior by inducing positive emotions and hedonic feelings (White, 1989).

Dopamine is the main neurotransmitter involved in the reward system. Food consumption stimulates the release of dopamine and generates a pleasant feeling associated with reward (Arias-Carrián, Stamelou, Murillo-Rodríguez, Menéndez-Gonzlez, & Pöppel, 2010). The regulation of the reward system is important for maintaining control over food ingestion (Volkow, Wang, & Baler, 2011). Two opposing theories that involve the reward system try to explain the development of obesity: hypersensitivity and hyposensitivity (C. Davis & Fox, 2008). The theory of hypersensitivity assumes that overeating is caused by an increased sensitivity to be rewarded by food as it is experienced as extremely pleasant. The availability of palatable food cannot be resisted (C. Davis, Strachan, & Berkson, 2004; Dawe & Loxton, 2004). The second theory of hyposensitivity involves the idea that activation of the reward system by food consumption is insufficient so that the individual will overeat to compensate the lack of reward (Comings & Blum, 2000; Wang et al., 2001).

Consumption of a diet high in fat and sugar was suggested to cause a decrease in dopamine D_2 receptor expression and a reduced dopamine turnover in animals models (Carlin, Hill-Smith, Lucki, & Reyes, 2013; J. F. Davis et al., 2008). A decrease in dopamine D_2 receptor density was also observed in Positron Emission Tomography (PET) studies with obese patients; subjects with the lowest D_2 receptor availability had the largest body mass index (Wang et al.,

2001). The decrease in D_2 receptor expression suggests that the compulsive behavior in obese patients or the vulnerability for addictive behavior is related to the decreased activation of the reward system or to a low number of dopamine D_2 receptors (Wang et al., 2001).

Knowledge gaps about the relation between compulsory food consumption and the involvement of dopamine still exist. Therefore, the main objective of this study was to investigate if consumption of a palatable, so-called cafeteria, diet would lead to a decreased sensitivity of the reward system in rats. To this purpose, the dopamine D_2 receptor availability was measured noninvasively by PET using the D_2 receptor antagonist [^{11}C]-raclopride in rats being chronically fed with a cafeteria diet and after receiving an additional challenge with highly palatable food.

Materials and Methods

Animals and Housing

Male Wistar rats (n=16), weighing 280-410 g at the beginning of the study, were obtained from Envigo (Horst, The Netherlands). Before the start of the experiment, the rats were habituated for one week. All rats were individually housed in standard cages. The room was controlled for temperature and humidity (20 ± 2 °C, 60%) and was kept under 12:12 light-dark cycle. The experiments were performed during the light phase. All experiments were approved by the local animal ethical committee (DEC number 5865C).

Study design

The rats were randomly divided in two groups: the control diet group (n=8) and the cafeteria diet group (n=8). Each rat underwent three [^{11}C]-raclopride PET scans for the assessment of in vivo dopamine D_2-receptor availability: 1) at baseline, i.e. one day before the start of diet, 2) after 4 weeks of diet, and 3) after a challenge with highly palatable sweetened condensed milk, 1-5 days after the second PET scan.

Diet

The cafeteria diet consisted of medium fat pellets (Hope Farms RMH-B knaagdier korrel, Arie Block Diervoeding, Woerden, NL; 4.8 kcal/g, 45% fat), supplemented with 15% sucrose solution and highly palatable food (chocolate; 5,4 kcal/g and cheese; 3,62 kcal/g), all *ad libitum*. Chocolate and cheese were changed daily. The rats of the experimental group were habituated to the new diet in three steps: on the first day the medium fat diet pellets were given in addition to the regular chow, on the second day only medium fat pellets were provided and on the third day the rat received the complete cafeteria diet, consisting of medium fat pellets, chocolate, cheese and sucrose solution. The control group was fed standard laboratory chow (Hope Farms, RMH-B knaagdier korrel, Arie Block Diervoeding, Woerden, NL; 3.7 kcal/g, 14% fat), ad libitum. Rats were maintained on the diet for 4 weeks. Food intake (weight of the consumed food) and body weight were monitored daily. Weight gain was calculated by subtracting the body weight at the start of the diet from the consecutive measurements. The caloric intake was determined by weighing the food daily and calculating the sum of all calories consumed using the known calories per gram of each food component.

Challenge

Prior to the last PET scan, the rats were offered highly palatable food, consisting of condensed sweetened milk (Friesche Vlag, Friesland Campina, the Netherlands, 3.21 kcal/g). To get familiarized with the taste, the rats received the sweetened condensed milk twice during the first 3 weeks of diet with a one-week interval. To prevent obstruction of the bottles, the sweetened condensed milk was diluted with water in a ratio of 1:3 (water: sweetened condensed milk). Three hours before the last PET scan, the food was removed. Prior the scan, the rats received sweetened condensed milk for 30 minutes.

PET imaging

Rats were anaesthetized with a mixture of isoflurane and medical air (5% induction and 1-2% maintenance) and positioned in the small animal PET camera

(Focus 220, Siemens Medical Solutions USA, Inc.) in a transaxial position with their heads in the field of view. After positioning the rats, [^{11}C]-Raclopride (21 ± 11 MBq) was injected intravenously. Injected volumes were approximately 0.5-0.9 ml. Simultaneously with the injection of [^{11}C]-Raclopride, an emission scan of 60 minutes was started. The emission scan was preceded by a transmission scan of 10 minutes with a Co-57 point source for corrections of attenuation and scatter by tissue. The PET data were iteratively reconstructed (OSEM2D, 4 subsets, 16 iterations) and separated into 21 frames (6 x 10s, 4 x 30s, 2 x 60s, 1 x 120s, 1 x 180s, 4 x 300s and 3 x 600s) using an attenuation-weighted two-dimensional ordered-subset expectation maximization algorithm. Data was corrected for random coincidences, scatter, radioactive decay and attenuation. Final images had a 256 x 256 x 95 matrix with a pixel width of 0.949 mm and slice thickness of 0.796 mm.

PET analyses

The reconstructed PET images were automatically co-registered to an in-house developed ^{11}C-raclopride brain template using PMOD 3.8 software (PMOD Technologies LLC, Switzerland), which allowed the use of a predefined volume-of-interest (VOI) map and the reporting of results in Paxinos stereotactic coordinates of the rat brain (Garcia et al., 2015). After co-registration, time activity curves (TACs) were generated for the left and right striatum and the cerebellum. The simplified reference tissue model (SRTM), using the cerebellum as the reference region, was used to estimate the non-displaceable binding potential (BP_{ND}) (Wu & Carson, 2002).

Statistical Analyses

Statistical analyses were performed using the program IBM SPSS Statistics® 23. Data were analyzed using the Generalized Estimation Equations model with “group” and “time” as predictive variables. The Independent working correlation matrix was selected according to the quasi-likelihood under the independence model information criterion value. The Wald test was used to report the *p*-values. Differences were considered statistically significant when $p<0.05$.

Results

Body weight and food intake

Pairwise comparison revealed that the body weight gain of the cafeteria diet group was significantly higher ($p<0.05$) than that of the control diet group (Figure 2). At the end of the experiment, the body weight gain of the cafeteria diet group was 19.5% higher than that of the control diet groups. The small dip in body weight gain at the end of the experiment was caused by the PET scans that were performed during these days. The pairwise comparison for the cumulative caloric intake showed that the cafeteria diet group had a significantly higher caloric intake ($p<0.05$) after the introduction of the complete cafeteria diet (i.e. at day 4 of the study) than the control diet group. The caloric intake at the end of the experiment was 9.9% higher in the cafeteria diet group when compared to the control diet group.

For the cafeteria diet group, the total caloric intake represented the calories from the medium fat pellets, chocolate, cheese and sugar water. Most calories were consumed via the medium fat diet (40.1±4.8%), followed by the cheese (23.8±2.2%), chocolate (23.4±2.7%) and sugar water (12.2±2.7%) (Figure 3).

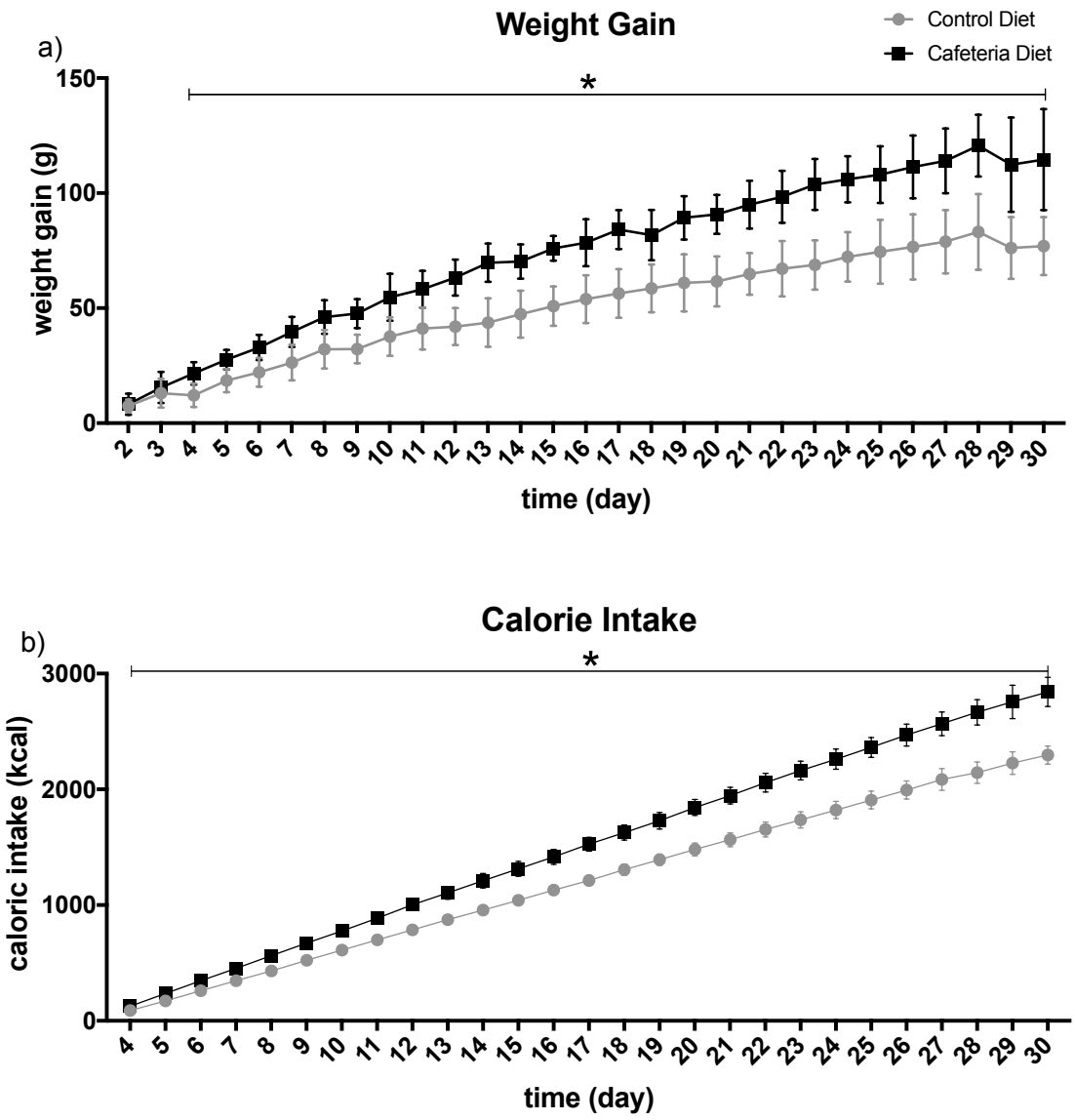

Figure 2: Weight gain (a) and cumulative caloric intake (b) of rats in the cafeteria diet group (n=8) and the control diet group (n=8) during a 4-week period. Statistically significant differences between groups are indicated by: * p< 0.05. The graphics represent the mean +/- 95%CI.

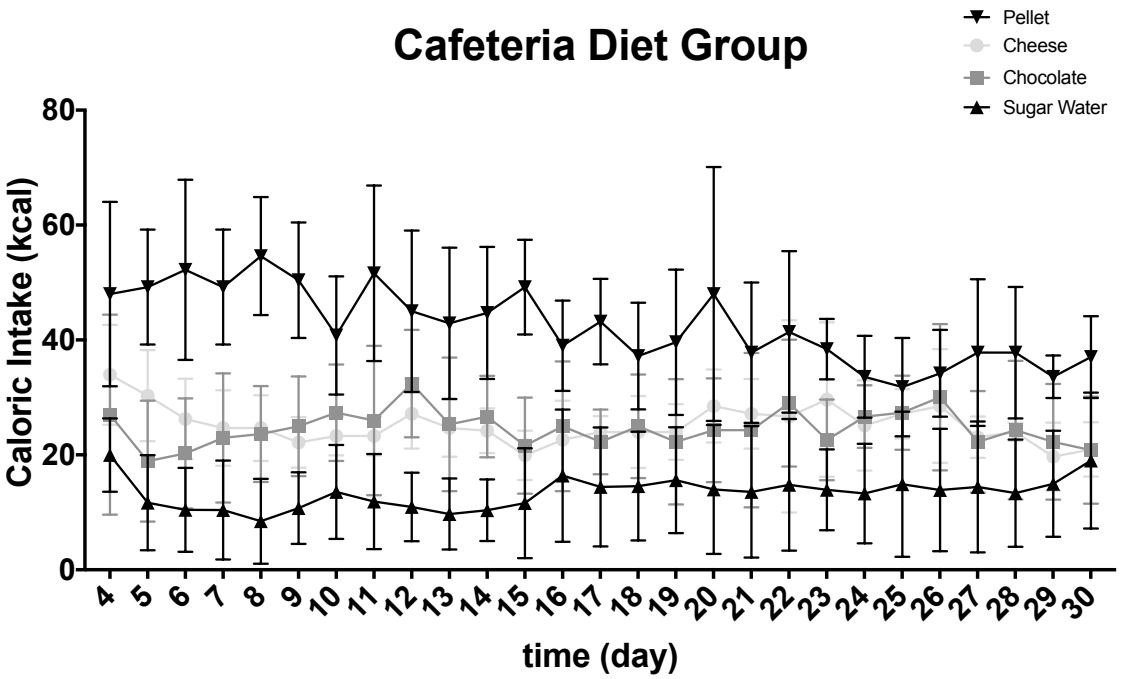

Figure 3: Chocolate, cheese, sugar water and medium fat pellet intake by the cafeteria diet group (in kcal) during a 4-week period. The graphics represent the mean +/- 95%CI.

[^{11}C]-raclopride PET

The analysis of the [^{11}C]-raclopride PET data was performed in two steps. We first determined if there was an effect of the diet on [^{11}C]-raclopride binding by comparing the baseline BP_{nd} with the BP_{nd} after the diet, and then determined the effect of the challenge by comparing the BP_{nd} after the diet with the BP_{nd} after the challenge. Data from one animal after the diet (high fat diet group) was excluded from the analysis, as it was considered to be an outlier by the ROUT (1%) and Grubb's (alpha=0.05) test (GraphPad Prism® 8). Two animals from the last time-point (after challenge, one for each diet group) were excluded, because the scan was made weeks later due to technical problems.

In the first analysis (baseline vs. after the diet), the pairwise comparisons showed no significant difference in [^{11}C]-raclopride BP_{nd} in the striatum between both groups at baseline (p=0.5). After 4-weeks of the experimental diet, the cafeteria diet group had a statistically significantly lower [^{11}C]-raclopride BP_{nd} in the striatum, when compared with the control diet group (mean difference = -

0.156; 95% CI = -0.285 – -0.0262; p=0.018, 14.7%). In addition, a statistically significant decrease in [^{11}C]-raclopride BP_{nd} was found between baseline and after the diet for the cafeteria diet group (mean difference = 0.149; 95% CI = -0.030 – 0.268; p=0.014, -13.8%), while no differences were found in the control diet group.

The second analysis (after diet vs. after challenge) showed a statistically significantly lower striatal BP_{nd} after the challenge in the cafeteria diet group, when compared to the control diet group (mean difference = 0.114; 95% CI = 0.0019 – 0.2256; p=0.046, 10.3%). No statistically significant differences were found in the [^{11}C]-raclopride BP_{nd} between the after diet and after challenge scan, for either the cafeteria diet or the control diet group.

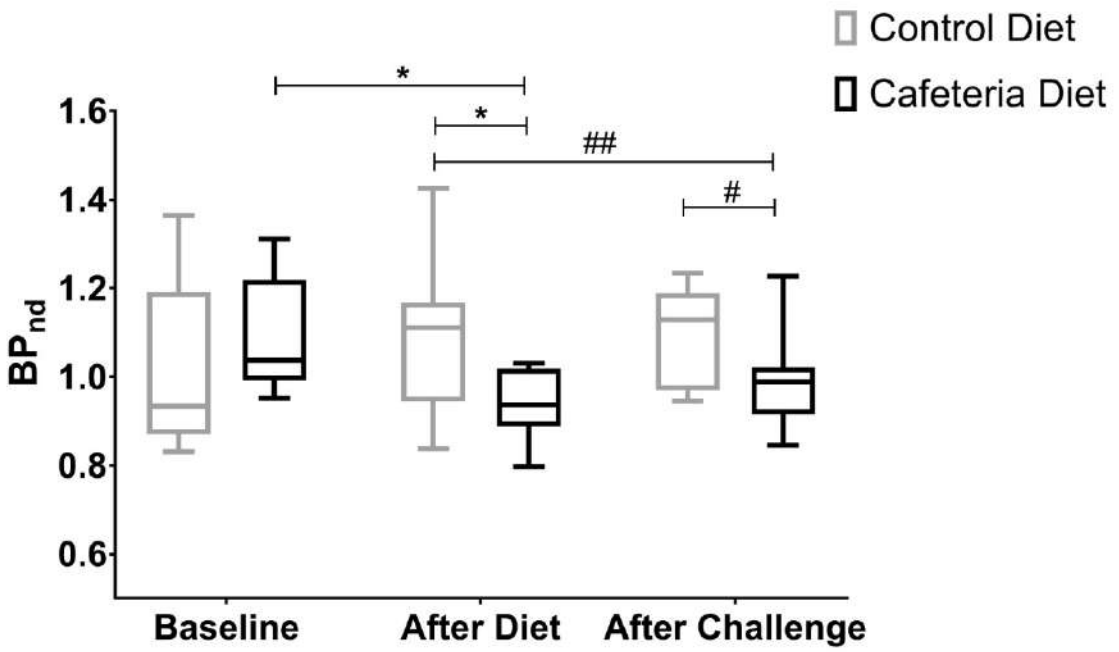

Figure 4: [^{11}C]-Raclopride PET imaging of dopamine D_2 receptor availability in striatum (expressed as BP_{nd}) of Wistar rats at baseline, after 4 weeks on control diet or cafeteria diet and after a subsequent challenge with condensed milk. Box plot and whiskers represents median and min-max values, respectively. Asterisks and hashtag represent the within-group effect *p<0.05, #p<0.05, ##<0.001,

Discussion

This longitudinal study demonstrated an effect of the consumption of cafeteria diet on D_2 receptor availability using [^{11}C]-raclopride PET. We found a decrease in the [^{11}C]-raclopride BP_{nd} in the cafeteria diet group after one month of diet, indicative of decreased D_2 receptor availability. The [^{11}C]-raclopride BP_{nd} was not affected by the challenge with highly palatable food.

The animals fed with the cafeteria diet showed a higher weight gain due to the higher caloric intake of the food. These results are in agreement with a previous study, in which rats were fed a similar diet (Lewis, Singh, & Youssef, 2019). The use of a cafeteria diet that contains a mix of sweet and savory food has been considered the best comparison with the diet in humans, as the animals have the free option to consume the aliments that are available for them (Sampey et al., 2011; Shafat, Murray, & Rumsey, 2009). The cafeteria diet does not offer the rats the control over the intake of macronutrients and micronutrients, as is possible for only high fat or high sugar diets, and is therefore causing a more severe metabolic phenotype (Leigh, Kendig, & Morris, 2019).

In addition to the body weight gain, the consumption of the cafeteria diet led to a decrease in the [^{11}C]-raclopride BP_{nd} in the striatum by 14%, revealing a decreased dopamine D_2 receptor availability. This decreased availability could be due to a reduction in the D_2 receptor density in the striatum or an increase in dopamine release by the presynaptic dopaminergic neurons. These results are in line with other studies using different techniques, like immunohistochemistry, and showed that a high fat diet and a high sugar diet can cause a decrease in the D_2 receptor expression or an increased dopamine release (Carlin et al., 2013; J. F. Davis et al., 2008; Johnson & Kenny, 2010). This effect is also found in [^{11}C]-raclopride PET studies in humans, when comparing obese with non-obese patients (Wang et al., 2001).

It has been previously shown that a diet high in fat and/or sugar can affect the [^{11}C]-raclopride BP_{nd}, but the effect of an additional challenge with high palatable food was only recently studied in humans (Eisenstein et al., 2020). In that study, no differences were found between the fasted and high-calorie food stimulated obese patients. In our study, the decreased [^{11}C]-raclopride BP_{nd} that was observed after the diet was not affected by the challenge with the highly

palatable food in either the control diet or the cafeteria diet group. As the [^{11}C]-raclopride BP_{nd} was not affected in the control diet group, the acute challenge with highly palatable food did apparently not result in a measurable release of dopamine, as this should have led to a reduction in the striatal [^{11}C]-raclopride binding. This could be related to the variability of the data due to the different responses from the animals to the challenge or the diet. In a previous study it was observed that a group of rats fed with a high-fat diet for 10 weeks could be divided in to a group of obesity prone and obesity resistance rats (Levin & Dunn-Meynell, 2000). In our study, however, no correlation (data not show) was found between the bodyweight, weight gain or caloric intake and the [^{11}C]-raclopride BP_{nd} of the 2 last scans (after the diet and after the challenge). The group size used in our study is however too small to make such a division. In future studies, it would be of interest to measure the dopamine release in addition to the [^{11}C]-raclopride BP_{nd} to better understand the processes that occur during the challenge or to change the challenge for something more effective, also the consume of the condensed milk could be measured.

Conclusion

Our study demonstrates that the dopamine D_2 receptor availability is affected by consumption of a high caloric diet, suggesting a role for the D_2 receptor in obesity. The challenge with highly palatable condensed milk did not affect the dopamine D_2 receptor availability in rats on a normal diet, so that we cannot conclude if dopamine release by palatable foot is blunted after a high caloric diet.

Acknowledgments

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References

Arias-Carrián, O., Stamelou, M., Murillo-Rodríguez, E., Menéndez-Gonzlez, M., & Pöppel, E. (2010, October 6). Dopaminergic reward system: A short integrative review. *International Archives of Medicine*, Vol. 3, p. 24. https://doi.org/10.1186/1755-7682-3-24

Barry, D., Clarke, M., & Petry, N. M. (2009, October 23). Obesity and its relationship to addictions: Is overeating a form of Addictive Behavior? *American Journal on Addictions*, Vol. 18, pp. 439–451. https://doi.org/10.3109/10550490903205579

Carlin, J., Hill-Smith, T. E., Lucki, I., & Reyes, T. M. (2013). Reversal of dopamine system dysfunction in response to high-fat diet. *Obesity*, *21*(12), 2513–2521. https://doi.org/10.1002/oby.20374

Comings, D. E., & Blum, K. (2000). Reward deficiency syndrome: Genetic aspects of behavioral disorders. *Progress in Brain Research*, *126*, 325–341. https://doi.org/10.1016/S0079-6123(00)26022-6

Davis, C., & Fox, J. (2008). Sensitivity to reward and body mass index (BMI): Evidence for a non-linear relationship. *Appetite*, *50*(1), 43–49. https://doi.org/10.1016/j.appet.2007.05.007

Davis, C., Strachan, S., & Berkson, M. (2004). Sensitivity to reward: Implications for overeating and overweight. *Appetite*, *42*(2), 131–138. https://doi.org/10.1016/j.appet.2003.07.004

Davis, J. F., Tracy, A. L., Schurdak, J. D., Tschöp, M. H., Lipton, J. W., Clegg, D. J., & Benoit, S. C. (2008). Exposure to Elevated Levels of Dietary Fat Attenuates Psychostimulant Reward and Mesolimbic Dopamine Turnover in the Rat. *Behavioral Neuroscience*, *122*(6), 1257–1263. https://doi.org/10.1037/a0013111

Dawe, S., & Loxton, N. J. (2004, May). The role of impulsivity in the development of substance use and eating disorders. *Neuroscience and Biobehavioral Reviews*, Vol. 28, pp. 343–351. https://doi.org/10.1016/j.neubiorev.2004.03.007

Eisenstein, S. A., Black, K. J., Samara, A., Koller, J. M., Dunn, J. P., Hershey, T., … Smith, G. I. (2020). Striatal Dopamine Responses to Feeding are Altered in People with Obesity. *Obesity*, *28*(4), 765–771. https://doi.org/10.1002/oby.22753

Garcia, D. V., Casteels, C., Schwarz, A. J., Dierckx, R. A. J. O., Koole, M., & Doorduin, J. (2015). A standardized method for the construction of tracer specific PET and SPECT rat brain templates: Validation and implementation of a toolbox. *PLoS ONE*, *10*(3), e0122363. https://doi.org/10.1371/journal.pone.0122363

Johnson, P. M., & Kenny, P. J. (2010). Dopamine D2 receptors in addiction-like reward dysfunction and compulsive eating in obese rats. *Nature Neuroscience*, *13*(5), 635–641. https://doi.org/10.1038/nn.2519

Kenny, P. J. (2011, February 24). Reward Mechanisms in Obesity: New Insights and Future Directions. *Neuron*, Vol. 69, pp. 664–679. https://doi.org/10.1016/j.neuron.2011.02.016

Leigh, S. J., Kendig, M. D., & Morris, M. J. (2019). Palatable western-style cafeteria diet as a reliable method for modeling diet-induced obesity in rodents. *Journal of Visualized Experiments*, *2019*(153). https://doi.org/10.3791/60262

Levin, B. E., & Dunn-Meynell, A. A. (2000). Defense of body weight against chronic caloric restriction in obesity- prone and -resistant rats. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *278*(1 47-1). https://doi.org/10.1152/ajpregu.2000.278.1.r231

Lewis, A. R., Singh, S., & Youssef, F. F. (2019). Cafeteria-diet induced obesity results in impaired cognitive functioning in a rodent model. *Heliyon*, *5*(3). https://doi.org/10.1016/j.heliyon.2019.e01412

Sampey, B. P., Vanhoose, A. M., Winfield, H. M., Freemerman, A. J., Muehlbauer, M. J., Fueger, P. T., … Makowski, L. (2011). Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: Comparison to high-fat

diet. *Obesity*, *19*(6), 1109–1117. https://doi.org/10.1038/oby.2011.18

Shafat, A., Murray, B., & Rumsey, D. (2009). Energy density in cafeteria diet induced hyperphagia in the rat. *Appetite*, *52*(1), 34–38. https://doi.org/10.1016/j.appet.2008.07.004

Volkow, N. D., Wang, G. J., & Baler, R. D. (2011, January). Reward, dopamine and the control of food intake: Implications for obesity. *Trends in Cognitive Sciences*, Vol. 15, pp. 37–46. https://doi.org/10.1016/j.tics.2010.11.001

Wang, G. J., Volkow, N. D., Logan, J., Pappas, N. R., Wong, C. T., Zhu, W., … Fowler, J. S. (2001). Brain dopamine and obesity. *Lancet*, *357*(9253), 354–357. https://doi.org/10.1016/S0140-6736(00)03643-6

WHO. (2016). Global Report on Diabetes. *Isbn*, *978*, 6–86. Retrieved from http://www.who.int/about/licensing/copyright_form/index.html

Wu, Y., & Carson, R. E. (2002). Noise reduction in the simplified reference tissue model for neuroreceptor functional imaging. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *22*(12), 1440–1452. https://doi.org/10.1097/01.WCB.0000033967.83623.34

CHAPTER 6

The influence of a high-fat diet on D_2 receptor availability and behavior in a rat model of Parkinson's disease.

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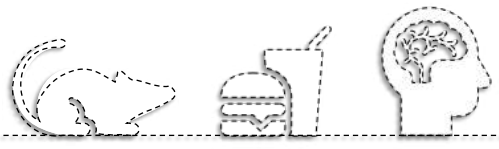


Abstract

Lifestyle can influence the incidence and progression of Parkinson's disease (PD). However, the underlying molecular mechanisms are still unknown. The objective of this study was to evaluate the effect of a high-fat diet (HFD) on the D_2 receptor availability in the brain and gut in a rat model of PD. Nine-weeks old male Wistar rats were fed with either a HFD (60% fat) or a standard diet (SD; 10% fat) for three months. The body weight and food intake were measured twice a week and a glucose tolerance test (GTT) was performed every month. After two months of diet the animals were submitted to stereotactic surgery, injecting a low dose of 6-hydroxydopamine (2x3µg) in the right striatum. Dynamic ^{11}C-raclopride PET scans were performed two days before and one month after the surgery, and the non-displaceble binding potential (BP_{ND}) ratio in the striatum (ipsilateral/contralateral) was measured. Behavior was evaluated with the cylinder, open field, object recognition and Y-maze test. After three months, animals on HFD had a greater weight gain and caloric intake than those on SD. The surgery caused a temporary decrease in bodyweight in both groups. No difference in the GTT between the SD and HFD groups were found. ^{11}C-raclopride PET imaging showed a significantly lower ipsilateral/contralateral BP_{ND} ratio in the striatum of the HFD group postsurgery than in both the SD and the HFD group presurgery ($p<0.05$). No significant difference was found between SD group presurgery and postsurgery. The results of the cylinder test showed a similar pattern as the PET scans (HFD presurgery vs. postsurgery: $p<0.05$), and no statistically significant differences were found in the outcome measures of the other behavioral tests. Postmortem analysis of the gut showed a statistically significant ($p<0.05$) reduction in the ^{11}C-raclopride uptake in the duodenum of animals in the HFD group. Concluding, this study indicates that the HFD exacerbated the effect of the neurotoxin 6-hydroxydopamine with respect to dopamine D_2 receptor availability and asymmetric paw use. Both observations could suggest a detrimental role of a HFD on the onset or progression of PD.

Introduction

Lifestyle choices, such as exercise, consumption of caffeine, smoking and alcoholism, have been suggested to modulate the progression of Parkinson's disease (PD) (Paul et al., 2019). Diet is another important factor that can influence PD development and progression (Mischley et al., 2017). For example, a Western diet (high intake of fat, sugar and red meat) increases the risk to develop PD, whereas a Mediterranean diet (high intake of fibers) decreases the risk (Mischley et al., 2017; Maraki et al., 2019). In addition, diseases associated with an unhealthy diet, like diabetes type 2, have been shown to increase the risk of developing Parkinson by 40% (Hu et al., 2007; Driver et al., 2008; Xu et al., 2011), although other studies have found the opposite (Palacios et al., 2011; Savica et al., 2012). Furthermore, some studies have shown that a pre-diabetic state could be sufficient to cause a more severe PD phenotype with accelerated disease progression and an increased risk of PD dementia (Bosco et al., 2012; Cereda et al., 2012).

A hallmark of PD is the loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNc), leading to a deficit in dopamine signalling in the projection areas, like the striatum (Poewe et al., 2017). Animal studies (post mortem) in a PD model in combination with a high fat diet showed an increase of dopamine depletion in the striatum and related regions (Choi et al., 2005; Morris et al., 2010; Bousquet et al., 2012). The consumption of high caloric food or obesity can also lead to a decrease in the availability of D_2 receptors (D_2R) in the striatum, as was observed in a ^{11}C-raclopride Positron Emission Tomography (PET) study in healthy and obese volunteers (Wang et al., 2001). The physiological changes that are associated with both diet and PD can also have behavioral effects, such as depression-like behavior and impaired learning (Greenwood and Winocur, 1990; Campos et al., 2013; Hassan et al., 2019).

One of the most common and earliest symptoms of PD is constipation (Postuma et al., 2013; Fasano et al., 2015), and it has been suggested that dopaminergic neurons located in the gut may also be affected. In a normal state, enteric D2R support the motility of food throughout the digestive tract (Zhi et al., 2006). It was reported that a decrease in peristaltic movements in a rat model of PD was associated with a reduced D2R expression in the proximal and distal colon (Colucci et al., 2012).

A few studies have tried to combine a model of PD with a model of diabetes type 2 or an prediabetic state in animals (Morris et al., 2010), but to the best of our knowledge, no attempts have been made to assess the long-term effects of this combination in vivo. PET is a non invasive technique used in clinical and pre-clinical research to measure physiological parameters longitudinally. In preclinical research, PET can help to reduce the number of animals required and improve the robustness of the study, because it allows comparison of the same animal before and after an intervention (Myers, 2001; Yao et al., 2012). In this study, ^{11}C-raclopride PET was used to evaluate the D_2R availability. We hypothesized that a high-fat diet will aggravate the features of PD. Therefore, this study aimed to determine if a high-fat diet augments the changes in D2R availability and enhances disease symptoms in the 6-hydroxydopamine (6-OHDA) model of PD in a longitudinal approach.

Material and methods

Animals

Nine-weeks old male Wistar rats, weighing 290-370 g at the beginning of the study, were obtained from Envigo (The Netherlands). After habituation to the animal facility for one week, the animals were housed individually to enable measurement of individual food intake and caloric intake. The housing room was controlled for temperature and humidity (T= 20±2 °C, humidity 60%) and kept under a 12:12 h light-dark cycle (lights on at 8 A.M.; lights off at 8 P.M.). The experiments were performed during the light phase. All experiments were approved by the National Committee on Animal Experiments (CCD license: AVD1050020173069) and the Animal Welfare Body of the University of Groningen (IvD study number 173069-01-002).

Study Design

An overview of the study is displayed in Figure 1. Animals were randomly divided into two groups: one group was fed with a high-fat diet (HFD; 60% fat, 20% protein, 20% carbohydrates, energy density of 5.21 kcal/g, Research Diets, U.S.) and the other group with a standard diet (SD; 10% fat, 20% protein, 70% carbohydrate, energy density of 3.82 kcal/g, Research Diets, U.S.) for three months. The body weight and

food intake were measured twice a week and a glucose tolerance test (GTT) to check for early signs of diabetes was performed every month. After two months of diet, animals were submitted to stereotactic injection of 6-OHDA in the right striatum. Dynamic ^{11}C-raclopride PET scans were performed two days before 6-OHDA injection (after two months of diet), and one month after the 6-OHDA injection. The cylinder test was performed 7 days after 6-OHDA injection and 3 days before the last PET scan. Open field, y-maze, and novel object recognition tests were performed 2 or 3 days before each PET scan. After the last PET scan, animals were terminated and enteric tissue samples were collected.

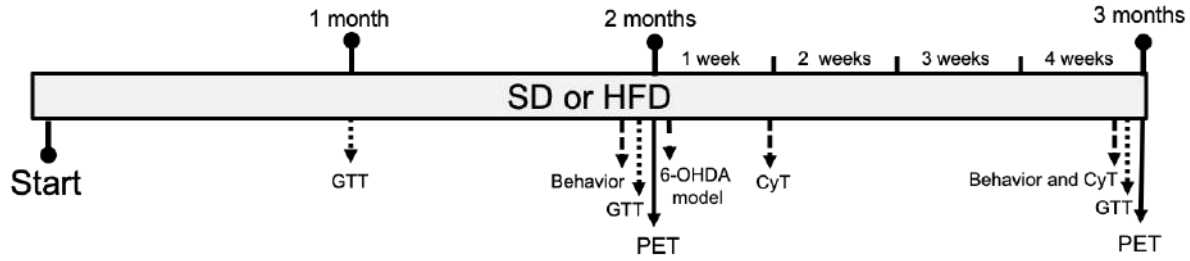


Figure 1: Study design. GTT – glucose tolerance test; SD – standard diet; HFD – high-fat diet; CyT – cylinder test; PET – positron emission tomography; Behavior – open field, object recognition and Y-maze test.

Glucose tolerance test (GTT)

Animals were deprived of food for 6 h prior to the glucose tolerance test. A glucose solution of 2g/kg body weight (total volume between 1.0-2.0 mL) was administered via oral gavage. Blood samples (droplets) were taken from the tail vein at five time points: immediately before and 30, 60, 90, and 120 min after glucose intake. Blood glucose levels (mmol/L) were measured with a Glucometer (TD-GLUCO; Ht One, The Netherlands).

Stereotactic surgery

After 2 months of diet, all animals were submitted to a stereotactic injection of 6-OHDA. First, animals were anaesthetized with isoflurane (5% induction, 1.5%-2.0% maintenance), placed in a stereotactic frame, and fixed with earbars to stabilize the

head. Heating pads were used to maintain the body temperature, and eye salve was used to prevent eye dehydration. Analgesia (Carpofren 1mg/kg) was injected subcutaneously before surgery. An incision was made along the medial line of the skull, and the skin and fascia were pushed aside to expose the skull. A solution of 3μg of 6-OHDA and 0.3% ascorbic acid in 0.5 μL saline was injected in two areas within the right striatum (coordinate 1: AP: +1.12, L: -2.6, V: -5 mm; coordinate 2: AP: +0.2, L: -3.0, V: -4.5 mm from Bregma) (Real et al., 2019) at a rate of 0.2 μL/min. After injection, the needle was left in place for 3 min to allow diffusion of the solution into the brain, and then the needle was slowly removed from the injection site. The incision was sutured and the animals were placed back in their cage. Another injection of analgesia was applied 24 h after surgery.

Open field test

The open field test was performed 8 days before and 21 days after intrastriatal 6-OHDA injection. A round, dark-floored area (60 cm diameter) was used as the open field arena in a dark room, with two light sources close to the arena. The animals were habituated to the experimental room for 1 h, after which they were individually allowed to explore the arena for 10 min. After the open field test, the animals were placed back in their home cage. The arena was thoroughly cleaned with 70% ethanol and wiped dry after each trial. The open field test was recorded on video, which was analyzed using Ethovision XT14 (Noldus, The Netherlands). The following parameters were assessed: velocity, distance traveled, time spent in the center, and time spent in the border zone of the arena.

Novel object recognition (NOR)

The novel object recognition test was used to assess the short-term memory of the animals 7 days before and 22 days after intrastriatal 6-OHDA injection. The same arena and light conditions were used as for the open field test. This test was performed 1 day after the open field test, so the animals were already habituated to the arena and thus could focus on the objects. For this test, similar objects were placed close to each other in the middle of the arena (at approximately the same distance from the wall as between each other) and fixed in place with tape. Each animal was left to explore the

arena and the objects for 10 min and was then placed back to its home cage. After 90 min, the animal was placed back in the arena, in which one object was replaced by another object. After each test, the arena was cleaned with 70% ethanol and wiped dry. The time the animal spent exploring each object was recorded and analysed using Ethovision XT14 (Noldus, The Netherlands). Exploration was defined as the nose of the animals being close to the object. The preference index (PI) was used as a measure of novel object recognition, and was defined as the time spent exploring the new object divided by the total time spent exploring both objects.

Y-maze

The animal was placed in the center of a Y-shaped arena, with three arms (50 cm each) at an angle of 120 degrees from each other. The animals were allowed to explore the arms freely for 8 min. After the test the animal was placed back in its home cage and the arena cleaned with 70% ethanol and wiped dry. The test was recorded on video and the number of spontaneous alternations between arms was counted automatically using Ethovision XT14 (Noldus, The Netherlands).

Cylinder test

The cylinder test was used to evaluate asymmetric paw use as a consequence of the damage caused by the unilateral injection of 6-OHDA. The test was performed 1 and 3 weeks after injection of 6-OHDA. The animals were placed individually in a transparent cylinder (diameter: 20 cm; height: 40 cm) with two mirrors behind it to create 360° vision, during normal light conditions. Animal behavior was recorded for 5 min, and the number of forepaw contacts with the cylinder were counted manually using Ethovision XT14 (Noldus, The Netherlands). Forepaw contact was defined as the placement of the whole palm on the glass, indicating its use for body support. To calculate the percentage of contralateral paw use, the number of times the animal used the left paw was divided by the total number of times the animal supported itself with one of its paws.

PET imaging

^{11}C-raclopride was used as tracer for PET imaging of dopamine D2R availability. ^{11}C-raclopride was synthesized by alkylation of S-(+)-O-desmethylraclopride (ABX, Radeberg, Germany) with ^{11}C-methyl triflate. The product had a molar activity higher than 25,000 GBq/mmol. PET scans were performed using a small animal PET scanner (Focus 220, Siemens Medical Solutions, USA). Prior to PET imaging, the animals were anesthetized using a mixture of isoflurane and oxygen (5% for induction, 2% for maintenance) and a cannula was inserted in the tail vein for tracer injection. Heating pads were used to maintain body temperature throughout the experiment and an eye salve was used to prevent dehydration of the eyes. After insertion of the tail vein cannula, the animals were placed in the PET camera in a prone position with their heads in the field of view. A transmission scan was performed using a ^{57}Co point source for attenuation and scatter correction. ^{11}C-raclopride (25.5±4.4 MBq) was injected over 1 min using an automatic injection pump at a speed of 1 mL/min. At the start of tracer injection, the acquisition of a 60-min dynamic PET scan was started. During the scan, temperature, heart rate and blood oxygen saturation were monitored.

Image processing and PET analysis

The 60-min emission scan was iteratively reconstructed into 21 frames (6 x 10s, 4 x 30s, 2 x 60s, 1 x 120s, 1 x 180s, 4 x 300s and 3 x 600s) using an attenuation-weighted two-dimensional ordered-subset expectation maximization algorithm (4 iterations and 16 subsets). Data was corrected for random coincidences, scatter, radioactive decay and attenuation. Final images had a 256 x 256 x 95 matrix with a pixel width of 0.632 mm and slice thickness of 0.796 mm. The reconstructed PET images were automatically co-registered to an in-house developed ^{11}C-raclopride brain template using PMOD 3.8 software (PMOD Technologies LLC, Switzerland), which allowed use of a predefined volume-of-interest (VOI) map and the reporting of results in Paxinos stereotactic coordinates of the rat brain (Garcia et al., 2015). Time activity curves (TACs) were generated for the left and right striatum and the cerebellum. The simplified reference tissue model 2 (SRTM2) with a fixed k_2' obtained from the contralateral striatum and the cerebellum as the reference region, was used to estimate the non-displaceable binding potential (BP_{ND}) (Wu and Carson, 2002).

Ex vivo biodistribution

After the final ^{11}C-raclopride PET scan, the animals were terminated under deep isoflurane anesthesia (cardiac perfusion) and duodenum, proximal and distal jejunum, proximal ileum and proximal and distal colon were dissected. The excised tissues were weighed and radioactivity was measured in a gamma-counter (Wizard 2480; Perkin-Elmer, U.S.A.). The amount of radioactivity in the tissues was then corrected for the injected dose and the weight of the tissue, and expressed as the percentage of injected dose per gram of tissue (%ID/g).

Statistical analysis

Statistical analyses of the outcome parameters of the glucose tolerance test, open field test, novel object recognition, Y-maze, and cylinder test and ^{11}C-raclopride PET were performed using the generalized estimating equation (GEE) model, using diet as the between-subject factor and time as the within-subject factor. Additionally, the interaction of diet and time was analyzed. Diet, time and the interaction between diet and time, were analyzed by pairwise comparisons after applying a Bonferroni correction for multiple comparisons. Biodistribution data was analyzed using an unpaired sample t-test to assess between-group effects. All data was analyzed using IBM SPSS 24 (IBM Corporation, U.S.). For all statistical analyses, a p-value below 0.05 was considered as statistically significant.

Results

Body weight and caloric intake

Statistical analysis revealed a main effect of time ($p<0.001$), but no main effect of diet on body weight gain. The analysis of the interactions between time and diet showed that weight gain was significantly higher in the HFD group than in the SD group at the first 6 and the last time point ($p<0.05$, Figure 2). There were statistically significant main effects of diet ($p=0.004$) and time ($p<0.001$), and a significant interaction between diet and time ($p<0.001$) on caloric intake. Pairwise between-group comparisons of the

interactions between time and diet showed that caloric intake was significantly higher in the HFD group than the SD group at all time points, except for day 74 and 78 (Figure 2). Overall the animals in the HFD group had 6% higher body weight, 19.5% more weight gain and 9.6% higher calorie intake than controls in the SD group. The small drop in weight gain presented in the graph is likely due to the anesthesia used for the PET scan and the surgical intervention.

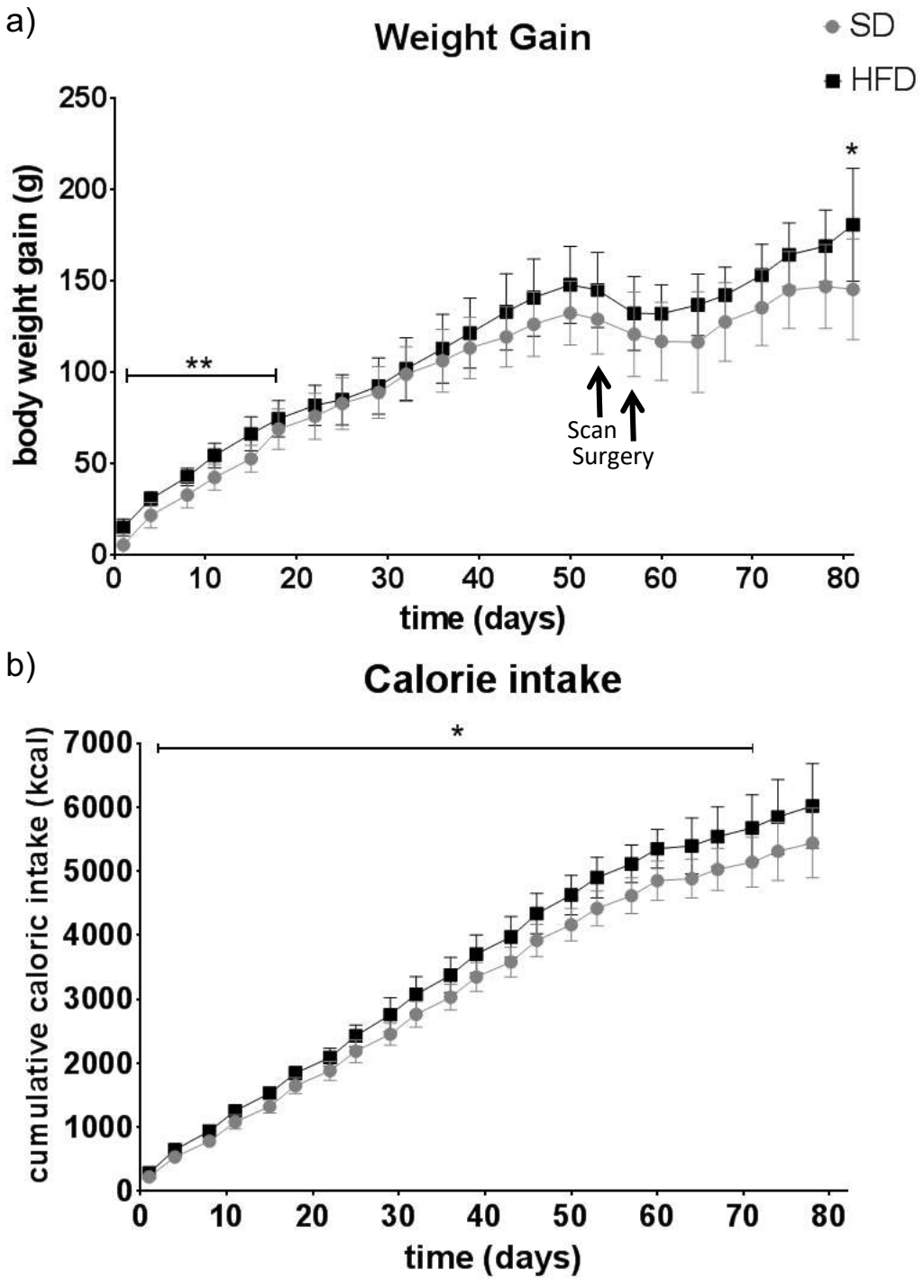


Figure 2: (a) Weight gain and (b) Calorie Intake for animals the SD and HFD group throughout the experiment. Asterisks represents a between group effect at a specific time point: *p<0.05, **p<0.01.

Glucose tolerance test (GTT)

Without a glucose challenge, basal blood glucose levels did not differ between groups at any time point (Figure 3a). Blood glucose levels after a glucose challenge are represented in Figure 3b-d and are expressed as percentage of the baseline value. Blood glucose levels showed a gradual decrease between 30 and 120 minutes after the glucose challenge. Consequently, statistical analysis showed a main effect of time (p<0.05) in the glucose tolerance test, but no main effect for diet. When analyzing the interactions between time and diet, there were no significant differences between groups at any time point (all p>0.05), indicating that the animals had not become prediabetic yet.

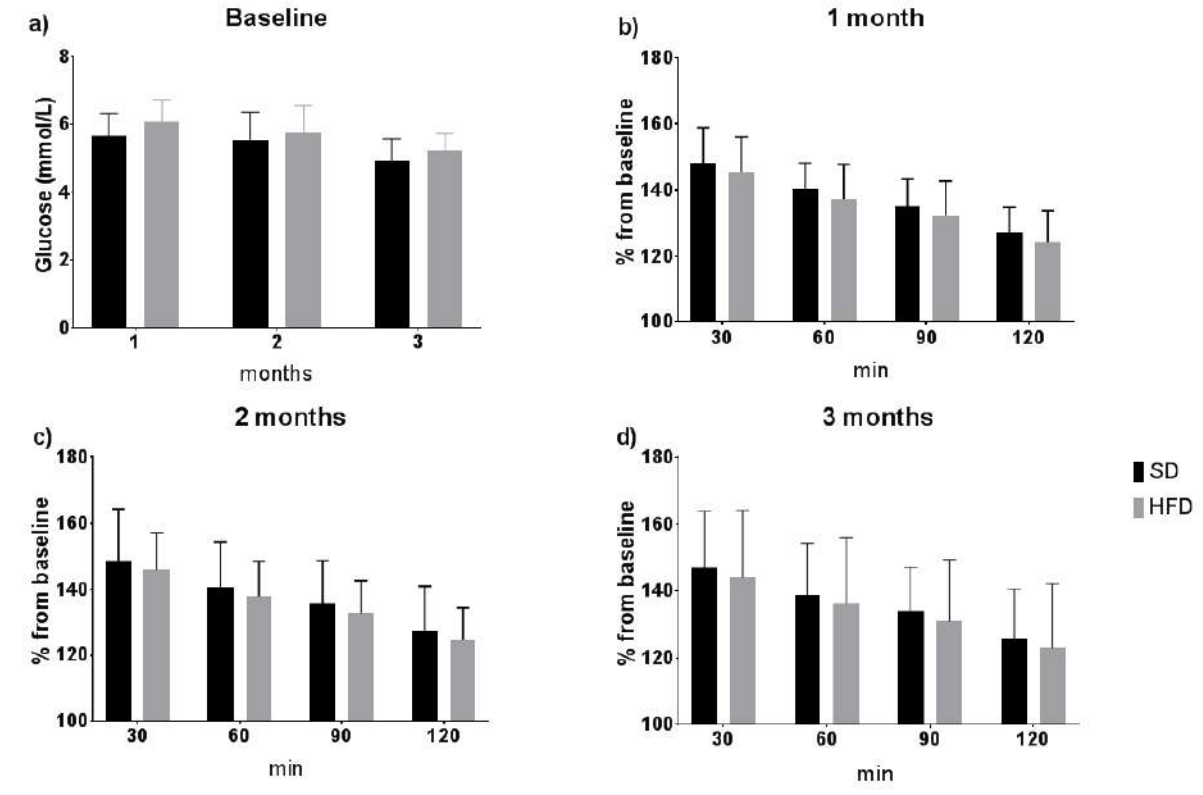


Figure 3: Glucose tolerance test at baseline (a) and after one (b), two (c) and three months (d) on a high fat diet (HFD) or standard diet (SD). The data is represented as

the glucose concentration (nmol/L) in the graph (a) and as percentage change from baseline in the graphs (b), (c) and (d).

Cylinder test

The cylinder test did not show any main effect of diet on the percentage of contralateral paw use (Figure 4). A statistically significant main effect of time was found, however, showing less use of the contralateral paw for support at 3 weeks after surgery when compared with 1 week after surgery (mean difference = 9.6; 95% CI = 3.2 – 15.9%; p=0.003). Interactions of diet x time showed that the contralateral paw use in the HFD animals 3 weeks after surgery was 34% lower than in both the HFD group (mean difference = 14.9%; 95% CI = 6.0 – 23.8%; p=0.001) and SD group (mean difference = 15.1%; 95% CI = 3.83 – 26.40%; p=0.001) 1 week after surgery. No other statistically significant differences between groups and time points were observed, although a tendency towards significance was found between the HFD and SD group at the last time point (p=0.062, 27%).

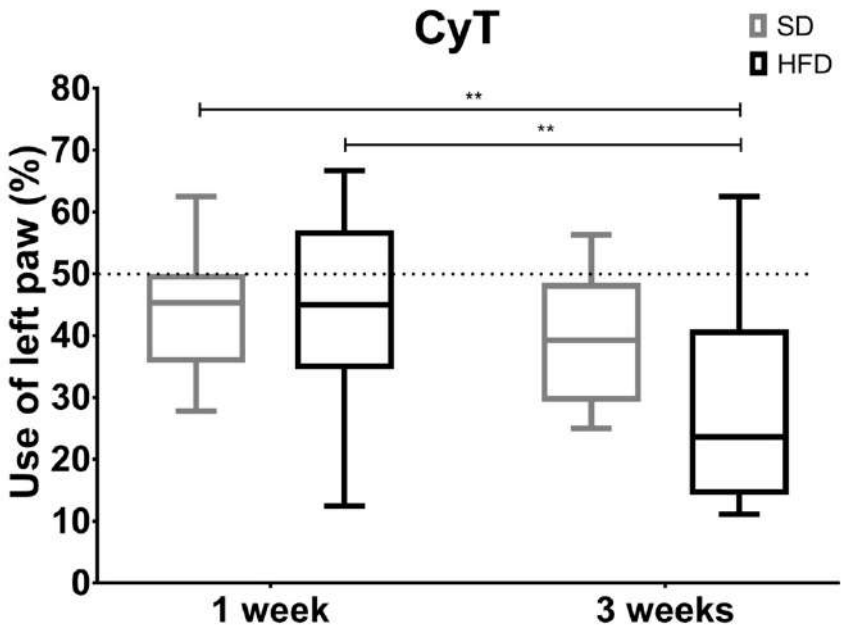
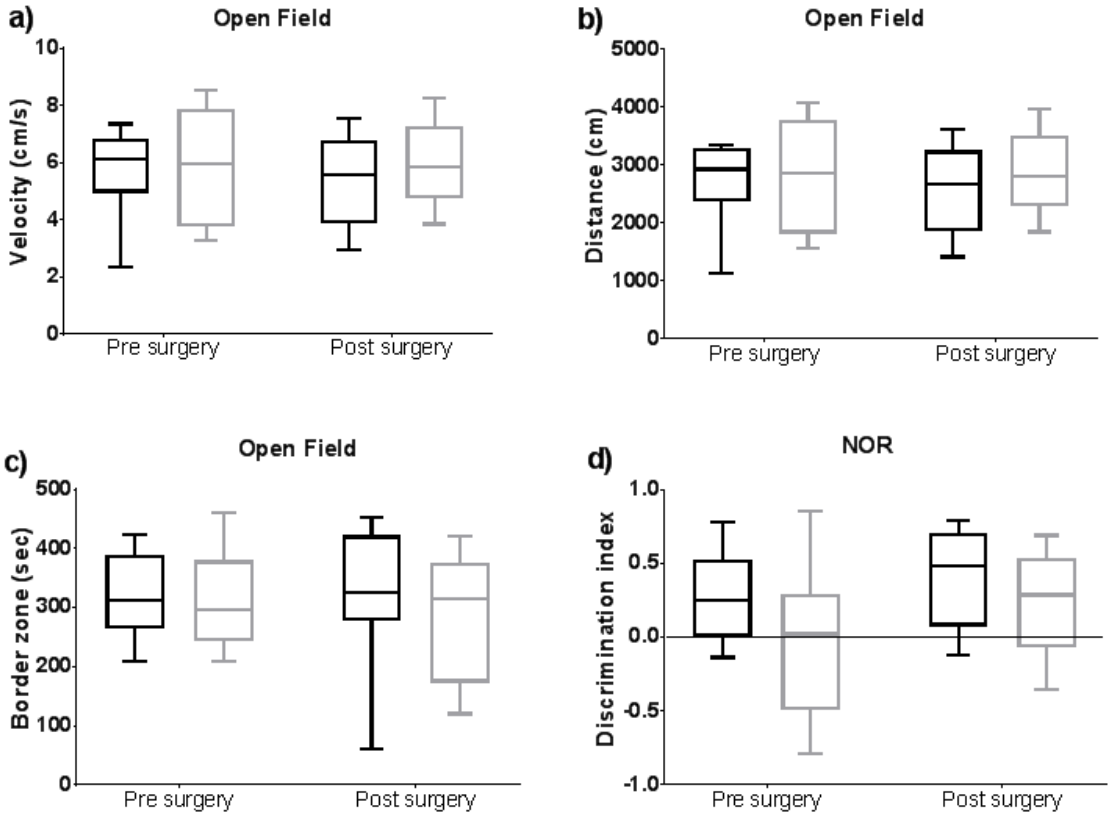


Figure 4: Percentage contralateral paw use in the cylinder test (CyT), 1 week and 3 weeks after injection of 6-OHDA in animals on a standard diet (SD) or a high fat diet (HFD). Box plots and whiskers represent the median, interquartile range and min-max

values, respectively. Asterisks represent the significant interactions between diet and time point: **p=0.001. The dashed line represents the equal use of the left and right paw (50%).

Behavioral measurements

No statistically significant main effects of diet or time, or any significant interaction between diet and time on the open field measures (i.e. velocity, distance traveled, time in center, and time in the border area) was found (p>0.05; Figure 5a-c). Additionally, there was no statistically significant effect of diet or time, or any significant interaction between diet and time on the short-term memory or spatial memory in the novel object recognition test (p>0.05; Figure 5d) and Y-maze tests (p>0.05; Figure 5e).



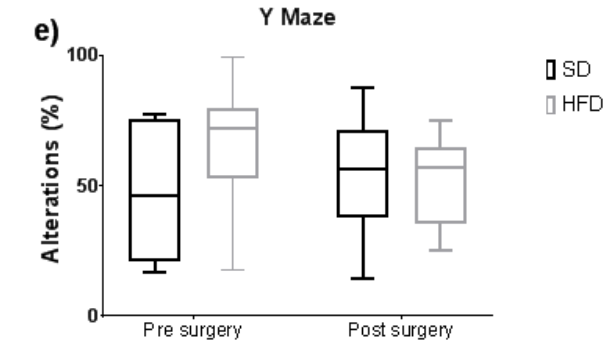


Figure 5: Results of behavioral tests. a) Velocity, b) distance, and c) time in border zone in the open field test; d) discrimination index in the novel object recognition (NOR) test, and e) percentage of alternations in the Y-maze test. Box plot and whiskers represents median, interquartile ranges and min-max values, respectively. The line in panel "d" represent a discrimination index of 0, when half of the time is spent exploring the old object and half of the time spent exploring the new object.

Striatal dopamine D_2 receptor availability

The ^{11}C-raclopride BP_{ND} was calculated using cerebellum as reference region. We first analyzed the striatum of each hemisphere separately and subsequently analyzed the ratio of tracer binding between the ipsilateral and contralateral striatum. No main effect of diet or time was found in the ipsilateral striatum. The variability of the data in the HFD group before the surgery could be the responsible for not finding a main effect of time (p=0.09). The interaction analysis showed that post-surgery tracer binding in the ipsilateral striatum was significantly decreased in both SD animals (mean difference = 0.14; 95% CI = 0.03 – 0.24, p=0.007) and HFD (mean difference = 0.133; 95% CI = 0.01 – 0.25, p=0.033), as compared to baseline ^{11}C-raclopride binding (Figure 6a).

In the contralateral striatum no main effect of time and diet was found. However, the interaction analysis showed that there was a significant effect of time in the SD group, with higher BP_{ND} before than after surgery (mean difference = 0.11; 95% CI = 0.011 – 0.22, p=0.03). In the HFD group, the difference in striatal [^{11}C]raclopride binding in the contralateral striatum between pre- and post-surgery scans approached significance (mean difference = -0.76; 95% CI = -0.15 – 0.0003, p=0.05) (Figure 6b). To exclude any confounding factors that could have affected ^{11}C-raclopride binding in

the contralateral striatum, the ratio of striatal tracer binding between hemispheres was assessed as well.

Statistical analysis of the striatal tracer binding ratio revealed a significant main effect of time, with a higher binding ratio before than after surgery (mean difference = 0.054; 95% CI = 0.015 -0.93; p=0.006), but no main effect of diet (p=0.35). Interestingly, when analyzing the interaction between time and diet, a statistically significantly lower tracer binding ratio in HFD animals after surgery was observed when compared with both the HFD (7.6%) and the SD group (8.5%) at before surgery (HFD post-surgery vs. SD pre-surgery: mean difference = -0.089; 95% CI = -0.156 – -0.022; p=0.009; HFD post-surgery vs. HFD pre-surgery: mean difference = -0.085; 95% CI = -0.009 – -0.020; p=0.009).

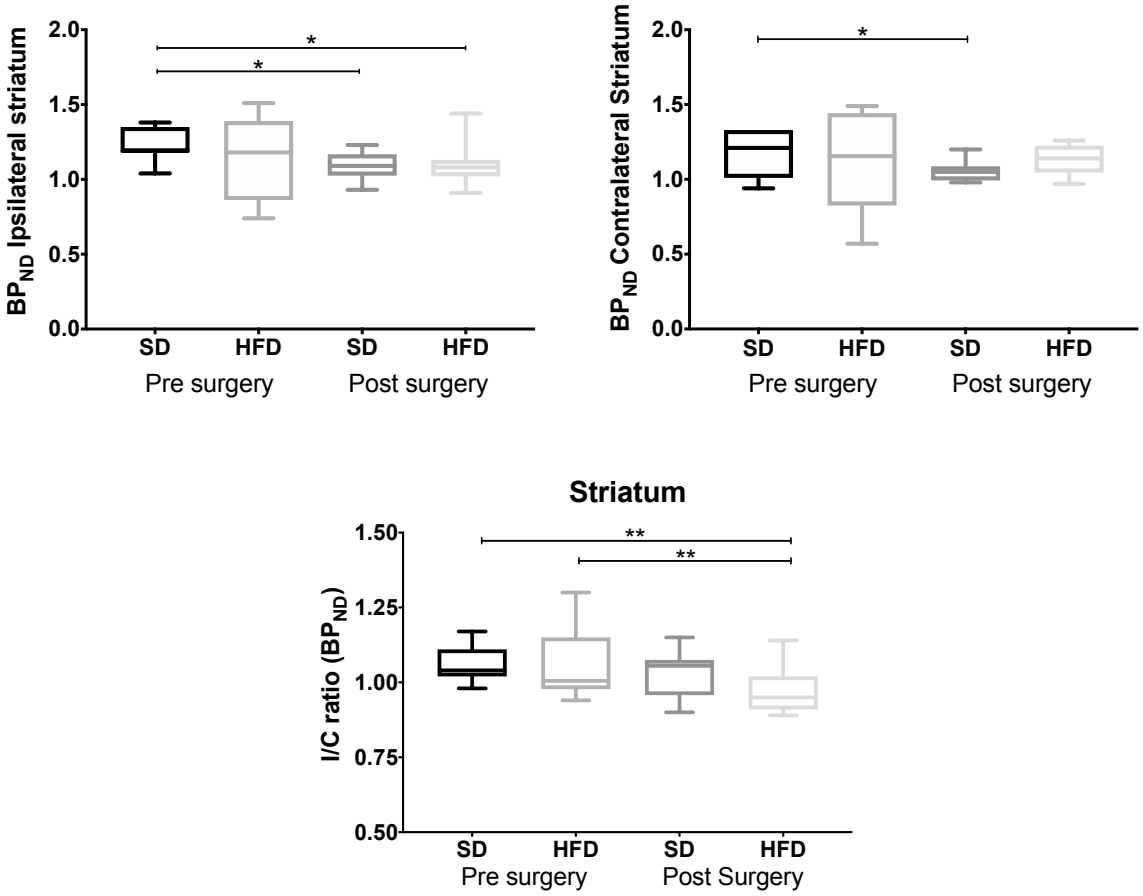


Figure 6: a) ^{11}C-raclopride BP_{ND} in the ipsilateral striatum and b) in the contralateral striatum. c) The ^{11}C-raclopride binding ratio between ipsilateral and contralateral striatum (I/C). ^{11}C-raclopride PET scans were acquired after the 2 months of standard or high-fat diet (SD or HFD – pre surgery) and after 3 month of diet and 1 month after

stereotactic injection of 6-hydroxydopamine in the right striatum (SD or HFD – post surgery). Box plot and whiskers represent the median, interquartile ranges and min-max values, respectively. Statistically significant interactions are indicated with asterisks: p<0.05,**p<0.001.

^{11}C-raclopride biodistribution in the gut

A statistically significant difference in ^{11}C-raclopride uptake in the duodenum between the SD and HFD group was found at 1 month after surgery, with a lower uptake in the HFD group (Mean difference: 0.91 %ID/g; 95% CI: 0.34 - 1.69%; p=0.027). For the other parts of the gut no statistically significant differences between diet groups were found (Figure 7).

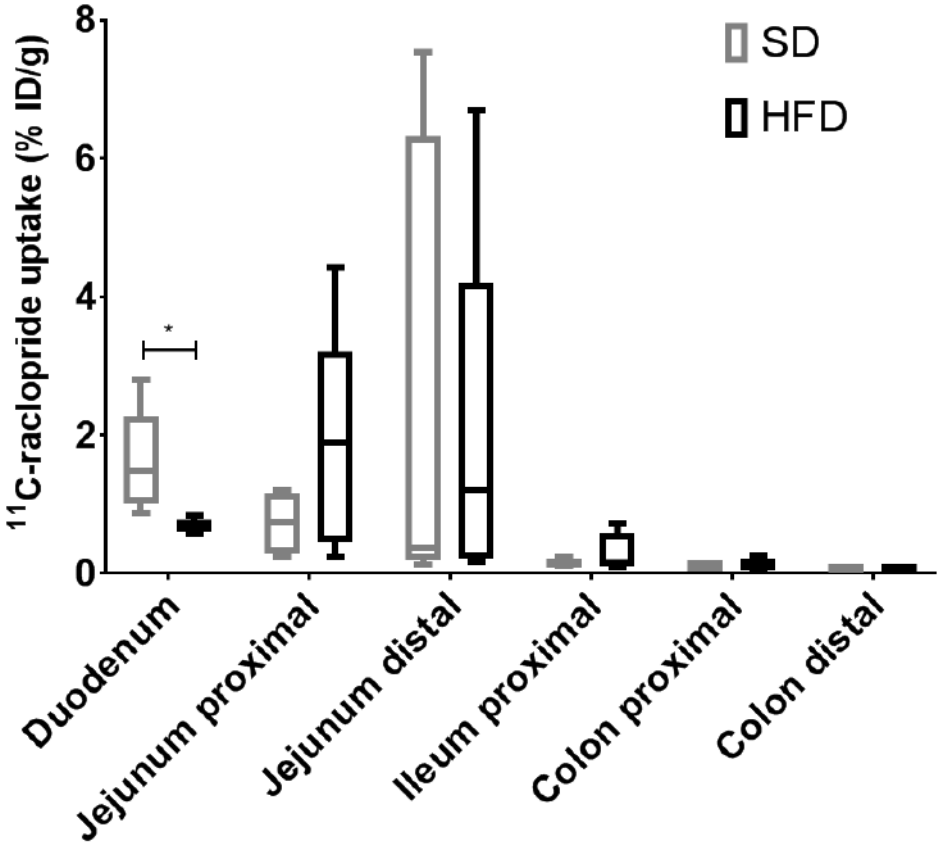


Figure 7: ^{11}C-raclopride uptake (%ID/g) in various parts of the gut of animals fed with a standard diet (SD) or a high-fat diet (HFD) for 3 months. Animals received in stereotactic injection of 6-hydroxydopamine in striatum after 2 months on the diet. Box

plot and whiskers represent the median, interquartile ranges and min-max values, respectively. Statistically significant differences between diet groups are indicate with an asterisk: * $p<0.05$, n=5.

Discussion

This study aimed to determine if a HFD would affect D_2R availability and exacerbate symptoms in the 6-OHDA model of PD. The main finding of this study was the significantly lower ipsilateral-to-contralateral striatal tracer binding ratio in animals on a HFD one month after the administration of 6-OHDA than before surgery. Additionally, these animals showed significantly more locomotor asymmetry in the cylinder test after surgery than before, but no effect on other behavioral parameters or the glucose levels was found. In the SD group, such within group differences were not observed.

The HFD led to higher caloric intake and, consequently, resulted in an increased weight gain. The observed weight gain was consistent with other studies that also found increased weight gain and caloric intake even when a lower percentage of fat was administrated to the animals (Morris et al., 2010; Marques et al., 2016). Some studies also added sucrose or fructose which increased the diabetic profile of the animals (Lozano et al., 2016; Marques et al., 2016). In our study, the weight gain did not differ between groups halfway through the experiment, which might be due to the PET scan and GTT tests. For the PET scan the animals had to be anesthetized and in the case of GTT the animal had to fast for a few hours. In contrast, caloric intake was significantly higher in the HFD group than in the SD group at all time points assessed. Our HFD model showed the expected changes in weight, although additional parameters could have been measured to better access the changes in the animals' physiology, like the percentage of fat and abdominal circumference of the animals (Moreno-Fernández et al., 2018).

The glucose tolerance test is a well established method to assess insulin resistance in rodents similar to the assessment in humans (Marques et al., 2016; Eyth et al., 2020). Contrary to our study, Marques et al. found differences in the insulin resistance in rats after 17 weeks of treatment with a 45% fat diet (Marques et al., 2016), which is probably due to the longer duration of the HFD. Assessment of insulin levels could have given a better impression of the glucose metabolism in the animals. It is

important, however, to note that we couldn't measure insulin levels due to the design of our study, as larger amounts of blood are necessary for this propose.

The cylinder test showed an effect of the HFD on asymmetric paw use 3 weeks after intrastriatal injection of 6-OHDA, indicating an effect of the diet on motor impairment. In a previous rodent study, 6-OHDA injection (total of 16 µg) in the striatum caused a 30% decrease in the contralateral paw use at approximately three weeks after neurotoxin injection (Boix et al., 2018), which is similar to our findings in the HFD group (-34%). The animals in the SD group did not show a decrease in contralateral paw use. This finding could be explained by the possibility that these animals were able to recover from the motor damage, as observed in other studies with a low dose of 6-OHDA (Maia et al., 2012). Apparently, the HFD had a similar aggravating effect on motor symptoms as the 2.7-fold higher 6-OHDA dose used in the study by Boix et al. Taking together, these results suggest that the HFD leads to a higher degree of motor impairment in the animals.

Other behavior parameters were not altered by the diet or 6-OHDA injection. In contrast with our study, a high-caloric diet was able to alter recognition memory in rats in another study, but in this study sucrose was used to induce obesity in the animals (Jurdak and Kanarek, 2009). Different diet manipulation can induce different phenotypes of behavior, but the mechanisms behind this phenomenon still need to be explored.

We found a relative decrease in D2R availability in the affected striatum as a consequence of the 6-OHDA injection in the HFD group only. This result underlines the detrimental role of high fat diet on the dopaminergic system during the recovery phase from the 6-OHDA injection. The 6 µg-dose used in this study can be considered a low dose, when compared to other studies that used doses like 10 µg (Maia et al., 2012), 16 µg (Boix et al., 2018), or even 24 µg. The latter dose was injected directly in substancia nigra (Zhou et al., 2017). This can explain the lack of any significant effects in the SD group over time in our study. Previous studies reported an effect of diabetes or HFD on the D_2R availability, as was demonstrated by a decrease in ^{11}C-raclopride binding in both humans and animal models (Wang et al., 2001; Thanos et al., 2008). Our results are in line with the work from Morris et al. that observed similar effects in a PD model (6-OHDA injected in the right medial forebrain bundle) fed with a HFD. In this work, post-mortem HPLC analysis demonstrated a depletion of dopamine in the striatum and substancia nigra in the HFD group, but not in the control group (Morris et

al., 2010). The effects of a HFD on D_2R availability in animal models of PD demonstrate how the simple lifestyle changes can influence processes in the brain in diseases like PD.

A HFD is known to have widespread effects on the gastrointestinal tract, including local tissue modifications to better absorb fat-rich food, microbiome dysbiosis and the induction of inflammation (Serino et al., 2012; Hamilton et al., 2015; Deshpande et al., 2019). While the expression of dopamine D_2R in the gastrointestinal tract is relatively low compared to other dopamine receptors, they appear to have an important effect on general gastrointestinal tract motility by affecting the efferent signalling of cholinergic neurons (Zhi et al., 2006). In this study, animals on a HFD had a significantly lower D_2R availability in the duodenum than animals on the SD, while other gut regions did not show any significant differences. It has been shown that a diet rich in fat decreases motility in the duodenum and severely modifies duodenum architecture (Soares et al., 2015). In addition, gastrointestinal tract motility was found to be affected by partial depletion of dopaminergic neurons in the substantia nigra after 6-OHDA injection, especially in distal regions of the gastrointestinal tract (Colucci et al., 2012). The changes found in this study are in line with these results and could be related to the dysregulation of the metabolic hormones secreted by the duodenum that regulate dopamine release and their effects on gastric emptying (De Araujo et al., 2012). However, as ^{11}C-raclopride uptake was only measured at one time point, it is not known if the changes in the duodenum are a consequence of the HFD or the 6-OHDA-induced lesion or both. Additionally, it is worth noting that the results showed a high variance in other gut regions, which could be related to the size of the sample. In the future perhaps the whole organs should be measured.

Conclusions

This is the first study using PET imaging to assess the effect of a HFD in a model of PD longitudinally. HFD was able to exacerbate the effect of the neurotoxin 6-hydroxydopamine with respect to asymmetric paw use and dopamine D_2R availability in the brain and gut. These results seem to implicate that the diet can induce changes in the gut that can aggravate injury in the brain, resulting in more severe motor symptoms. Such mechanisms could also be involved in the onset and progression of

PD. Because of its translational character, PET would be an attractive tool to investigate this hypothesis in PD patients as well.

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References

Boix J, von Hieber D, Connor B (2018) Gait analysis for early detection of motor symptoms in the 6-ohda rat model of parkinson's disease. Front Behav Neurosci 12:39 Available at: http://www.ncbi.nlm.nih.gov/pubmed/29559901 [Accessed March 19, 2020].

Bosco D, Plastino M, Cristiano D, Colica C, Ermio C, De Bartolo M, Mungari P, Fonte G, Consoli D, Consoli A, Fava A (2012) Dementia is associated with insulin resistance in patients with Parkinson's disease. J Neurol Sci 315:39–43.

Bousquet M, St-Amour I, Vandal M, Julien P, Cicchetti F, Calon F (2012) High-fat diet exacerbates MPTP-induced dopaminergic degeneration in mice. Neurobiol Dis 45:529–538 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21971528 [Accessed March 19, 2020].

Campos FL, Carvalho MM, Cristovão AC, Je G, Baltazar G, Salgado AJ, Kim YS, Sousa N (2013) Rodent models of Parkinson's disease: Beyond the motor symptomatology. Front Behav Neurosci.

Cereda E, Barichella M, Cassani E, Caccialanza R, Pezzoli G (2012) Clinical features of Parkinson disease when onset of diabetes came first A case-control study. Neurology 78:1507–1511 Available at: http://www.ncbi.nlm.nih.gov/pubmed/22539572 [Accessed March 19, 2020].

Choi JY, Jang EH, Park CS, Kang JH (2005) Enhanced susceptibility to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity in high-fat diet-induced obesity. Free Radic Biol Med 38:806–816 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15721991 [Accessed March 19, 2020].

Colucci M, Cervio M, Faniglione M, De Angelis S, Pajoro M, Levandis G, Tassorelli C, Blandini F, Feletti F, De Giorgio R, Dellabianca A, Tonini S, Tonini M (2012) Intestinal dysmotility and enteric neurochemical changes in a Parkinson's disease rat model. Auton Neurosci Basic Clin 169:77–86 Available at: http://www.ncbi.nlm.nih.gov/pubmed/22608184 [Accessed March 4, 2020].

De Araujo IE, Ferreira JG, Tellez LA, Ren X, Yeckel CW (2012) The gut-brain dopamine axis: A regulatory system for caloric intake. Physiol Behav 106:394–399.

Deshpande NG, Saxena J, Pesaresi TG, Carrell CD, Ashby GB, Liao MK, Freeman LR (2019) High fat diet alters gut microbiota but not spatial working memory in early middle-aged Sprague Dawley rats. PLoS One 14.

Driver JA, Smith A, Buring JE, Gaziano JM, Kurth T, Logroscino G (2008) Prospective cohort study of type 2 diabetes and the risk of parkinson's disease. Diabetes Care 31:2003–2005.

Eyth E, Basit H, Smith CJ (2020) Glucose Tolerance Test. StatPearls Publishing. Available at: http://www.ncbi.nlm.nih.gov/pubmed/30422510 [Accessed April 16, 2020].

Fasano A, Visanji NP, Liu LWC, Lang AE, Pfeiffer RF (2015) Gastrointestinal dysfunction in Parkinson's disease. Lancet Neurol 14:625–639.

Garcia DV, Casteels C, Schwarz AJ, Dierckx RAJO, Koole M, Doorduin J (2015) A standardized method for the construction of tracer specific PET and SPECT rat brain templates: Validation and implementation of a toolbox. PLoS One 10:e0122363 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25823005 [Accessed March 23, 2020].

Greenwood CE, Winocur G (1990) Learning and memory impairment in rats fed a high saturated fat diet. Behav Neural Biol 53:74–87.

Hamilton MK, Boudry G, Lemay DG, Raybould HE (2015) Changes in intestinal barrier function and gut microbiota in high-fat diet-fed rats are dynamic and region dependent. Am J Physiol - Gastrointest Liver Physiol 308:G840–G851 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25747351 [Accessed March 23, 2020].

Hassan AM, Mancano G, Kashofer K, Fröhlich EE, Matak A, Mayerhofer R, Reichmann F, Olivares M, Neyrinck AM, Delzenne NM, Claus SP, Holzer P (2019) High-fat diet induces depression-like behaviour in mice associated with changes in microbiome, neuropeptide Y, and brain metabolome. Nutr Neurosci 22:877–893 Available at:

http://www.ncbi.nlm.nih.gov/pubmed/29697017 [Accessed March 19, 2020].
Hu G, Jousilahti P, Bidel S, Antikainen R, Tuomilehto J (2007) Type 2 diabetes and the risk of Parkinson's disease. Diabetes Care 30:842–847 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17251276 [Accessed March 19, 2020].
Jurdak N, Kanarek RB (2009) Sucrose-induced obesity impairs novel object recognition learning in young rats. Physiol Behav 96:1–5.
Kosari S, Badoer E, Nguyen JCD, Killcross AS, Jenkins TA (2012) Effect of western and high fat diets on memory and cholinergic measures in the rat. Behav Brain Res 235:98–103.
Lozano I, Van Der Werf R, Bietiger W, Seyfritz E, Peronet C, Pinget M, Jeandidier N, Maillard E, Marchioni E, Sigrist S, Dal S (2016) High-fructose and high-fat diet-induced disorders in rats: Impact on diabetes risk, hepatic and vascular complications. Nutr Metab 13:15 Available at: http://www.nutritionandmetabolism.com/content/13/1/15 [Accessed April 1, 2020].
Maia S, Arlicot N, Vierron E, Bodard S, Vergote J, Guilloteau D, Chalon S (2012) Longitudinal and parallel monitoring of neuroinflammation and neurodegeneration in a 6-hydroxydopamine rat model of Parkinson's disease. Synapse 66:573–583 Available at: http://doi.wiley.com/10.1002/syn.21543 [Accessed February 18, 2020].
Maraki MI, Yannakoulia M, Stamelou M, Stefanis L, Xiromerisiou G, Kosmidis MH, Dardiotis E, Hadjigeorgiou GM, Sakka P, Anastasiou CA, Simopoulou E, Scarmeas N (2019) Mediterranean diet adherence is related to reduced probability of prodromal Parkinson's disease. Mov Disord 34:48–57.
Marques C, Meireles M, Norberto S, Leite J, Freitas J, Pestana D, Faria A, Calhau C (2016) High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. Adipocyte 5:11–21 Available at: www.tandfonline.com/kadi [Accessed April 1, 2020].
Mischley LK, Lau RC, Bennett RD (2017) Role of Diet and Nutritional Supplements in Parkinson's Disease Progression. Available at: https://doi.org/10.1155/2017/6405278 [Accessed March 2, 2020].
Moreno-Fernández S, Garcés-Rimón M, Vera G, Astier J, Landrier JF, Miguel M (2018) High fat/high glucose diet induces metabolic syndrome in an experimental rat model. Nutrients 10.
Morris JK, Bomhoff GL, Stanford JA, Geiger PC (2010) Neurodegeneration in an animal model of Parkinson's disease is exacerbated by a high-fat diet. Am J Physiol - Regul Integr Comp Physiol 299:R1082.
Myers R (2001) The biological application of small animal PET imaging. Nucl Med Biol 28:585–593.
Palacios N, Gao X, Mccullough ML, Jacobs EJ, Patel A V., Mayo T, Schwarzschild MA, Ascherio A (2011) Obesity, diabetes, and risk of Parkinson's disease. Mov Disord 26:2253–2259.
Paul KC, Chuang YH, Shih IF, Keener A, Bordelon Y, Bronstein JM, Ritz B (2019) The association between lifestyle factors and Parkinson's disease progression and mortality. Mov Disord 34:58–66.
Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkmann J, Schrag AE, Lang AE (2017) Parkinson disease. Nat Rev Dis Prim 3:1–21.
Postuma RB, Gagnon JF, Pelletier A, Montplaisir J (2013) Prodromal autonomic symptoms and signs in Parkinson's disease and dementia with Lewy bodies. Mov Disord 28:597–604 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23554107 [Accessed March 19, 2020].
Real CC, Doorduin J, Kopschina Feltes P, Vallez Garcia D, de Paula Faria D, Britto LR, de Vries EF (2019) Evaluation of exercise-induced modulation of glial activation and dopaminergic damage in a rat model of Parkinson's disease using [(11)C]PBR28 and [(18)F]FDOPA PET. J Cereb Blood Flow Metab 39:989–1004.
Savica R, Grossardt BR, Ahlskog JE, Rocca WA (2012) Metabolic markers or conditions preceding Parkinson's disease: A case-control study. Mov Disord 27:974–979.
Serino M et al. (2012) Metabolic adaptation to a high-fat diet is associated with a change in the

gut microbiota. Gut 61:543–553.
Sherwin E, Sandhu K V., Dinan TG, Cryan JF (2016) May the Force Be With You: The Light and Dark Sides of the Microbiota–Gut–Brain Axis in Neuropsychiatry. CNS Drugs 30:1019–1041.
Soares A, Beraldi EJ, Ferreira PEB, Bazotte RB, Buttow NC (2015) Intestinal and neuronal myenteric adaptations in the small intestine induced by a high-fat diet in mice. BMC Gastroenterol 15:3 Available at: http://bmcgastroenterol.biomedcentral.com/articles/10.1186/s12876-015-0228-z [Accessed March 19, 2020].
Thanos PK, Michaelides M, Piyis YK, Wang GJ, Volkow ND (2008) Food restriction markedly increases dopamine D2 receptor (D2R) in a rat model of obesity as assessed with in-vivo μPET imaging ([11C] raclopride) and in-vitro ([3H] spiperone) autoradiography. Synapse 62:50–61 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17960763 [Accessed April 1, 2020].
Wang GJ, Volkow ND, Logan J, Pappas NR, Wong CT, Zhu W, Netusll N, Fowler JS (2001) Brain dopamine and obesity. Lancet 357:354–357.
Wu Y, Carson RE (2002) Noise reduction in the simplified reference tissue model for neuroreceptor functional imaging. J Cereb Blood Flow Metab 22:1440–1452.
Xu Q, Park Y, Huang X, Hollenbeck A, Blair A, Schatzkin A, Chen H (2011) Diabetes and risk of Parkinson's disease. Diabetes Care 34:910–915.
Yao R, Lecomte R, Crawford ES (2012) Small-animal PET: What is it, and why do we need it? J Nucl Med Technol 40:157–165 Available at: http://tech.snmjournals.org/cgi/doi/10.2967/jnmt.111.098632 [Accessed March 19, 2020].
Zhi SL, Schmauss C, Cuenca A, Ratcliffe E, Gershon MD (2006) Physiological modulation of intestinal motility by enteric dopaminergic neurons and the D2 receptor: Analysis of dopamine receptor expression, location, development, and function in wild-type and knock-out mice. J Neurosci 26:2798–2807.
Zhou X, Doorduin J, Elsinga PH, Dierckx RAJO, de Vries EFJ, Casteels C (2017) Altered adenosine 2A and dopamine D2 receptor availability in the 6-hydroxydopamine-treated rats with and without levodopa-induced dyskinesia. Neuroimage 157:209–218 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28583881 [Accessed March 19, 2020].

CHAPTER 7

A high-fat diet induces exacerbated neuroinflammation and altered gut microbiota in a preclinical model of Parkinson's disease

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Abstract

Lifestyle factors, such as diet, are reported to contribute to the development and progression of Parkinson's disease and thus could be an attractive target for disease prevention. Therefore, we aimed to evaluate the impact of a high-fat diet on the composition of the gut microbiota, development of neuroinflammation and disease symptoms in a rat model of Parkinson's disease.

Methods: Wistar rats were fed with either a high-fat diet (HFD) or a standard diet (SD) for three months. Body weight and food intake were measured twice a week. After two months of diet, the rats were submitted to a unilateral stereotactic injection of a low dose of 6-OHDA (6µg) in the right striatum to induce degeneration of dopaminergic neurons. [^{11}C]-PBR28 PET scans were performed to assess neuroinflammation after two months of diet and one month after the 6-OHDA injection. Feces were collected at baseline, after two months of the diet and one month after the 6-OHDA injection for microbiome analysis. Systemic cytokine levels were assessed in plasma at the last time point.

Results: Between-group comparison showed an increased body weight (p=0.01) in animals on a HFD when compared to SD controls, but did not reveal any significant effects of the diet in the cylinder test or PET imaging. Within-group comparison in the HFD group, however, demonstrated a decrease in left paw use, as assessed by the cylinder test (p=0.001), and an increase in [^{11}C]-PBR28 uptake in the right striatum 1 month after 6-OHDA injections as compared to the assessments before surgery. Within group analysis in the SD group, on the other hand, showed that [^{11}C]-PBR28 uptake had significantly increased bilaterally in *globus pallidus* and thalamus 1 month after 6-OHDA injection, but not in striatum. The HFD was also associated with changes in the microbiota over time, including altered abundance of *Allobaculum*, *Muribaculaceae* and *Erysipelotrichaceae*, all of which correlated with left paw use or [^{11}C]-PBR28 uptake. No significant changes in plasma cytokine levels were observed.

Conclusion: This study suggests the documented impact of a HFD on neuroinflammation may be mediated by the gut microbiome in the 6-OHDA rat model of Parkinson's disease and suggests it is independent of peripheral inflammation.

Introduction

Parkinson's disease (PD), one of the most common neurodegenerative disorders, is characterized by motor symptoms like tremor, rigidity, bradykinesia, and postural instability (clinical phenotype) (Moustafa et al., 2016) and non-motor symptoms, such as mood and behavioral disorders, constipation, hyposmia and pain (Poewe, 2008). A hallmark of Parkinson's disease is the progressive aggregation of α-synuclein, which induces neuroinflammation and eventually leads to degeneration of dopaminergic neurons (Braak et al., 2003; Dickson et al., 2009; Poewe, 2008). Ample evidence suggests that inflammatory processes are directly or indirectly involved in the onset and progression of the disease (Troncoso-Escudero, Parra, Nassif, & Vidal, 2018). Identification of pro-inflammatory pathways has promoted investigation of how neuroinflammation can be controlled to slow disease progression and control disease burden.

One of the main regulators of immune function and increased systemic cytokine production is the gut microbiota (Iler, Pappalardo, & Hafler, 2016). For this reason, the interaction between the brain and the gut has been the topic of intense research on the prevention of neurodegenerative diseases. Interestingly, the gut microbiota of patients with PD is often characterized by a deficiency in the Firmicutes family, including *Lactobacillaceae* and *Enterococcaceae*, and an enrichment of *Enterobacteriaceae* and *Bifidobacterium*. These changes in gut microbiota disrupt the production of short chain fatty acids (SCFAs) and lipopolysaccharide (LPS) (Adams et al., 2019; Unger et al., 2016), each of which have a significant effect on mucosal immune function. SCFAs play critical roles in immune regulation and can have pro- and anti-inflammatory properties (Li et al., 2018). In contrast, LPS is a well-described endotoxin associated with the induction of a pro-inflammatory immune response (X. Liu et al., 2018). Several reports link endotoxins with neurodegenerative diseases and, while the mechanism remains speculative, evidence indicates that the decrease of endotoxin responses can lead to reduction in neuroinflammation (Batista, Gomes, Candelario-Jalil, Fiebich, & de Oliveira, 2019; Brown, 2019). Similarly, it has been suggested that SCFAs can exert neuroprotective effects, although the exact effect on PD development is unclear (Mulak, 2018).

One of the main factors that determines microbiota composition is diet. A recent meta-analysis (Qu, Chen, Xu, & Sun, 2019) concluded that dietary fat intake, in particular high cholesterol and arachidonic acid consumption, is an important predictive risk factor of PD. Interestingly, both a low-fat and ketogenic diet adhered to for 8 weeks was shown to improve motor and non-motor symptoms in PD (Phillips, Murtagh, Gilbertson, Asztely, & Lynch, 2018). These findings are supported by studies in animal models, demonstrating exaggerated

degeneration of dopaminergic neurons in the nigrostriatal pathway in rats fed with a high-fat diet prior to induction of PD (Morris, Bomhoff, Stanford, & Geiger, 2010).

Despite mounting evidence that a high-fat diet is clinically associated with PD and exacerbates disease progression in animal models, few studies have moved beyond these observations to provide mechanistic insight. Given the profound impact of diet on the microbiota, and its ability to control neuroinflammatory mechanisms, we hypothesize that a high-fat diet contributes to the development of PD via modulation of microbiota. As such, we aimed to evaluate the impact of a high-fat diet on the composition of microbiota, development of neuroinflammation and severity of motor symptoms, in the frequently used 6-hydroxydopamine (6-OHDA) rat model of PD. Neuroinflammation was assessed using PET imaging with [^{11}C]-PBR28, and gut microbiota and cytokine levels in blood were analyzed.

Material and methods

Animals

Nine-week old male Wistar rats, weighing 290-370 g, were obtained from Envigo (The Netherlands) and habituated to the animal facility for one week. In order to measure food intake accurately, animals were individually housed during the study. The room was controlled for temperature and humidity (T=20 ± 2 °C, humidity 60%) and was kept under a 12:12 hour light-dark cycle (lights on 7 A.M.; lights off 8 P.M.). All experiments were performed during the light phase. All experiments were approved by the National Committee on Animal Experiments (CCD license: AVD1050020173069) and the Institutional Animal Care and Use Committee of the University of Groningen (IvD study number 173069-01-002).

Study Design

An overview of the study design can be seen in Figure 1. Rats were fed with either a high-fat diet (HFD) or a standard diet (SD) for three months. Body weight and food intake were measured twice a week throughout the entire experiment. Food intake was converted into caloric intake. After two months of diet, the rats were submitted to stereotactic injection of a low dose of 6-OHDA. Static [^{11}C]-PBR28 PET scans were performed 3 days before and 1 month after the 6-OHDA injection. Feces was collected before the start of diet (baseline), after 2 months of diet (before 6-OHDA injection) and after 3 months of diet (i.e. 1 month after 6-OHDA injection). The cylinder test was performed at 1 and 3 weeks after surgery, and blood was collected immediately prior to the last PET scan.

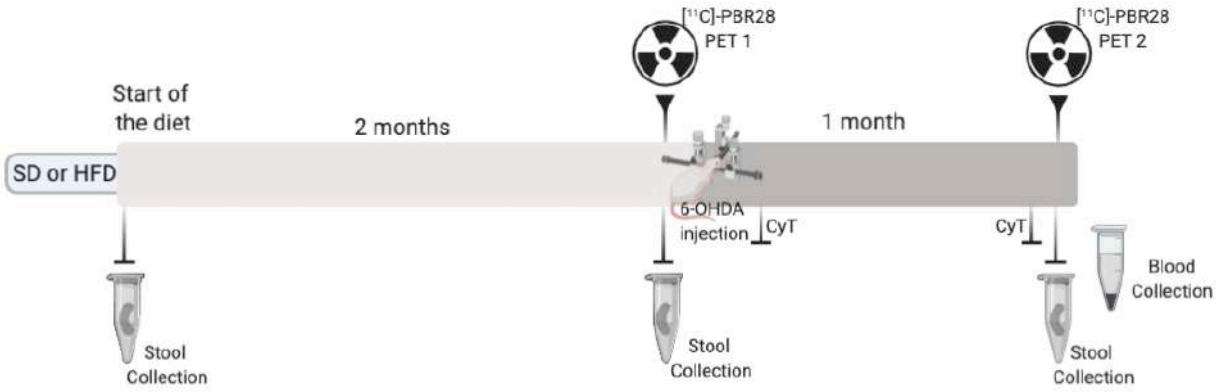


Figure 1: Study design. Abbreviations: SD standard diet, HFD high-fat diet, CyT: Cylinder test. (Made in ©BioRender - biorender.com)

Diet

The animals were randomly divided in two groups: one group was fed with a HFD (60% fat, 20% protein, 20% carbohydrates, energy density of 5.21 kcal/g, Research Diets, U.S.), the other group with a SD (10% fat, 20% protein, 70% carbohydrate, energy density of 3.82 kcal/g, Research Diets, U.S.), for 12 weeks.

Stereotactic surgery

After 2 months of diet, all animals were submitted to stereotactic injection of 6-OHDA. Rats were anaesthetized with isoflurane (5% induction, 1.5%-2.0% maintenance) mixed with oxygen, placed in a stereotactic frame and fixed with ear bars. Analgesia (Carpofren, 1 mg/kg) was applied subcutaneously, an incision was made along the medial line of the skull, the skin and fascia were pushed aside, and two holes were drilled in the skull for the unilateral injections. 6-OHDA dissolved in saline (6µg/µL) was injected in two areas in the right striatum with the following the coordinates from Bregma: coordinate 1: AP: +1.12, L: -2.6, V: -5 mm; coordinate 2: AP: +0.2, L: -3.0, V: -4.5 mm (Real et al., 2019). A total volume of 0.5 µL (3 µg) was injected at each coordinate at a rate of 0.1 µL/min. After administration, the needle was left in place for 3 min to prevent efflux of the solution, and then slowly removed from the injection site. The incision site was sutured and the animals were placed back in their cage. A second injection of analgesic was given 24 h after surgery to prevent discomfort due to surgery.

Cylinder test

The cylinder test was used to evaluate asymmetric paw use as a consequence of the unilateral injection of 6-OHDA. The test was performed one and three weeks after surgery. In the light phase, rats were placed in a transparent cylinder (diameter: 20 cm; height: 40 cm) with two mirrors behind it to create 360° vision. Animal behavior was recorded for 5 min, and the number of forepaw contacts with the cylinder was counted manually. Forepaw contact was defined as the placement of the whole paw on the glass, indicating its use for body support. To calculate the percentage of left paw use, the number of times the animal used the left paw was divided by the total number of forepaw contacts (Real et al., 2019).

[^{11}C]-PBR28 PET imaging

[^{11}C]-PBR28 scans were performed with a dedicated small animal PET scanner (Focus 220, Siemens Medical Solutions, USA). Before tracer injection, anesthesia was induced with 5% isoflurane and maintained with 2% isoflurane in oxygen. After anesthesia induction, a tail cannula was inserted in the lateral tail vein and a solution of [^{11}C]-PBR28 in saline (mean ± SD: 40.2 ± 11.2 MBq; 1 – 1.5mL; specific activity always higher than 25,000 GBq/mmol) was then injected as a bolus. The animal was placed back in its caged and allowed to wake up. After 30 min, the animal was anesthetized again, placed in the PET scanner and a transmission scan was performed with a ^{57}Co source for correction of attenuation and scatter. A 30-min emission scan was started 45 min after tracer injection.

Images were reconstructed (OSEM2D, 4 iterations and 16 subsets) after correction for attenuation, scatter, random coincidences and radioactive decay, and co-registered automatically to a [^{11}C]-PBR28 rat brain template using PMOD 4.004 (PMOD technologies LLC, Switzerland). A predefined set of regions of interest (ROI's) were placed on each PET image and the radioactivity concentrations in the following regions were extracted: striatum (right and left separately), *globus pallidus* (right and left separately), *substantia nigra* and thalamus. The average tracer uptake in the ROI's (in kBq/cc) was corrected for the injected tracer dose and the bodyweight and expressed as standardized uptake value (SUV).

Microbiota analysis

Feces samples were collected directly from the rectum to prevent contamination and were immediately stored at -80 ^{o}C. Samples were collected before the start of the experimental diet, and after two and three months of the diet.

DNA extraction and PCR amplification

DNA was extracted from feces samples using the double bead-bester procedure adapted from Yu and Morrison (2004) and QIAamp DNA Stool Minikit guidelines (Qiagen, Hilden, Germany). DNA yield and quality were assessed using a NanoDrop UV Spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and diluted to a working concentration of 100 ng/μL. DNA samples were amplified by PCR using 1 μL of 10 μM 341f forward primer, 25 μL ThermoFisher Phire HS II Master Mix, 22 μl DNase free water, 1 μL of 10 μM 806r barcoded reverse primer and 1 μL DNA template (100 ng/μL). The samples were denatured at 98 °C for 30 s, and amplified in 31 cycles at 98 °C for 5 s, 50 °C for 5 s and 72 °C for 10 s. Samples were kept at 72 °C for 1 min and subsequently at 4 °C until collection. Amplification was confirmed using agarose gel electrophoresis.

PCR products were purified using AMPure XP magnetic beads (Beckman Coulter, Brea California, USA) added at a ratio of 1:2. The resulting supernatant was discarded and the pelleted beads were washed with 80% ethanol and allowed to air-dry before DNA was removed from the bead with 52.5 μL 10 mM Tris HCl (pH 8.5). Purified DNA was quantified using the Qubit quantification kit (ThermoFisher Scientific) with a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, California, USA) and diluted to a working concentration of 2 mM with Tris-EDTA buffer.

Sequencing and bioinformatics

Sequencing was performed using a MiSeq Benchtop Next Generation Sequencer (Illumina) according to the manufacturer's guidelines. The paired-end sequencing data received from Illumina software were processed with PANDAseq (version 2.5) (Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012) and QIIME (version 1.7.0) software (Caporaso et al., 2010). Readouts with a quality score below 0.9 were discarded by the PANDAseq software to increase the quality of the sequence readouts. De novo OTU-picking was performed without chimera filtering with Greengenes (version 13.5) as reference database. Data were visualized and graphs produced using Genomics Workbench 12 kindly provided by Qiagen and GraphPad prism v8.4.2.

Cytokine analyses

During anesthesia before the PET scan, blood samples were collected into microtubes. Plasma was separated by centrifugation and stored at –80 °C prior to analysis. Plasma concentrations (in pg/mL) of IFN-γ, IL-10, IL-13, IL-1β, IL-4, IL-5, IL-6, KC/GRO and TNF-α were determined using V-PLEX Proinflammatory Panel 2 Rat Kit (Meso Scale Diagnostics, Rockville, MD, U.S.A.). 1:5 was used as the dilution for the samples.

Statistical analysis

All data, except microbiota and cytokine data, was statistically analyzed using the generalized estimating equation (GEE), with diet (SD vs. HFD) and time (before vs. 1 month after 6-OHDA injection) as between- and within-subject factors, respectively. Dietary treatment, time point of assessment, and interaction between diet and time were analyzed by pairwise comparisons, applying post-hoc Bonferroni correction for multiple comparisons. All data was analyzed using IBM SPSS 23 software (IBM Corporation, U.S.). Microbiota-related data was analyzed using a mixed-effect model with Tukey's post-hoc correction for multiple comparisons. Associations between microbe of interest and neuroinflammation or behavior were assessed using Spearman's correlation in GraphPad Prism v8.0. For cytokine analysis, an unpaired t-test was performed using GraphPad Prism v8.0. For samples without detectable cytokine levels, the results were reported as the half of the lowest limit of detection, as this imputation method was shown to be robust and well established (Gupta et al., 2017). For all statistics $p<0.05$ was considered statistically significant.

Results

HFD increases bodyweight and causes physiological changes

A statistically significant increase in bodyweight of the HFD group relative to the SD group was found for the last time point (MD = 49.5 g; 95% CI 12.0 - 86.9 g; p=0.010; Figure 2). Post-mortem inspection of the rat liver revealed pronounced hepatosteatosis in all HFD exposed animals (insert in Figure 2).

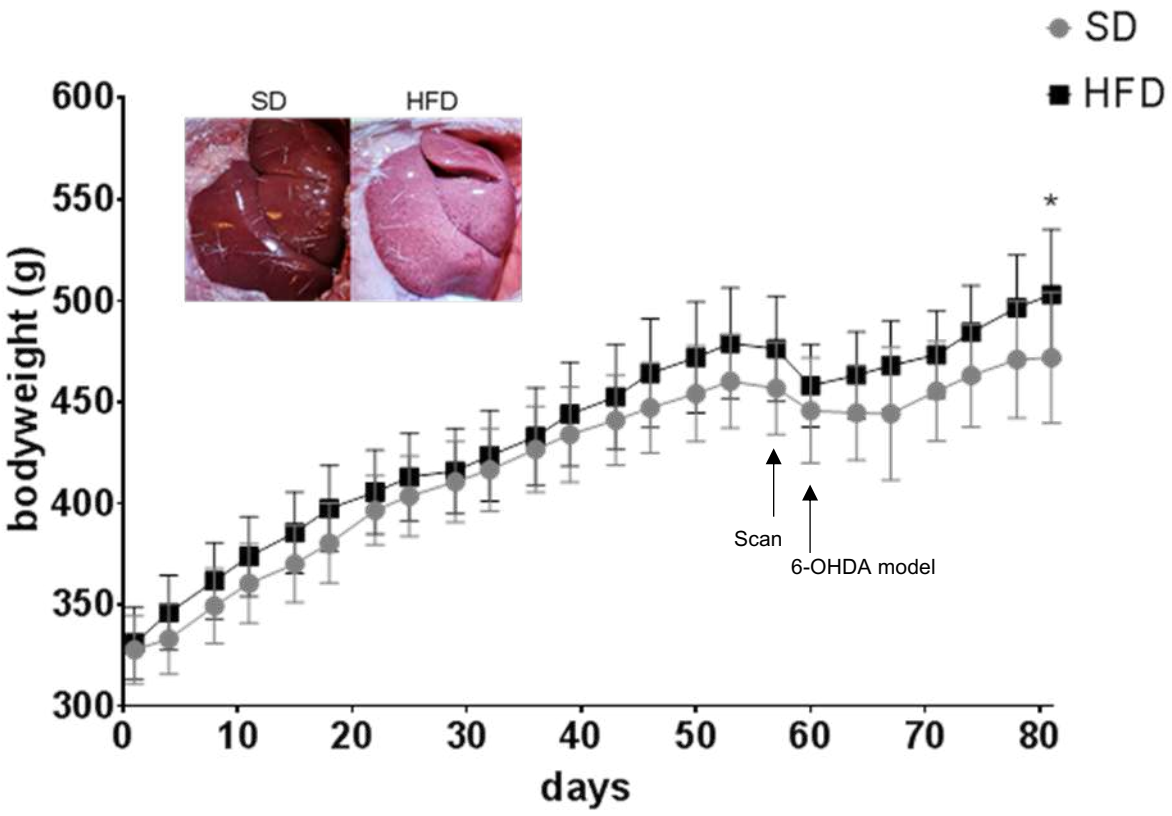


Figure 2: Physiological changes. Body weight change of rats fed with a standard diet (SD) or a high-fat diet (HFD) during the experiment. The small drop in bodyweight around day 60 is due to the PET scan and the surgical intervention (striatal injection of 6-OHDA). The insert shows representative pictures of the livers of animals fed with SD and HFD. A statistically significant difference is represented as *p<0.05.

HFD aggravates locomotor asymmetry

Statistical analysis of the cylinder test showed no main effect of the diet on the animal's forepaw preference. However, there was a significant main effect of time (p=0.003). When analyzing the interaction between time and dietary treatment, a significant decrease in the use of the left paw between 1 week and 3 weeks after 6-OHDA injection was observed in the HFD group (MD = 14.9; 95% CI = 6.0 – 23.8%; p=0.001). In the SD group, no statistically significantly difference between time points was found. No statistically significant difference between SD and HFD was found at any time point (Data not shown).

[^{11}C]-PBR28 PET reveals neuroinflammation in several areas of the brain

[^{11}C]-PBR28 PET was performed to study the effect of the HFD on neuroinflammation caused by the 6-OHDA injection. The right striatum showed a main effect of time ($p < 0.001$),

but not of the diet. Post-hoc analysis showed a significantly higher tracer uptake in the right striatum in the HFD group 1 month after 6-OHDA injection than in the HFD group (MD = -0.12; 95% CI = -0.21 – -0.02; p=0.005) and the SD group (MD = -0.10, 95%CI = -0.019 - -0.14, p = 0.013) before 6-OHDA injection. No significant differences were found in the left striatum (Figure 3a). Averaged PET images of striatal [^{11}C]-PBR28 uptake are presented in Figure 3d.

To reduce the effect of intra-individual differences in tracer delivery to the brain, the ratio of the [^{11}C]-PBR28 uptake between right and left striatum was also determined. Statistical analysis with the GEE revealed a significant main effect of time (p=0.002), but no main effect of the diet. When analyzing the interaction between time and diet, a significantly higher right/left striatal [^{11}C]-PBR28 uptake ratio was observed in HFD animals after the 6-OHDA injections when compared to the SD animals (MD = -0.24; 95% CI = -0.45 - -0.03; p=0.012) and HFD animals (MD = -0.24 95% CI = -0.45 - -0.02; p=0.022) before the 6-OHDA injections (Figure 3b).

To determine if regions that share projections with the striatum present signs of neuroinflammation, we also analyzed the left *globus pallidus*, right *globus pallidus*, *substantia nigra* and thalamus (Figure 3c). When analyzing the *globus pallidus* in each hemisphere separately, there was a significant time effect for the left *globus pallidus* (p<0.001), but not a main effect of the diet. When analyzing the interaction between time and dietary treatment, a significantly higher [^{11}C]-PBR28 uptake was found in the SD group before 6-OHDA injection when compared to the SD group (MD = -0.09; 95% CI = -0.15 – -0.037; p<0.001) and the HFD group after 6-OHDA injection (MD = -0.08; 95% CI = -0.16 – -0.007; p=0.047). In the right *globus pallidus* these effects were even more pronounced, with a strong main effect of time (p<0.001) and interaction between time and dietary treatment (SD before vs. SD after 6-OHDA injection: MD = -0.12; 95% CI = -0.23 – -0.01; p=0.017; SD before vs. HFD after 6-OHDA injection: MD = -0.15, 95% CI = -0.27 – -0.02; p=0.011). For the *substantia nigra*, no main effect of time was found, but only a main effect of the diet, with a lower [^{11}C]-PBR28 uptake in animals on the HFD (p=0.043). No interactions between time and diet were found. In the thalamus, a main effect of time (p=0.001), but not of the diet, was observed. When analyzing the interaction between time and dietary treatment, a significantly higher [^{11}C]-PBR28 uptake is found in the SD group after than before 6-OHDA injection (MD = -0.09; 95% CI = -0.14 – -0.05; p<0.001). No significant differences were observed in the HFD group.

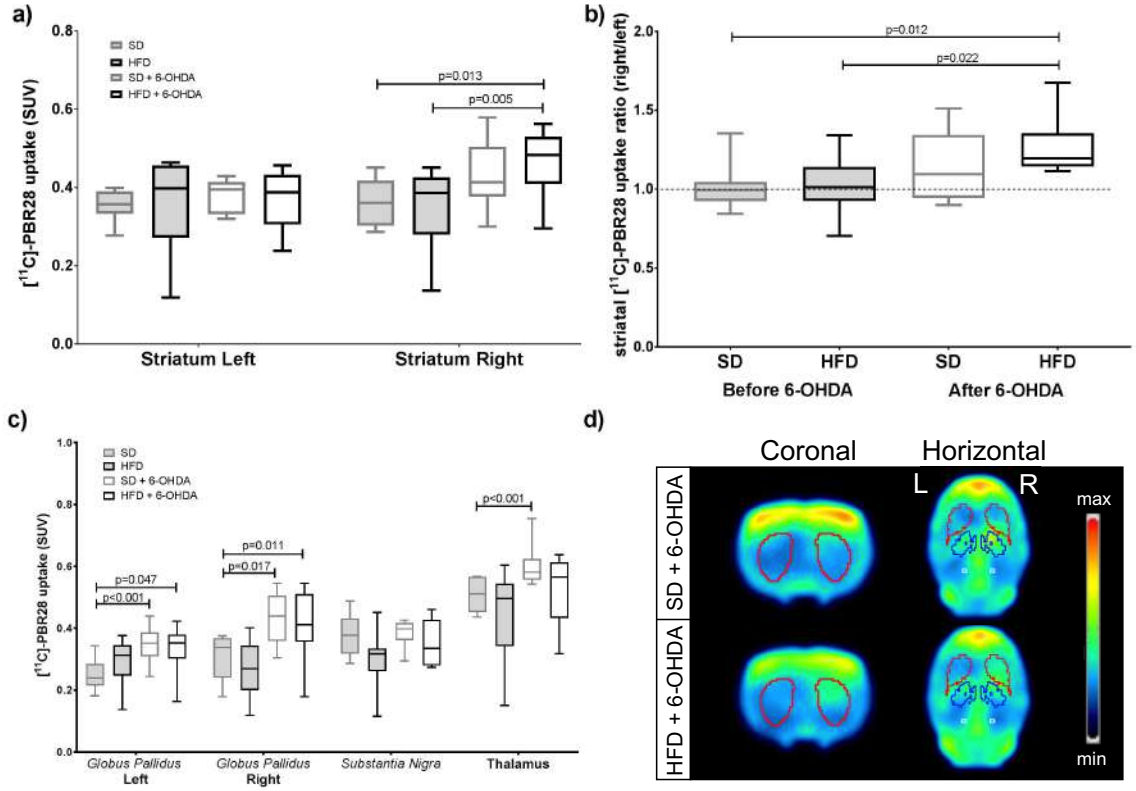


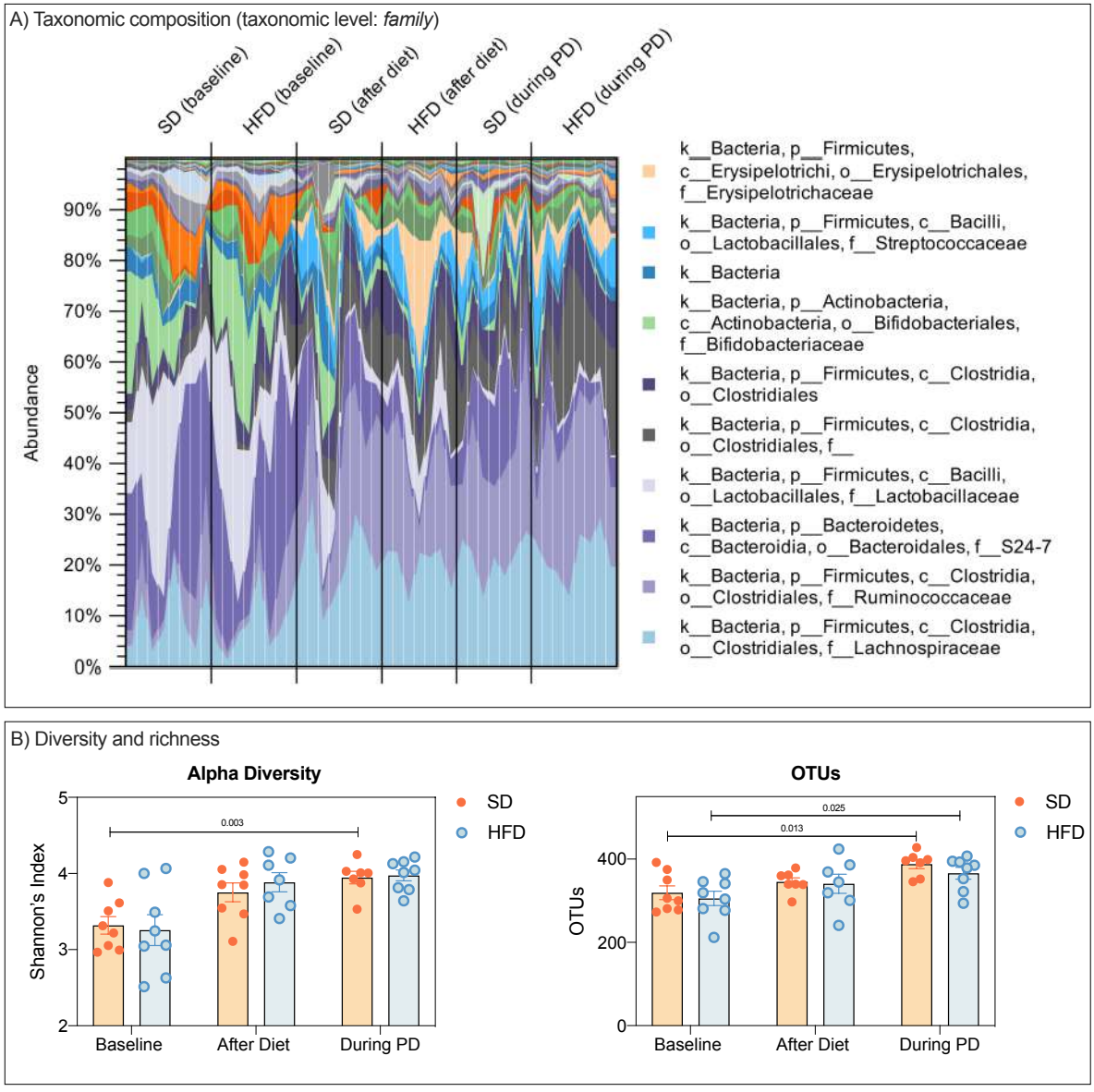
Figure 3: [^{11}C]-PBR28 PET results. Longitudinal changes in [^{11}C]-PBR28 uptake (SUV) in the brains of animals fed a standard diet (SD) or a high-fat diet (HFD) are shown. All animals were injected with 6-OHDA in the right striatum at 2 months after the start of the diet. a) [^{11}C]-PBR28 uptake in left and right striatum, expressed as SUV. b) Ratio of [^{11}C]-PBR28 uptake between right and left striatum. c) [^{11}C]-PBR28 uptake (SUV) in the regions connected to the striatum, in particular *globus pallidus* (right and left), *substantia nigra* and thalamus. d) Coronal and horizontal [^{11}C]-PBR28 PET images (averaged group images) of rats fed for 3 months with SD or HFD that were injected with 6-OHDA at 2 months after the start of the diet. ROIs representation: red= striatum, blue = *globus pallidus*, white = *substantia nigra*. Data is represented as median, interquartile ranges and min-max values.

HFD changes the gut microbiota profile

To investigate the effect of the HFD and 6-OHDA injection on the gut microbiota, we collected feces from the SD and HFD groups at three different time points: before diet (baseline), after 2 and 3 months of diet. The 3 month time point is 1 month after 6-OHDA injection. Changes in microbiota were (a) specific to each diet, and (b) reflective of time and therefore independent of dietary intervention. Taxonomically, the microbiota of both groups was dominated by the Firmicutes phylum and a high baseline abundance of families of *Lactobacillaecae* and *Muribaculaceae* (Figure 4A). Both experimental groups started with comparable baseline

diversity and richness (Figure 4B). The SD induced a significant increase in alpha diversity with time ($p<0.01$ baseline vs 1 month after 6-OHDA injection). There was no significant change in alpha diversity in the HFD group. In contrast, the Operational Taxonomic Units (OTUs) of both groups increase from baseline to month 3 (SD: $p=0.013$, HFD: $p=0.025$), likely reflecting maturation of the resident microbiota with increasing age of the rats.

Despite no differences in alpha diversity and OTUs between groups, the composition of the microbiota differed between the SD and HFD groups, with clear clustering of the groups (control and high fat diet) observed both after 2 and 3 months of the diet (Figure 4C).



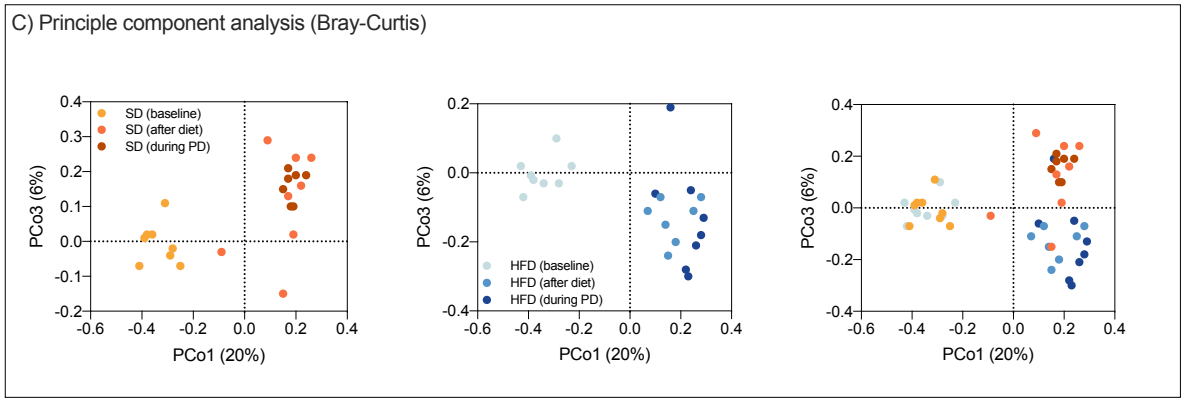


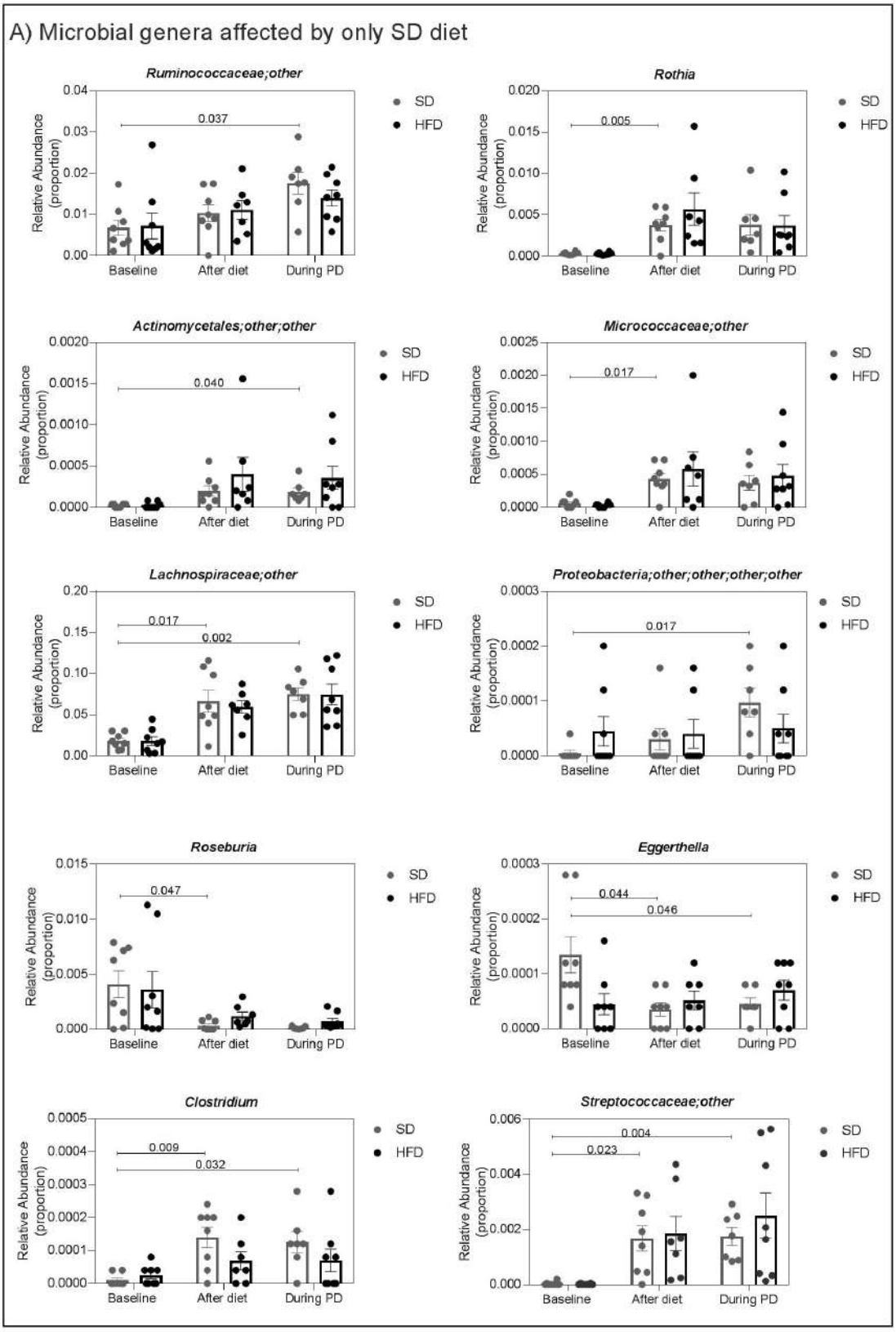
Figure 4: Feces analysis of microbiota diversity. Analysis of microbiota diversity was assessed for the HFD and SD at different time points. A) Taxonomic composition (Family), B) Diversity and richness. C) Principle component analysis (Bray-Curtis). Graphs represent mean ± SEM.

The overall changes in the 50 most abundant microbial genera are visualized in Supplementary data 1A. First, changes that occurred longitudinally in response to both diets were determined to identify changes that were considered a reflection of time/maturity. Decreases in the relative abundance of *Lactobacillus* were observed following both dietary interventions, whilst increases in *Clostridiales*;f_;g_, *Ruminococcaeae*;g_, *Ruminococcus*, *Lachnospiraceae*;other, *Lactococcus*, *Coprococcus* and *Christensellaceae*;g_ were also observed (Supplementary data 2A).

To identify longitudinal changes in microbial genera that were restricted to each diet, we performed a mixed effect model with *time x treatment* as factors (Figure 5A, 5B). In case two p-values are given, both follow-up time points were significantly different from baseline. Otherwise, only 1 follow-up time point showed a significant difference. Increases in *Ruminococcaceae;other* (p=0.037), *Rothia* (p=0.005), *Actinomycetales;other;other* (p=0.040), *Micrococcaceae;other* (p=0.017), *Lachnospiraceae;other* (p=0.017, p=0.002), Protobacteria (p=0.017), *Clostridium* (p=0.009, p=0.032), and *Streptococcaceae;other* (p=0.023, p=0.004) relative to baseline were all identified following the SD. These increases were coupled with decreases in *Roseburia* (p=0.047) and *Eggerthell*a (p=0.044, p=0.046). In contrast, the HFD group showed profound losses in the Bacteroidales order (p=0.045, p=0.036), *Prevotella* (p=0.025), *Muribaculaceae* (p=0.015, p=0.020) and *Lactobacillaceae;other* (p=0.011). These losses were coupled with an increase in *Facklamia* (p=0.033), *Dehalobacterium* (p=0.018), *Blautia* (p=0.031), *Streptococcus* (p=0.032, p=0.049) and *Mogibacteraceae;g_* (p=0.015, p=0.020).

We identified five microbial genera that were differentially expressed following dietary intervention (before 6-OHDA), with the HFD group showing a higher abundance of *Allobaculum* (p=0.005), *Erysipelotrichaceae;g_* (p<0.0001), *Mucipirilum* (p=0.004) and

*Bacteroidales;f_;g_* (p=0.002) and a lower abundance of *Muribaculaceae* compared to the SD group (p<0.0001) (Figure 6A).



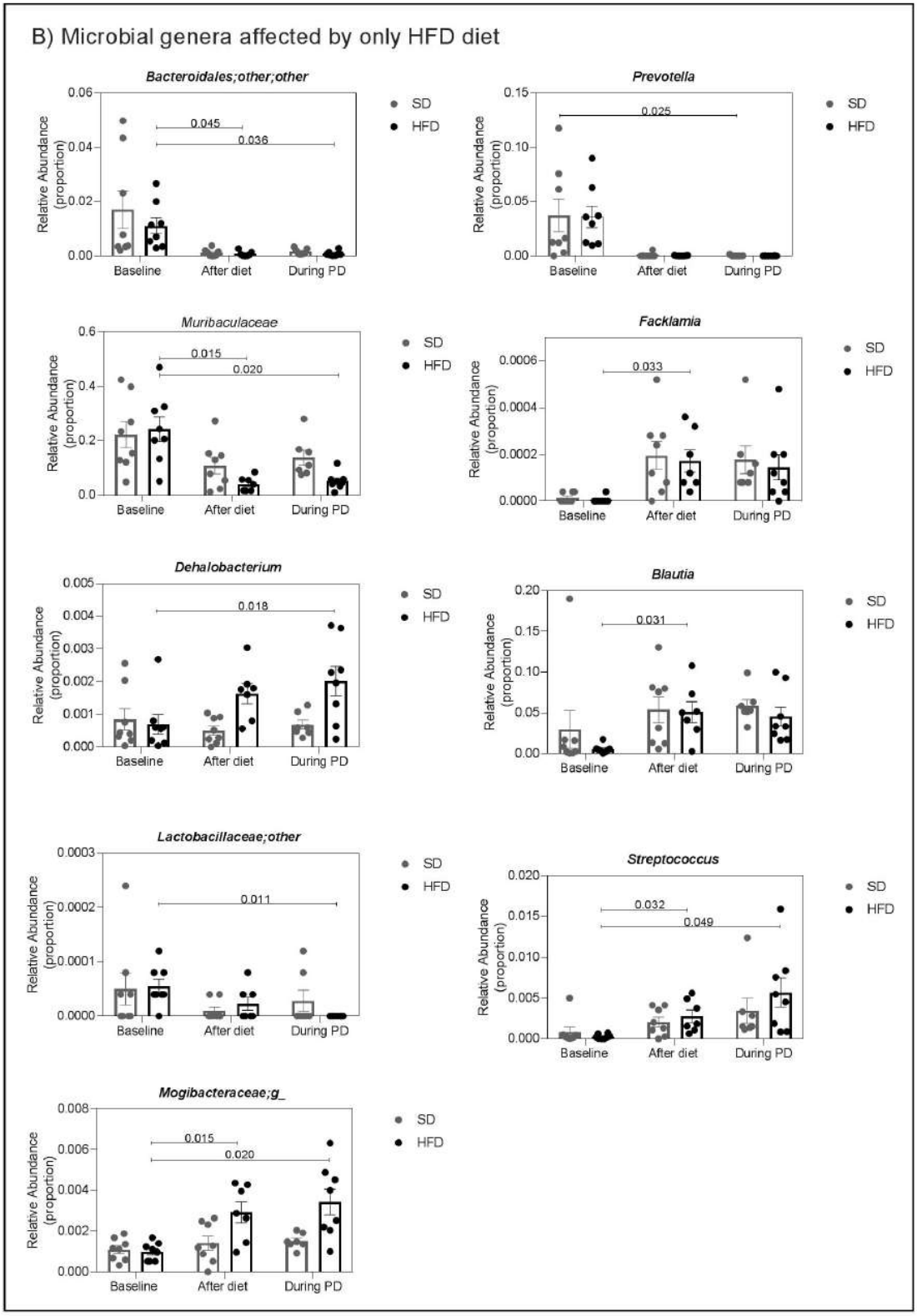


Figure 5: Changes in Microbiota genera due to diet. A) Genera changes in the SD group. B) Genera changes in the HFD group. Data is represented as mean ± SEM. Gray represents the SD group and black the HFD group.

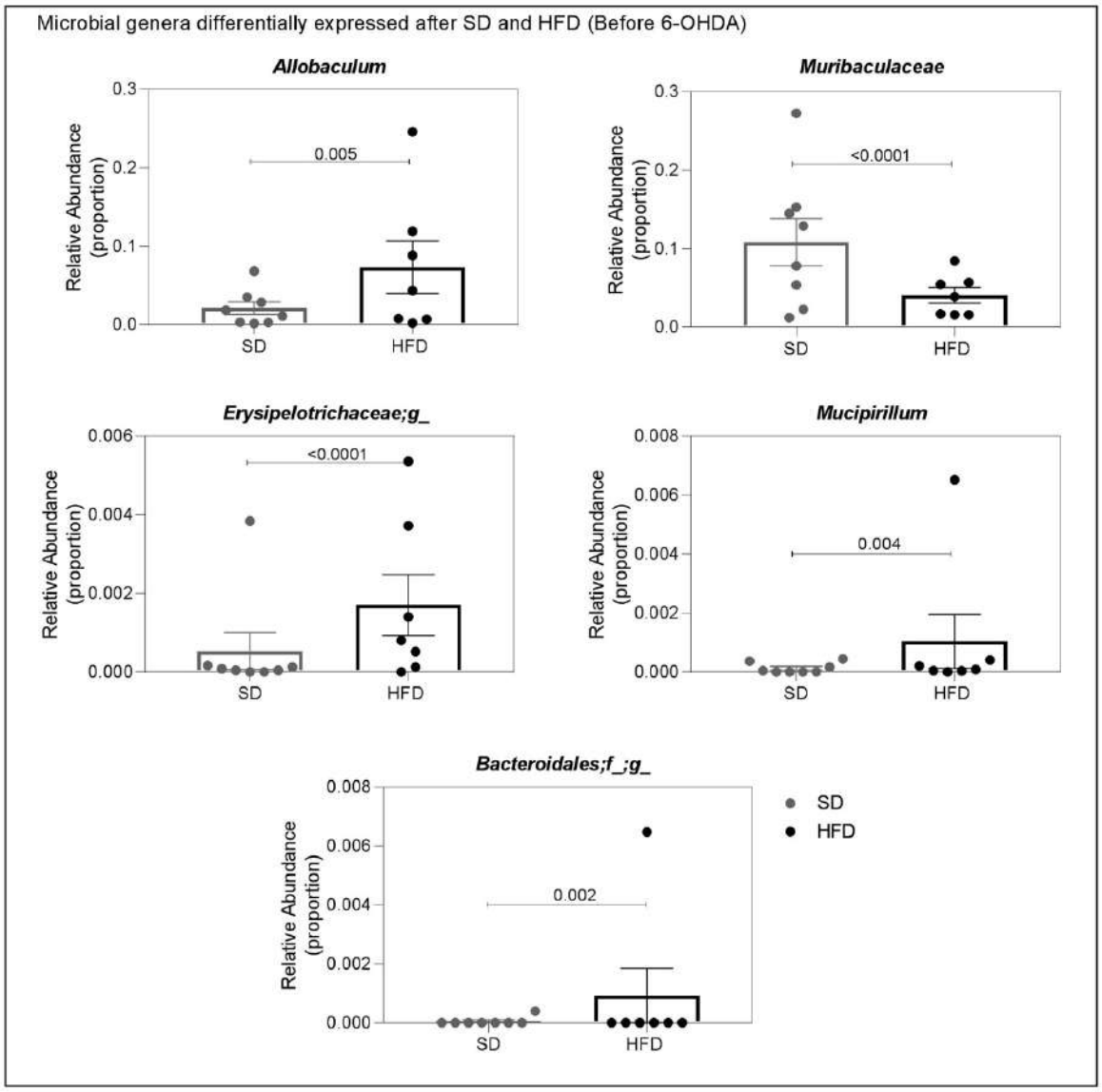
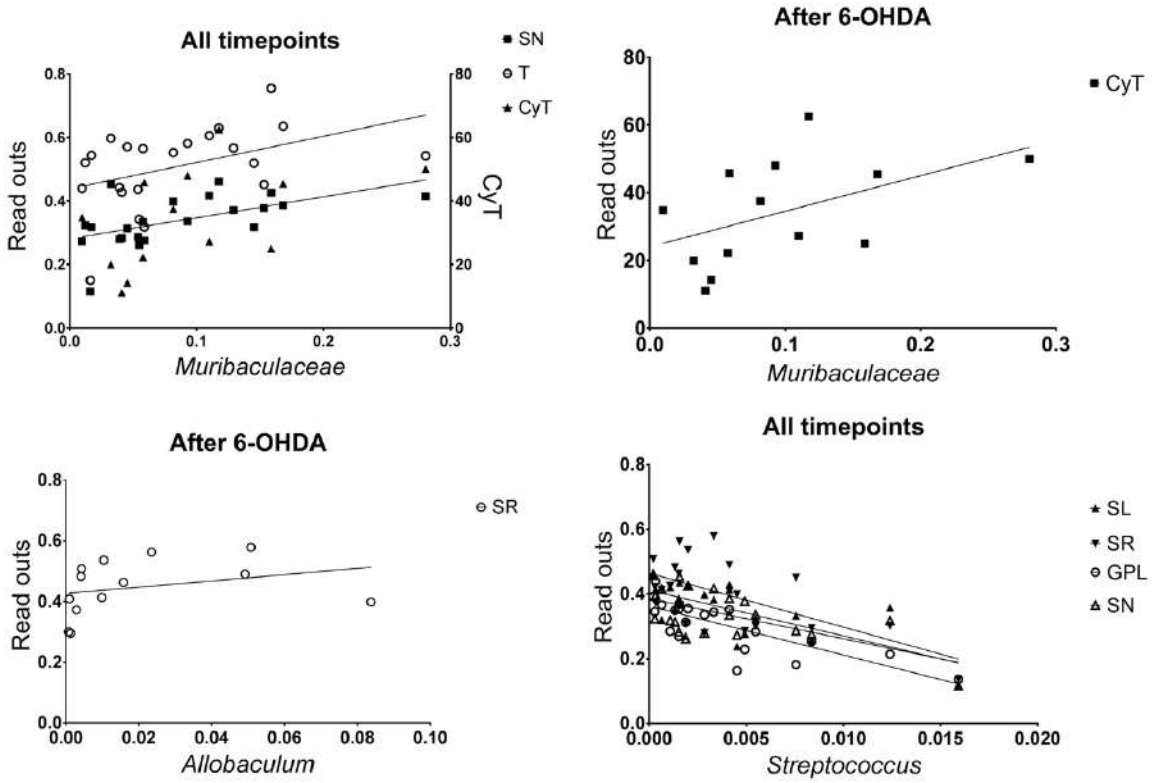


Figure 6: Changes in Microbiota genera due to diet. Microbial genera changes over time due to diet are shown. Microbial genera with differential abundance after diet (before 6-OHDA). Data is represented as mean ± SEM. Gray represents the SD group and black the HFD group.

HFD-induced microbiota changes correlate with neuroinflammation

To understand the functional impact of these microbial changes on the effects of the diet and 6-OHDA injection, correlation analyses between the microbial genera that were differentially expressed between the SD and HFD group, and read outs from [^{11}C]-PBR28 PET and the cylinder test were performed (Figure 7). Data from both the HFD and the SD group acquired after 2 months (just before 6-OHDA injection), or 3 months on diet (1 month after 6-OHDA injection), or the combined data from both time points were used in the analysis. Spearman correlations and p-values of significant correlations are summarized in Table 1. Positive

correlations were observed between *Muribaculaceae* abundance and [^{11}C]-PBR28 uptake in *substantia nigra* and thalamus, or asymmetric forepaw use when combining the data from both time points and also between *Muribaculaceae* abundance and asymmetric forepaw use when using only the 3-month data. A positive correlation was also observed between *Allobaculum* abundance and [^{11}C]-PBR28 uptake in right striatum when analyzing only the 3-month time point data. Negative correlations were found between *Streptococcus* abundance and [^{11}C]-PBR28 uptake in left and right striatum, left *globus pallidus* and *substantia nigra* when analyzing data from both time points. When only 2-month time was analyzed the left striatum and *globus pallidus* showed a significant correlation with *Streptococcus* abundance. Also, atthe 3-month time point, only the left *globus pallidus* showed a significant correlation with *Streptococcus* abundance. Negative correlations were also observed between *Dehalobacterium* abundance and [^{11}C]-PBR28 uptake in right and left *globus pallidus* when the combined data of both time points were used. This correlation persisted for the right *globus palllidus*, when only the data of the last time point were used. But a positive correlation between striatum right and the read out was found when analyzing only after diet. Also, a significant correlation was observed between *Mogibacteraceae* and [^{11}C]-PBR28 uptake in left *globus pallidus* when analyzing the combined data from both time points.



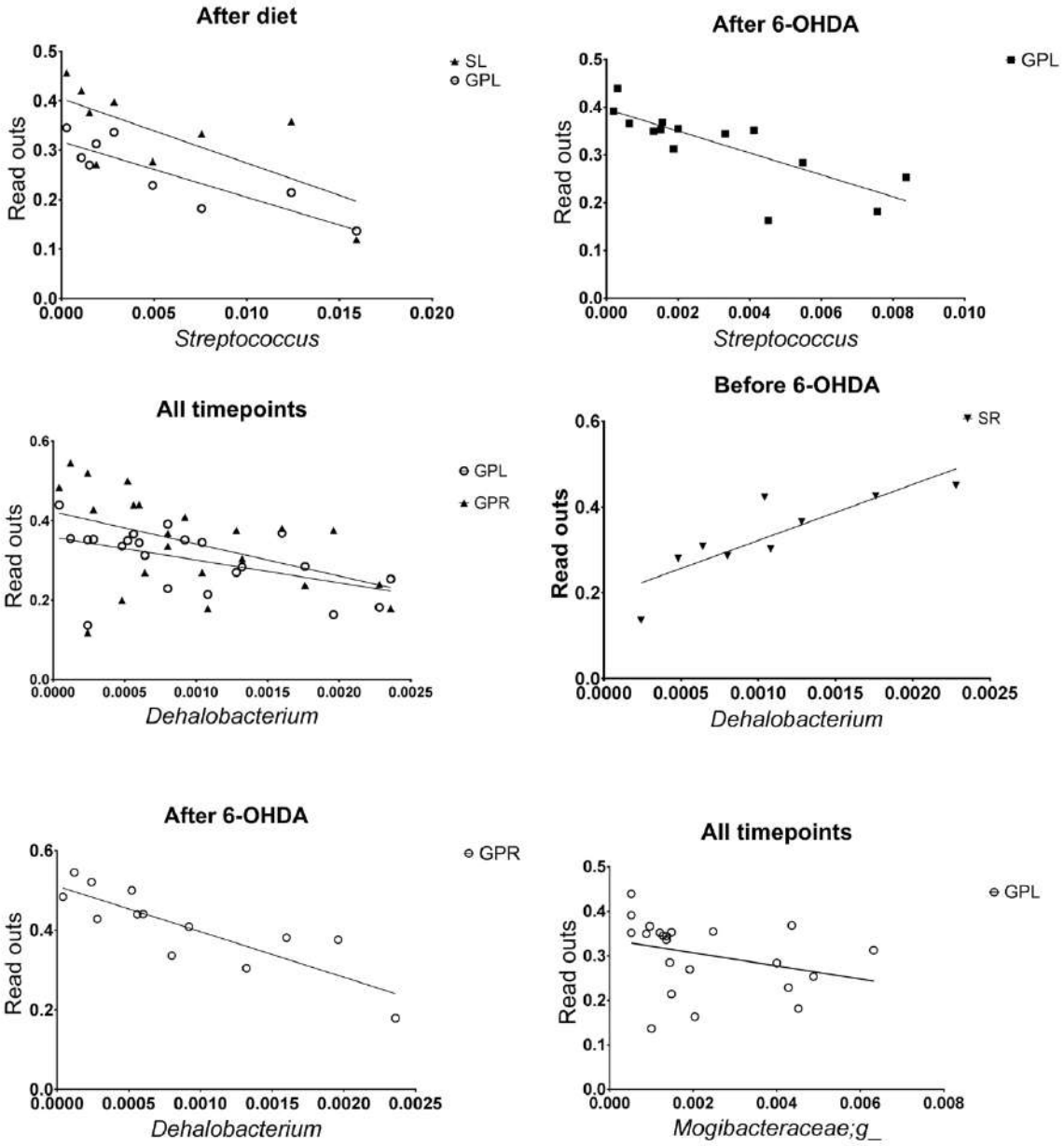


Figure 7: **Correlation analyses.** Correlations of the cylinder test or [^{11}C]-PBR28 PET readouts with the relative abundance of several microbiota. SR= striatum right, SL= striatum left, GPR= *globus pallidus* right, GPL= *globus pallidus* left, SN= *substantia nigra*, T= thalamus, CyT= cylinder test.

Table 1. Correlations between microbiota abundance and behavioral and neuroinflammatory parameters (data acquired after 2 months (before 6-OHDA); or 3 months (after 6-OHDA) on diet; or the combined data from both time points). Microbiome specific genera correlations with cylinder test and [^{11}C]-PBR28 PET outcomes are shown in this table. Correlations are presented as Spearman's correlation coefficient (r) and their probability (p-value).

	Muribaculaceae	*Allobaculum*	*Streptococcus*	*Dehalobacterium*	*Mogibacteraeceae*;g
Striatum Left			All data: r=-0.58 p=0.004 2 months only: r=-0.73 p=0.03		
Striatum Right		3 months only: r=0.60 p=0.03	All data: r=-0.46 p=0.03	2 months only: r=-0.94 p<0.001	
Globus Pallidus Left			All data: r=-0.73 p<0.001 2 months only: r=-0.83 p=0.008 3 months only: r=-0.83 p=0.007	All data: r=-0.49 p=0.02	All data: r=-0.46 p=0.03
Globus Pallidus Right				All data: r=-0.49 p=0.02 3 months only: r=-0.86 p=0.003	
Substantia Nigra	All data: r=0.60 p=0.003		All data: r=-0.42 p=0.049		
Thalamus	All data: r=0.46 p=0.03				
CyT week 3	All data: r=0.61 p=0.02 3 months only: r=0.61 p=0.02				

No evidence of peripheral inflammation

Analyses of cytokines in plasma at the 3-month time point showed no statically significant differences between the SD and HFD group for IFN-γ, IL-10, IL-4, KC/GRO and TNF-α ($p>0.05$; Figure 8). The levels of IL-13, IL1 β, IL-5, IL-6 in plasma were below the detection limit of the analysis method for both groups and therefore could not be assessed.

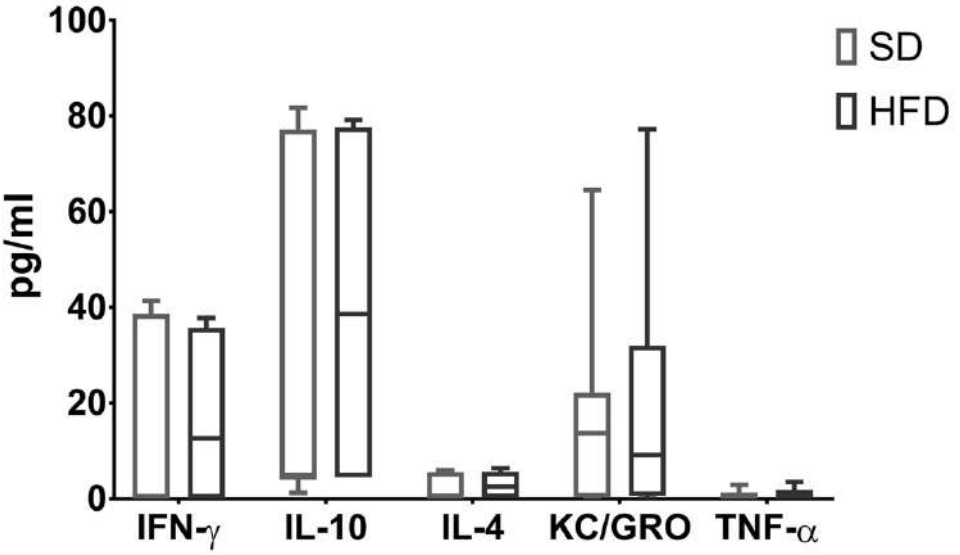


Figure 8: Profiling of pro- and anti-inflammatory cytokines (IFT-y, IL-10, IL-4, KC/GRO, TNF-alpha) in the plasma of rats fed with HFD or SD for 3 months, 1 month after injection of 6-

OHDA in the right striatum. Data is represented as median, interquartile ranges and min-max values.

Discussion

This study demonstrated the consequences of a HFD on disease development in the 6-OHDA animal model of PD. In particular, we showed that the HFD caused an increase in body weight and a fatty liver, and aggravated 6-OHDA induced locomotor asymmetry and neuroinflammation in the right striatum, while plasma cytokine levels remained unchanged. Interestingly, the HFD affected the composition of gut microbiota. Our findings support the hypothesis that a diet high in fat has detrimental effects on the brain and motor symptoms in in PD and suggest that this phenomenon is mediated by diet-induced changes in the microbiota.

Previous studies showed that a HFD can induce a significant body weight gain, starting from two to eight weeks after the start of the diet (Da Silva Rocha et al., 2019; Papazoglou, Jean, Gertler, Taouis, & Vacher, 2015). However, comparison between studies is difficult due to differences in the animal's age at the start of the diet (Nistiar et al., 2012), duration of the dietary protocol and the dietary composition of the chow. In our study, we only observed significant differences in bodyweight between the SD and HFD group after 3 months. This delayed effect could be due to the PET scan and stereotactic surgery after eight weeks of diet, which induced transient mild-to-moderate loss of body weight. However, *postmortem* analysis revealed pronounced hepatosteatosis in animals treated with the HFD, confirming that the HFD affected the health of the animals. This indicates that future studies on HFD should not only take body weight into account, but also include other metabolic and morphometric parameters, such as percentage of body fat, in order to have a specific measure of HFD-dependent body fat deposition.

In this study, we aimed to investigate the effects of the HFD on what we consider the beginning of dopaminergic depletion. Therefore, only a low dose of 6-OHDA was used to induce dopaminergic neurotoxicity without complete loss of motor function. According to literature, this dose is not enough to generate permanent dopaminergic damage, since animals recover from the neuronal damage and regain their normal functions at two months after 6-OHDA injection (Maia et al., 2012). The cylinder test was performed in our study as a method to identify locomotor asymmetry of the forepaws and thus to confirm the induction of dopaminergic nerve damage in this unilateral rat model for PD (Real et al., 2019). Our findings showed that the HFD exacerbated the asymmetry in forepaw use, suggesting that the HFD provoked a more severe PD phenotype.

This more severe PD phenotype was also observed by PET imaging with the neuroinflammation marker [^{11}C]-PBR28. Animals exposed to the HFD showed a significant increase in neuroinflammation in various brain areas. In our study, neuroinflammation was particularly exacerbated by the HFD in striatum. This region is primarily responsible for motor control, but also for motor learning, executive functions and behavior, and emotions (Lanciego, Luquin, & Obeso, 2012). Several publications report the involvement of the immune system and neuroinflammation in PD (Benkler et al., 2012; Kannarkat, Boss, & Tansey, 2013), but its relation with diet is not clear yet. Targeting neuroinflammation may be relevant to slow down the progression of PD (Hirsch & Hunot, 2009). Therefore, knowing which factors, besides alpha-synuclein depositions, are affecting neuroinflammatory processes in PD is important to enable the development of new therapies. As a potential mechanism for exacerbated neuroinflammation we investigated the gut microbiota composition.

We identified increases in the relative abundance of *Allobaculum*, *Dehalobacterium*, *Streptoccocos*, *Mogibacteraceae;_g* and a decrease in *Muribaculaceae* abundance after 3 months of HFD, as well as a correlation of their relative abundance with neuroinflammation or motor symptoms in our PD model. Interestingly, these findings support clinical observations, showing that the relative abundance of these bacteria is altered in subjects following a HFD and in obese people (Lee, Han, & Yim, 2015). Of the bacteria affected by a HFD, the changes in *Allobaculum* are of particular interest due to the influence on mucosal barrier function, a critical factor in gut-brain communication (Lee et al., 2015). Increased *Allobaculum* has been reported in people following a HFD, but also in a preclinical model of streptozocine-induced diabetes mellitus type 2 (S. Liu, Qin, & Wang, 2019)(Chan et al. 2016) and an animal model of PD (Perez-Pardo et al., 2018). In the model of PD with increased *Allobaculum* abundance, Toll-like receptor 4 (TLR4) proved critical for developing the PD phenotype (Perez-Pardo et al., 2018). There is also contradictory evidence, suggesting that the abundance of this bacterium decreases following a HFD (Qiao, Sun, Xie, Shi, & Le, 2014).

In addition to *Allobaculum*, we also showed a significant increase in the abundance of *Mogibacteraceae*, a microbe reported to be in higher abundance in obese people (Duvallet, Gibbons, Gurry, Irizarry, & Alm, 2017), as well as in mice fed with a HFD that were prone to develop dementia (Muhomah, Nishino, Katsumata, Haoming, & Tsuruta, 2019; Sanguinetti et al., 2018). While evidence suggests a contribution of *Mogibacteraceae* to the development of Alzheimer's disease (inverse relationship), its potential contribution to PD has yet to be identified (Gerhardt & Mohajeri, 2018). Similarly, while some preclinical evidence suggests PD is associated with decreased *Muribaculaceae* abundance, as observed following a HFD, its mechanistic role in PD remains unclear (Muhomah et al., 2019; Sanguinetti et al., 2018). *Dehalobacterium* is reported to be less abundant in obese patients (Peters et al., 2018), negatively associated with higher body mass index (Fu et al., 2015) and reduced in animals

fed with HFD (Chan et al., 2016). In contrast to the literature, the HFD in our study induced an increase in *Dehalobacterium*, which was correlated with neuroinflammation in *globus pallidus*. This discrepancy may reflect the integration of the two models (PD and HFD) used in our study.

In addition to chronic dysbiosis, acute infectious events involving the microbiota have been speculated to underpin PD pathogenesis. For example, a case of severe acute Parkinsonism associated with Streptococcal infection was reported in 2005 (McKee & Sussman, 2005). This observation was suggested to involve the Toll-like receptor 4, TLR4, which is reported to govern intestinal barrier function and local immune response (de Kivit, Tobin, Forsyth, Keshavarzian, & Landay, 2014). In line with this finding, our study showed that a HFD induced a significant increase in fecal *Streptococcus*. This could be due to a higher bile salt production and a lower amount of bile resistant bacteria, therefore higher number of *streptococceae*, but had a negative correlation with the read outs. However, this and other microbial changes were not strong enough to induce any profound effect on peripheral cytokine levels in our study.

As described, we focused on microbiota composition as a mediator for the impact of HFD on PD. One proposed mechanism is HFD-induced LPS production, resulting in the hyperactivation of TLR4 (Kim, Gu, Lee, Joh, & Kim, 2012), consequently increasing innate immune activation, barrier dysfunction, LPS translocation and peripheral inflammation (Manco, Putignani, & Bottazzo, 2010). Studies demonstrated that the absence of TLR4 (model of TL4 deficiency mice) prevents loss of striatal dopaminergic neurons and reduces the progress of neuroflammation associated with PD (Campolo et al., 2019). Thus, TLR4-mediated inflammation can be linked to intestinal and/or brain inflammation (Perez-Pardo et al., 2017).

The microbes that were most significantly associated with neuroinflammation and motor symptoms were gram positive and thus their influence on LPS production is limited. Although we did not measure LPS production, we evaluated the cytokines in the blood as peripheral inflammatory biomarkers. Our results suggest that the changes in the microbiota were not able to promote proinflammatory pathways that were detectable in peripheral blood. It is plausible that that the microbial changes we observed caused only local intestinal inflammation. However, it is important to note that the mechanisms by which the microbiota aggravates PD development are unclear. As an alternative mechanism to the circulating cytokines, altered SCFA production could be responsible. It has been shown that circulating SCFA are decreased in PD patients (Unger et al., 2016)

Direct communication between the gut and the brain via vagal nerve signaling might also be a possible mechanism driving gut-dependent PD mechanisms (Forsythe, Bienenstock, & Kunze, 2014), with mounting evidence supporting a crucial role of this pathway in various

aspects of neurophysiology and behavior (Fülling, Dinan, & Cryan, 2019). These processes can influence the progression of neurodegenerative diseases (Guzman-Martinez et al., 2019), and may be a risk factor for PD development and progression (Chen et al., 2014).

Conclusion

This study suggests a detrimental effect of HFD on inflammatory processes in the brain and the gut microbiome in the 6-OHDA rat model of Parkinson's disease. Just HFD alone can cause unique changes in the microbiota and these changes align with microbial signatures associated with PD patients. The findings are not correlated with the cytokine levels in the blood, suggesting gut-brain communication via other mediators like SCFA, or a direct interaction between the brain and gut via the vagal nerve. Our findings are (in some ways) consistent with microbial changes reported in high fat diet studies and obesity. Diet being a possible risk factor for PD is supported by the association of PD with microbiota instability. Thus, microbiota may provide a targetable and modifiable biomarker for early detection and prevention of PD.

Conflict of interest statement

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

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Supplementary data

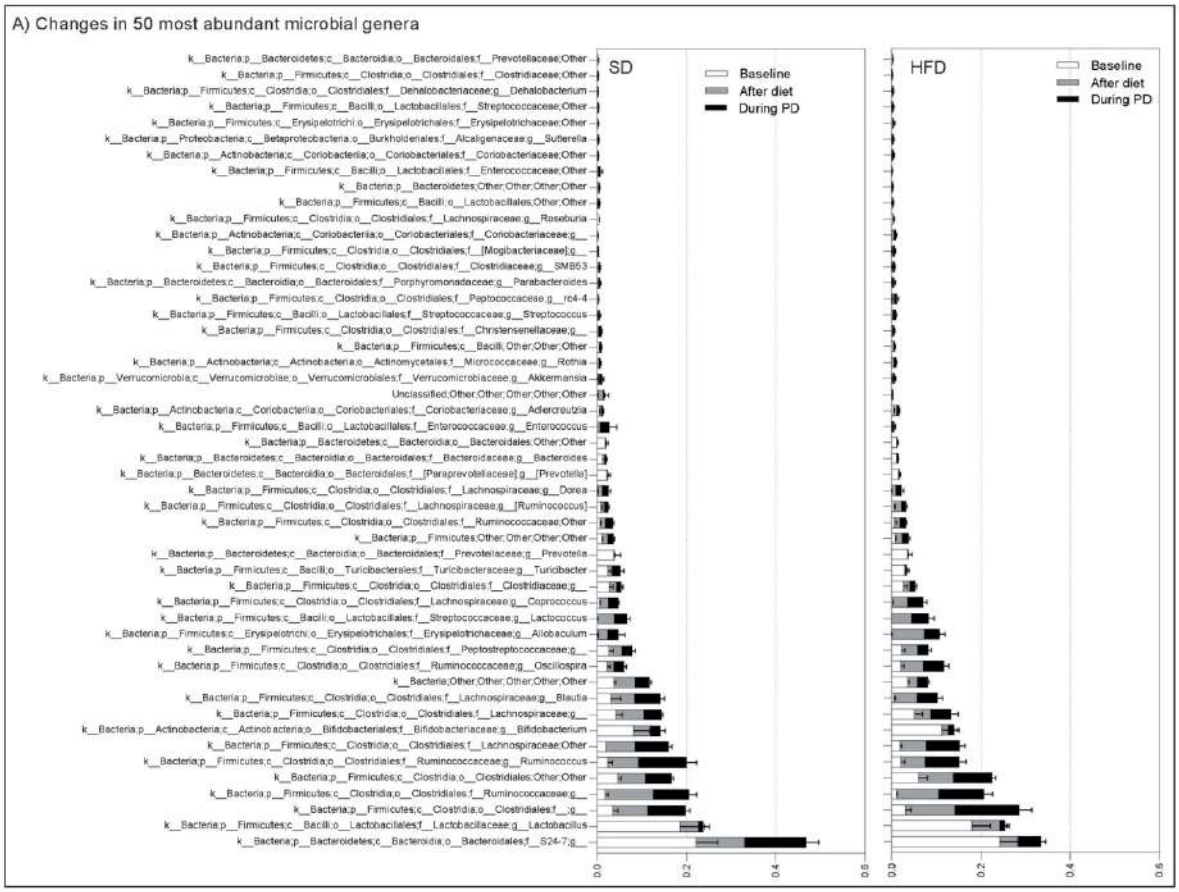
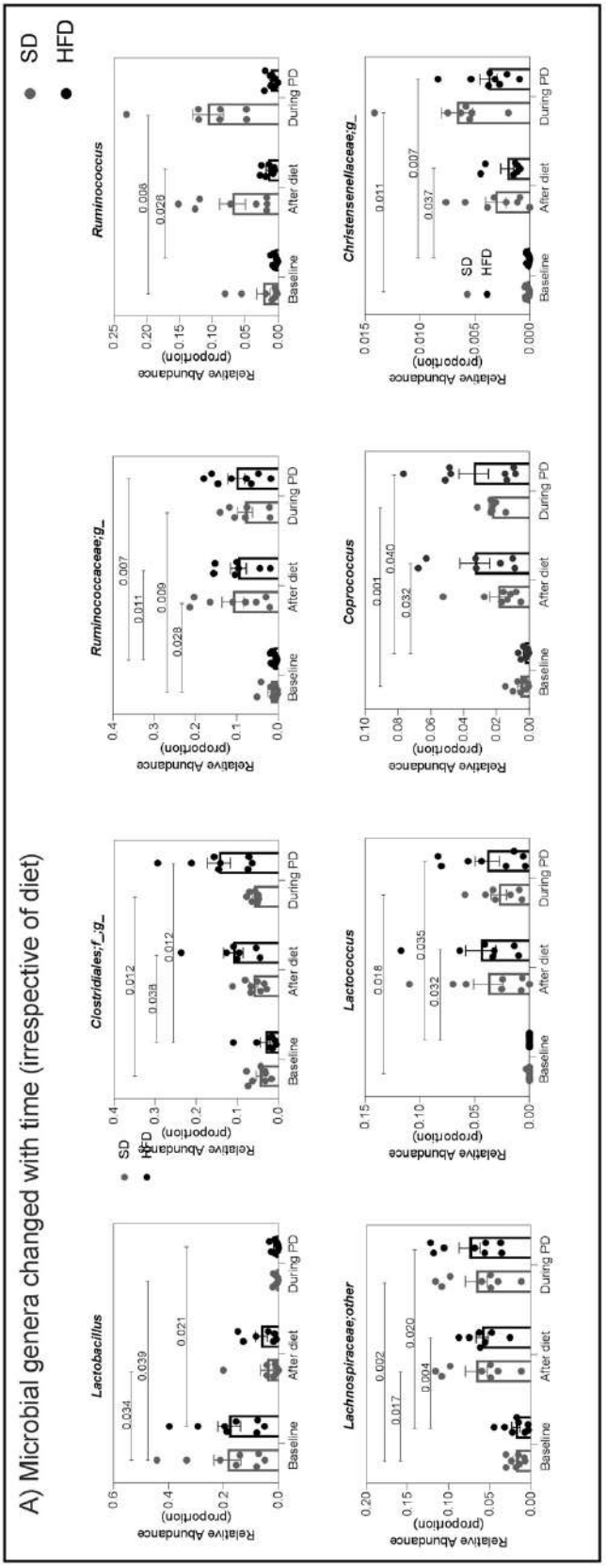


Figure Supplementary data 1A: Changes in Microbiota genera due to time and not diet. Longitudinal changes of microbiota genera were analyzed for HDF and SD at different time points. A) Changes in the 50 most abundant microbial genera. Data is represented as mean ± SEM.



Supplementary data 2: Changes in Microbiota genera due to time and not diet. Longitudinal changes of microbiota genera were analyzed for HDF and SD at different time points. A) Microbial genera changes with time (irrespective of diet). Data is represented as mean ± SEM.

References

Adams, B., Nunes, J. M., Page, M. J., Roberts, T., Carr, J., Nell, T. A., … Pretorius, E. (2019). Parkinson's disease: A systemic inflammatory disease accompanied by bacterial inflammagens. *Frontiers in Aging Neuroscience*, *10*(JUL), 210. https://doi.org/10.3389/fnagi.2019.00210

Batista, C. R. A., Gomes, G. F., Candelario-Jalil, E., Fiebich, B. L., & de Oliveira, A. C. P. (2019, May 1). Lipopolysaccharide-induced neuroinflammation as a bridge to understand neurodegeneration. *International Journal of Molecular Sciences*, Vol. 20. https://doi.org/10.3390/ijms20092293

Benkler, M., Agmon-Levin, N., Hassin-Baer, S., Cohen, O. S., Ortega-Hernandez, O. D., Levy, A., … Shoenfeld, Y. (2012, April 14). Immunology, autoimmunity, and autoantibodies in parkinson's disease. *Clinical Reviews in Allergy and Immunology*, Vol. 42, pp. 164–171. https://doi.org/10.1007/s12016-010-8242-y

Braak, H., Del Tredici, K., Rüb, U., De Vos, R. A. I., Jansen Steur, E. N. H., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of Aging*, *24*(2), 197–211. https://doi.org/10.1016/S0197-4580(02)00065-9

Brown, G. C. (2019, September 13). The endotoxin hypothesis of neurodegeneration. *Journal of Neuroinflammation*, Vol. 16, p. 180. https://doi.org/10.1186/s12974-019-1564-7

Campolo, M., Paterniti, I., Siracusa, R., Filippone, A., Esposito, E., & Cuzzocrea, S. (2019). TLR4 absence reduces neuroinflammation and inflammasome activation in Parkinson's diseases in vivo model. *Brain, Behavior, and Immunity*, *76*, 236–247. https://doi.org/10.1016/j.bbi.2018.12.003

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Chen, J., Guan, Z., Wang, L., Song, G., Ma, B., & Wang, Y. (2014). Meta-analysis: Overweight, obesity, and Parkinson's disease. *International Journal of Endocrinology*, Vol. 2014. https://doi.org/10.1155/2014/203930

Da Silva Rocha, V., Gonçalves Claudio, E. R., Da Silva, V. L., Cordeiro, J. P., Domingos, L. F., Da Cunha, M. R. H., … Leopoldo, A. S. (2019). High-fat diet-induced obesity model does not promote endothelial dysfunction via increasing leptin/Akt/eNOS signaling. *Frontiers in Physiology*, *10*(MAR). https://doi.org/10.3389/fphys.2019.00268

de Kivit, S., Tobin, M. C., Forsyth, C. B., Keshavarzian, A., & Landay, A. L. (2014). Regulation of intestinal immune responses through TLR activation: Implications for pro- and prebiotics. *Frontiers in Immunology*, Vol. 5. https://doi.org/10.3389/fimmu.2014.00060

Dickson, D. W., Braak, H., Duda, J. E., Duyckaerts, C., Gasser, T., Halliday, G. M., … Litvan, I. (2009). Neuropathological assessment of Parkinson's disease: refining the diagnostic criteria. *The Lancet Neurology*, Vol. 8, pp. 1150–1157. https://doi.org/10.1016/S1474-4422(09)70238-8

Duvallet, C., Gibbons, S. M., Gurry, T., Irizarry, R. A., & Alm, E. J. (2017). Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. *Nature Communications*, *8*(1), 1–10. https://doi.org/10.1038/s41467-017-01973-8

Forsythe, P., Bienenstock, J., & Kunze, W. A. (2014). Vagal pathways for microbiome-brain-gut axis communication. *Advances in Experimental Medicine and Biology*, *817*, 115–133. https://doi.org/10.1007/978-1-4939-0897-4_5

Fu, J., Bonder, M. J., Cenit, M. C., Tigchelaar, E. F., Maatman, A., Dekens, J. A. M., … Zhernakova, A. (2015). The gut microbiome contributes to a substantial proportion of the variation in blood lipids. *Circulation Research*, *117*(9), 817–824. https://doi.org/10.1161/CIRCRESAHA.115.306807

Fülling, C., Dinan, T. G., & Cryan, J. F. (2019, March 20). Gut Microbe to Brain Signaling: What Happens in Vagus…. *Neuron*, Vol. 101, pp. 998–1002. https://doi.org/10.1016/j.neuron.2019.02.008

Gerhardt, S., & Mohajeri, M. H. (2018, June 1). Changes of colonic bacterial composition in

parkinson's disease and other neurodegenerative diseases. *Nutrients*, Vol. 10. https://doi.org/10.3390/nu10060708

Gupta, D., Wen, J. C., Huebner, J. L., Stinnett, S., Kraus, V. B., Tseng, H. C., & Walsh, M. (2017). Cytokine biomarkers in tear film for primary open-angle glaucoma. *Clinical Ophthalmology*, *11*, 411–416. https://doi.org/10.2147/OPTH.S125364

Guzman-Martinez, L., Maccioni, R. B., Andrade, V., Navarrete, L. P., Pastor, M. G., & Ramos-Escobar, N. (2019). Neuroinflammation as a common feature of neurodegenerative disorders. *Frontiers in Pharmacology*, Vol. 10. https://doi.org/10.3389/fphar.2019.01008

Hirsch, E. C., & Hunot, S. (2009, April). Neuroinflammation in Parkinson's disease: a target for neuroprotection? *The Lancet Neurology*, Vol. 8, pp. 382–397. https://doi.org/10.1016/S1474-4422(09)70062-6

Kannarkat, G. T., Boss, J. M., & Tansey, M. G. (2013). The role of innate and adaptive immunity in parkinson's disease. *Journal of Parkinson's Disease*, Vol. 3, pp. 493–514. https://doi.org/10.3233/JPD-130250

Kim, K. A., Gu, W., Lee, I. A., Joh, E. H., & Kim, D. H. (2012). High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway. *PLoS ONE*, *7*(10), e47713. https://doi.org/10.1371/journal.pone.0047713

Lanciego, J. L., Luquin, N., & Obeso, J. A. (2012). Functional neuroanatomy of the basal ganglia. *Cold Spring Harbor Perspectives in Medicine*, *2*(12). https://doi.org/10.1101/cshperspect.a009621

Lee, S. M., Han, H. W., & Yim, S. Y. (2015). Beneficial effects of soy milk and fiber on high cholesterol diet-induced alteration of gut microbiota and inflammatory gene expression in rats. *Food and Function*, *6*(2), 492–500. https://doi.org/10.1039/c4fo00731j

Li, M., van Esch, B. C. A. M., Wagenaar, G. T. M., Garssen, J., Folkerts, G., & Henricks, P. A. J. (2018). Pro- and anti-inflammatory effects of short chain fatty acids on immune and endothelial cells. *European Journal of Pharmacology*, *831*, 52–59. https://doi.org/10.1016/j.ejphar.2018.05.003

Liu, S., Qin, P., & Wang, J. (2019). High-Fat Diet Alters the Intestinal Microbiota in Streptozotocin-Induced Type 2 Diabetic Mice. *Microorganisms*, *7*(6), 176. https://doi.org/10.3390/microorganisms7060176

Liu, X., Yin, S., Chen, Y., Wu, Y., Zheng, W., Dong, H., … Zhao, P. (2018). LPS-induced proinflammatory cytokine expression in human airway epithelial cells and macrophages via NF-κB, STAT3 or AP-1 activation. *Molecular Medicine Reports*, *17*(4), 5484–5491. https://doi.org/10.3892/mmr.2018.8542

ller, M., Pappalardo, J. L., & Hafler, D. A. (2016). The Human Functional Genomics Project: Understanding Generation of Diversity. *Emerg. Infect. Dis*, *7*, 258–268. https://doi.org/10.1016/j.cell.2016.10.040

Maia, S., Arlicot, N., Vierron, E., Bodard, S., Vergote, J., Guilloteau, D., & Chalon, S. (2012). Longitudinal and parallel monitoring of neuroinflammation and neurodegeneration in a 6-hydroxydopamine rat model of Parkinson's disease. *Synapse*, *66*(7), 573–583. https://doi.org/10.1002/syn.21543

Manco, M., Putignani, L., & Bottazzo, G. F. (2010, December 1). Gut microbiota, lipopolysaccharides, and innate immunity in the pathogenesis of obesity and cardiovascular risk. *Endocrine Reviews*, Vol. 31, pp. 817–844. https://doi.org/10.1210/er.2009-0030

Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G., & Neufeld, J. D. (2012). *PANDAseq: PAired-eND Assembler for Illumina sequences*. https://doi.org/10.1186/1471-2105-13-31

McKee, D. H., & Sussman, J. D. (2005). Case report: Severe acute Parkinsonism associated with streptococcal infection and antibasal ganglia antibodies. *Movement Disorders*, *20*(12), 1661–1663. https://doi.org/10.1002/mds.20641

Morris, J. K., Bomhoff, G. L., Stanford, J. A., & Geiger, P. C. (2010). Neurodegeneration in an animal model of Parkinson's disease is exacerbated by a high-fat diet. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *299*(4), R1082. https://doi.org/10.1152/ajpregu.00449.2010

Moustafa, A. A., Chakravarthy, S., Phillips, J. R., Gupta, A., Keri, S., Polner, B., … Jahanshahi, M. (2016, September 1). Motor symptoms in Parkinson's disease: A unified framework. *Neuroscience and Biobehavioral Reviews*, Vol. 68, pp. 727–740. https://doi.org/10.1016/j.neubiorev.2016.07.010

Muhomah, T. A., Nishino, N., Katsumata, E., Haoming, W., & Tsuruta, T. (2019). High-fat diet reduces the level of secretory immunoglobulin A coating of commensal gut microbiota. *Bioscience of Microbiota, Food and Health*, *38*(2), 55–64. https://doi.org/10.12938/bmfh.18-027

Mulak, A. (2018). A controversy on the role of short-chain fatty acids in the pathogenesis of Parkinson's disease. *Movement Disorders*, *33*(3), 398–401. https://doi.org/10.1002/mds.27304

Nistiar, F., Racz, O., Lukacinova, A., Hubkova, B., Novakova, J., Lovasova, E., & Sedlakova, E. (2012). Age dependency on some physiological and biochemical parameters of male Wistar rats in controlled environment. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, *47*(9), 1224–1233. https://doi.org/10.1080/10934529.2012.672071

Papazoglou, I. K., Jean, A., Gertler, A., Taouis, M., & Vacher, C.-M. (2015). Hippocampal GSK3β as a Molecular Link Between Obesity and Depression. *Molecular Neurobiology*, *52*(1), 363–374. https://doi.org/10.1007/s12035-014-8863-x

Perez-Pardo, P., Dodiya, H. B., Engen, P. A., Forsyth, C. B., Huschens, A. M., Shaikh, M., … Keshavarzian, A. (2018). Role of TLR4 in the gut-brain axis in Parkinson's disease: A translational study from men to mice. *Gut*, *68*(5), 829–843. https://doi.org/10.1136/gutjnl-2018-316844

Perez-Pardo, P., Kliest, T., Dodiya, H. B., Broersen, L. M., Garssen, J., Keshavarzian, A., & Kraneveld, A. D. (2017). The gut-brain axis in Parkinson's disease: Possibilities for food-based therapies. *European Journal of Pharmacology*, *817*, 86–95. https://doi.org/10.1016/J.EJPHAR.2017.05.042

Peters, B. A., Shapiro, J. A., Church, T. R., Miller, G., Trinh-Shevrin, C., Yuen, E., … Ahn, J. (2018). A taxonomic signature of obesity in a large study of American adults. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-28126-1

Phillips, M. C. L., Murtagh, D. K. J., Gilbertson, L. J., Asztely, F. J. S., & Lynch, C. D. P. (2018). Low-fat versus ketogenic diet in Parkinson's disease: A pilot randomized controlled trial. *Movement Disorders*, *33*(8), 1306–1314. https://doi.org/10.1002/mds.27390

Poewe, W. (2008). Non-motor symptoms in Parkinson's disease. *European Journal of Neurology*, *15*(s1), 14–20. https://doi.org/10.1111/j.1468-1331.2008.02056.x

Qiao, Y., Sun, J., Xie, Z., Shi, Y., & Le, G. (2014). Propensity to high-fat diet-induced obesity in mice is associated with the indigenous opportunistic bacteria on the interior of Peyer's patches. *Journal of Clinical Biochemistry and Nutrition*, *55*(2), 120–128. https://doi.org/10.3164/jcbn.14-38

Qu, Y., Chen, X., Xu, M.-M., & Sun, Q. (2019). Relationship between high dietary fat intake and Parkinson's disease risk: a meta-analysis. *Neural Regeneration Research*, *14*(12), 2156. https://doi.org/10.4103/1673-5374.262599

Real, C. C., Doorduin, J., Kopschina Feltes, P., Vallez Garcia, D., de Paula Faria, D., Britto, L. R., & de Vries, E. F. (2019). Evaluation of exercise-induced modulation of glial activation and dopaminergic damage in a rat model of Parkinson's disease using [(11)C]PBR28 and [(18)F]FDOPA PET. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *39*(6), 989–1004. https://doi.org/10.1177/0271678X17750351

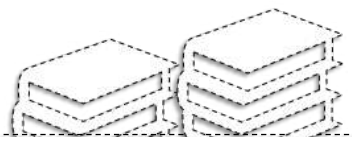
Sanguinetti, E., Collado, M. C., Marrachelli, V. G., Monleon, D., Selma-Royo, M., Pardo-Tendero, M. M., … Iozzo, P. (2018). Microbiome-metabolome signatures in mice genetically prone to develop dementia, fed a normal or fatty diet. *Scientific Reports*, *8*(1), 1–13. https://doi.org/10.1038/s41598-018-23261-1

Troncoso-Escudero, P., Parra, A., Nassif, M., & Vidal, R. L. (2018, October 15). Outside in: Unraveling the role of neuroinflammation in the progression of Parkinson's disease. *Frontiers in Neurology*, Vol. 9. https://doi.org/10.3389/fneur.2018.00860

Unger, M. M., Spiegel, J., Dillmann, K. U., Grundmann, D., Philippeit, H., Bürmann, J., … Schäfer, K. H. (2016). Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. *Parkinsonism and Related Disorders*, *32*, 66–72. https://doi.org/10.1016/j.parkreldis.2016.08.019

CHAPTER 8

Discussion and future perspectives



This thesis aimed to investigate several characteristic features of Parkinson's disease (PD) and the impact of diet thereon, using different animal models. The investigated features included neuroinflammation, changes in the dopaminergic and purinergic system and alterations in microbiota and behavior. This thesis was divided in three parts. In the first part, the purinergic system was reviewed (Chapter 2) and investigated in a zebrafish model for PD (Chapter 3). The second part addressed the feasibility of Positron Emission Tomography (PET) imaging in adult zebrafish (Chapter 4). In the third part, the effect of a high-fat diet was studied in healthy rats (Chapter 5) and a rat model for PD (Chapter 6 and 7). This chapter briefly discusses the relation between the results described in this thesis and the future perspectives in basic science and clinical research.

Treatment for Parkinson's disease

PD is a common neurodegenerative disorder that lacks proper treatment without side effects. Our limited understanding of the basic mechanisms of the disease before the onset of disease symptoms and the late diagnosis complicate the development of a curative treatment. The investigation of drug targets, or even completely new pathways, that differentiate from the classical dopaminergic approach should contribute to the identification of new biomarkers for early diagnosis and therapeutic interventions.

One of these approaches could be the investigation of the purinergic system, which is already being explored as a potential target for pharmacological and non-pharmacological treatment of PD. At present, one drug targeting the purinergic system has already been approved as add-on treatment for PD: the selective adenosine A_{2A} receptor antagonist istradefylline. This drug was approved based on results of different clinical trials with more than 4000 patients in total. In general, these trials showed a decrease in daily off symptoms, which occur when levodopa is not working well, in istradefylline treated PD patients, as compared to placebo treated patients (Chen & Cunha, 2020). Other drugs targeting the adenosine A_{2A} receptor, like preladenant, vipadenant and caffeine, have been tested as treatment for PD, however overall results were disappointing (Nazario, da Silva, & Bonan, 2017; Zhou et al., 2017). The influence of adenosine on the dopaminergic system and the success of istradefylline still support the search for purinergic receptor-targeting drugs (receptor antagonists), since

adenosine is capable of negatively modulating dopamine receptors through direct and indirect pathways (Tóth, Antal, Bereczki, & Sperlágh, 2019). Another strategy would be to target both the adenosine and the dopamine systems by a drug that is able to simultaneously bind to A_{2A} and D_2 receptors. Such a bispecific drug would have increased specificity, since the drug will only act on specific heterodimers. As a result, this could lead to a reduction in the dose needed and, consequently, the side effects (Soriano et al., 2009). This approach has been investigated for a long time, but only recently a dual targeted drug from the class of indolylpiperazinylpyrimidines was shown to have promising *in vitro* and *in vivo* results (Shao et al., 2018; Soriano et al., 2009). Problems related to the size of the molecule and the fast dissociation from the receptor heterodimer are still a challenge in this field (Carli et al., 2017).

Non-pharmacological approaches in the treatment of PD are usually related to the improvement of quality of life of patients through interventions like physical activity, diet, music therapy, and acupuncture (Ahn, Chen, Bredow, Cheung, & Yu, 2017). In animal studies, physical exercise was shown to induce a reduction in adenosine receptor expression, reinforcing the idea that the neuroprotective effect of physical activity is probably through a reduction of the antagonistic effect of adenosine on dopamine signalling (Clark et al., 2014). Diet is already known to have an impact on neuroprotection and neurodegeneration (Bianchi, Herrera, & Laura, 2019). Different types of diet can reduce or accelerate the progression of PD (Seidl, Santiago, Bilyk, & Potashkin, 2014). The beneficial effects of nutrients, like vitamins and antioxidants, on neuroinflammation was also found (Kurtys et al., 2019, 2018).

Additional efforts to find new drugs or new ways to slowdown the progression of PD are necessary to improve the quality of life for PD patients. Taking in consideration that a purinergic drug and lifestyle intervention can modulate disease progression and/or symptoms, it is important to explore the specific underlying mechanisms. This could help shedding some light on the pathways that are responsible for the onset of the disease and the course of disease progression before the diagnosis of patients.

Zebrafish as a model in basic PD research

There are different methodologies to investigate a disease. The most frequently used are observational research with or without the collection of biological samples in

humans, intervention studies and mimicking (part of the) disease symptoms in animal models. One of these animal models is the zebrafish. Zebrafish can be easily maintained in the laboratory and the production of large amounts of eggs can be induced in all periods of the year. This animal also has all major organs that are present in vertebrates, like the heart, eyes, kidneys, intestines, brain and pancreas. Moreover, zebrafish also have most of the neurotransmission systems that are present in rodents and primates (Rahman Khan & Sulaiman Alhewairini, 2019). About 80% of the genes related with human disease have an equivalent in zebrafish, which offers the opportunity to target specific disease-related genes, turn them on or off, or introduce foreign genes (Howe et al., 2013).

Because of these and other characteristics, zebrafish have already become an important animal model in basic research, in which they are mainly used to unravel disease mechanisms and to discover new potential targets for treatment. In **Chapter 3,** we translated the well-known 6-hydroxydopamine (6-OHDA) brain injection model of PD from rodents to the zebrafish. This procedure is challenging because of the size of the animal and the fact that it needs to be fast (the injection is done with the animal under stereomicroscope being just rinsed on the gills), but it can be reproduced. This model showed behavioural changes at various time points and there is a dose-dependent relation between 6-OHDA exposure and the features of PD. Our results showed that 6-OHDA injection in zebrafish caused an increase in dopamine D_2 receptor expression, while the adenosine A_{2A} receptor density initially was not affected by 6-OHDA, but decreased over time in 6-OHDA-treated animals. It has previously been described that adenosine and dopamine receptors are connected because they can form heterodimers (A_{2A}/D_2) in other species (Beggiato et al., 2014; Ferré & Ciruela, 2019). However, in zebrafish this is not known yet. For a more comprehensive understanding of chapter 3 findings, quantification of D_2 and A_{2A} receptor protein levels, availability and functionality of these receptors and elucidation of their status of heterodimerization should be investigated in order to identify if both receptors are similarly affected by 6-OHDA in zebrafish as in rodents (Antonelli et al., 2006; Fernández-Dueñas et al., 2015; Zhou et al., 2017). Enzymatic activity involved in the control of adenosine levels on extracellular medium can be easily investigated in zebrafish, as these enzymes are already used to investigate purinergic signalling in several pathological conditions (Altenhofen et al., 2018; Capiotti et al., 2016). In addition, pharmacological modulation of the zebrafish model of PD with purinergic

antagonists and agonists could provide more insight in the complex interaction between the purinergic system and other neurotransmitter systems, like the cholinergic, glutamatergic, GABAergic, cannabinergic and serotoninergic systems (Burnstock, 2008; Moreno et al., 2018; Ribeiro, Cunha, Correia-de-Sa, & Sebastiao, 1996; Tóth et al., 2019). These would give a better overview of all the factors that are involved in the degeneration of the dopaminergic system and may provide some hints for new therapeutic targets.

PET imaging of Zebrafish

PET is a versatile technique for studying biochemical and physiological processes in living organisms. So far, PET has mainly been applied in relatively large species like humans, non-human primates and rodents, as the application can be challenging in smaller animal models. Some studies report the use of nuclear imaging techniques in zebrafish, but only in dead animals or without recovery from anaesthesia (Bufkin, 2015; Dorsemans et al., 2017; Henderson et al., 2019). Efforts to establish truly *in vivo* PET imaging in zebrafish has not been published yet. In this thesis, we investigated the feasibility to perform PET experiments with living adult zebrafish **(Chapter 4).** The first challenge faced during the establishment of a procedure to obtain PET images was related to the concern of the use of a liquid in PET scanners, which was solved by ceiling the falcon tube contain the zebrafish with parafilm. The second challenge was to have sufficient time to capture images and keep the animals alive under anaesthesia. For this purpose, different concentrations of anaesthetic were tested. The third challenge was the impossibility to use a recirculating water system as this would cause movement of the fish and thus blurring of the images. The fourth challenge was to keep the water on an acceptable temperature for animals. To overcome the challenges, the technical training of researchers was essential in order to complete the procedure quick and efficiently. In this thesis we demonstrated that PET imaging in living zebrafish is possible. This zebrafish model could be complimentary to the models in rodents and *in vitro* analyses. Zebrafish can be used as an avatar, CRISPR and mutants, and considered as a tool to optimize, for example, a therapy dose for patients and predict tumour response (Costa et al., 2020).

In this thesis, the experiments were performed by the same researcher, but with training and more standardization this technique could be disseminated to other PET

centres as well. To implement this technique at different sites, my goal for the next years is to invest in the development of this field by establishing worldwide collaborations.

The development of new drugs and diagnostics can benefit from the combination of high-resolution PET/CT and PET/MRI with the zebrafish model. The main benefits are a fast throughput, since multiple zebrafish can be imaged at the same time with only a small amount of radiotracer, and a relatively low cost for breeding and maintaining of the animals. Unfortunately, we can only use the zebrafish in the adult stage when the size reaches around 3-4 cm, because of the resolution of the current PET cameras (0.7 - 1.5 mm). The small amount of blood in adult zebrafish and the limited time of anaesthesia will preclude kinetic modelling studies and the collection of multiple blood samples to perform metabolites analysis. On the other hand, the small size is an advantage, since the whole body of the animal can be scanned and therefore the overall distribution of the tracer is clear.

Another possibility that can be implemented in the field of molecular imaging is the use of zebrafish larvae (until five days post fertilization). Larvae can be incubated with the tracer and accumulated radioactivity can be detected with equipment such as a gamma counter or autoradiography. Thus, zebrafish larvae could fill the gap between cell studies and *in vivo* imaging studies. This approach can be used for binding studies, to measure specific binding of the tracer using various antagonists or agonists that compete with it. The advantage of using larvae until five days after fertilization is that they are not considered to be experimental animals yet, according to Central Authority for Scientific Procedures on Animal application in the Netherlands (not all the countries have the same legislation) and thus lengthy administrative procedures to obtain approval for the studies is not required. Moreover, the independent feeding of the larvae (yolk supplies) reduces the time and the costs of research even more. In this stage of life, the larvae have already developed all the organs and neurotransmitter systems, thus could be an add value from the studies with cells. One example is the use of zebrafish larvae as an avatar for studies with radiotherapy. In an article by Costa and colleagues, zebrafish were used as a tool to optimize the therapeutic dose for patients and predict tumour response. The zebrafish larvae were injected with colorectal cancer cells from biopsies of patients and different radiation doses were applied (Costa et al., 2020).

Because of the challenges associated with PET imaging in zebrafish, concerning the need to obtain a new license from the Central Authority for Scientific Procedures on Animals to perform these experiments and the limited amount of time to complete this PhD thesis, the final experiments in this thesis were performed in rats.

The effect of diet on the dopaminergic system and its impact on Parkinson's disease

Diet influences different processes in the body: satiety, pleasure, reward and necessity, among others. The role of food has expanded with human evolution, as nowadays food also plays a critical role in societal status (Luca, Perry, & Di Rienzo, 2010). The type of diet also changed with evolution, as the availability of an "easy meal" has increased the consumption of fast food over the world (De Vogli, Kouvonen, & Gimeno, 2014). The reward system does not only have an important role in the modulation of what we eat and how much we eat, but is also associated with addiction, motivation and mood disorders (Arias-Carrián, Stamelou, Murillo-Rodríguez, Menéndez-Gonzlez, & Pöppel, 2010). The excessive consumption of highly palatable food and the disbalance of the reward system can cause obesity (Kenny, 2011). The consumption habits of the Western society, culminating in a so-called the Western diet, have resulted in an increase in the number of diabetic patients. For research purposes, the Western diet can be translated to animals and is referred to as the cafeteria diet, consisting of highly palatable food (with high concentrations of sugar) and high fat chow (Lutz & Woods, 2012). With this diet, dopamine release is increased chronically and causes addictive behaviour, similar to what is observed in drugs abuse (Macedo, Freitas, & Torres, 2016). Because of the potentially detrimental impact of bad eating habits on society, it is important to know the processes that are affected by the Western diet, so that we can understand how diet can influence the development of diseases like diabetes. Such knowledge could also help in the development of new pharmacological strategies. In our longitudinal study described in **Chapter 5**, the effect of a cafetaria diet on the dopaminergic system was investigated. The observed decrease of dopamine D_2 receptor availability after cafeteria diet could be the result of a diet-induced increase in dopamine release or a decrease in expression of the D_2 receptor. The challenge with highly palatable food, which was expected to result in a blunted dopamine response in those animals that were exposed to the cafeteria diet,

did not show any effect. Possibly the timing of the PET scan or the intensity of the challenge was not adequate to observe any effect of the challenge.

After investigation of the effects of a cafeteria diet on dopamine D_2 receptor availability in healthy animals, research was continued by exposing an animal model of PD to a high-fat diet, to mimic the effects of a bad lifestyle on a number of PD disease characteristics (**Chapter 6 and 7**). Diet is hypothesized to influence the onset and progression of PD (Seidl et al., 2014). ^{11}C-raclopride and ^{11}C-PBR28 PET scans were performed to investigate the effect of diet on the dopaminergic system and on neuroinflammation, respectively, in the 6-OHDA model of PD. Imaging results were correlated with behavioural parameters and changes in microbiota. Overall, the results demonstrated a detrimental role of the high-fat diet on the severity of dopaminergic abnormalities and neuroinflammation. These effects could have been triggered by the observed changes in gut microbiota. Fortunately, diet is an easily modifiable risk factor. If prospective patients modify their eating habits and stop overeating, not only obesity could be prevented, but also the onset and progression of PD could be delayed or even prevented. However, this will not be easy, as many PD patients are suffering from depression, anxiety and other stressful situations that can lead to an overeating pattern. Understanding the basic mechanisms of the effects of diet in these patients can help in disease management. An interesting observation in our study was the correlation between changes in microbiota and glial activation in the brain. In the future, it may become possible to identify different types of disease just from the microbiota composition or from certain bacteria. If so, screening programs for the early identification of high-risk subjects could be started. This would enable preventive intervention. Nowadays, it is still difficult identify such early biomarkers due to the lack of standardization across the studies. For better results, large cohort studies with a diverse ethnic profile and standard protocols are necessary (Lavelle & Hill, 2019). Perhaps the LifeLines cohort in Groningen could provide the required data for the identification of predictive microbiota signatures.

The link between neurodegenerative disorders and diabetes type 2, or a pre-diabetic state, supports the hypothesis that treatments for diabetes, like metformin and glucagon-like peptide 1 (GLP-1), may also be applied in patients with neurodegenerative disease (Elbassuoni & Ahmed, 2019; Paudel, Angelopoulou, Piperi, Shaikh, & Othman, 2020). The mechanisms of action of metformin are related to the balance between survival and death in cells, signalling pathways that are also

connected to neurodegenerative diseases (Rotermund, Machetanz, & Fitzgerald, 2018). Animal studies report that metformin and GLP-1 are capable of reducing disease severity in animal models of PD (Bayliss et al., 2016; Lu et al., 2016; Patil, Jain, Ghumatkar, Tambe, & Sathaye, 2014; Zhang, Zhang, Li, & Hölscher, 2018). There is not enough data from clinical studies to conclude if metformin can be used as an treatment against PD (Paudel et al., 2020). However, the daily subcutaneous injection of GLP-1 was shown to have positive effects on motor and non-motor symptoms in PD patients, that did not persist after treatment was stopped (Athauda & Foltynie, 2016; Athauda et al., 2018).

To elucidate the effect of diet on the purinergic system of PD patients, PET studies with tracers like ^{11}C-preladenant, targeting the adenosine A_{2A} receptor, would be extremely interesting. The purinergic system is involved in different pathways related to inflammation, insulin resistance, hypothalamic control of feeding and control of white and brown adipocytes that directly affect obesity (Burnstock & Gentile, 2018). Such studies could help the development of an intervention with an A_{2A} antagonist that could benefit PD patients. Future PET studies with tracers such as ^{11}C-raclopride, ^{11}C-PBR28 and ^{11}C-preladenant in PD patients with different lifestyles can help clarifying the involvement and interrelationship of these system in PD. These PET studies can be combined with, amongst others, studying microbiota, cytokine and α-synuclein production, to complete the picture.

Concluding remarks

The world's obese population is increasing and so are the associated comorbidities. A link between obesity and neurodegenerative diseases has already been found. The search for knowledge about the basic pathways involved and potential interventions based on changes in lifestyle can help to prevent or delay neurodegenerative diseases. Increased understanding of the early disease stages could also facilitate the development of new treatments and diagnostic tools. My contribution to this field included investigating the relation between obesity and PD in an animal model in longitudinal studies, using PET. This PET approach enabled the combination of studying behaviour, microbiota, dopaminergic and purinergic response, and (neuro)inflammation. In addition, my work opened the possibility to investigate

diseases such as PD in a zebrafish model, including the use of PET as an assessment tool.

References

Ahn, S., Chen, Y., Bredow, T., Cheung1, C., & Yu, F. (2017). Effects of Non-PharmacologicalTreatments on Quality of Life in Parkinson's Disease: A Review. *Journal of Parkinson's Disease and Alzheimer's Disease*, *4*(1), 1–10. https://doi.org/10.13188/2376-922x.1000021

Altenhofen, S., Nabinger, D. D., Pereira, T. C. B., Leite, C. E., Bogo, M. R., & Bonan, C. D. (2018). Manganese(II) Chloride Alters Nucleotide and Nucleoside Catabolism in Zebrafish (Danio rerio) Adult Brain. *Molecular Neurobiology*, *55*(5), 3866–3874. https://doi.org/10.1007/s12035-017-0601-8

Altman, R. D., Lang, A. E., & Postuma, R. B. (2011). Caffeine in Parkinson's disease: A pilot open-label, dose-escalation study. *Movement Disorders*, *26*(13), 2427–2431. https://doi.org/10.1002/mds.23873

Antonelli, T., Fuxe, K., Agnati, L., Mazzoni, E., Tanganelli, S., Tomasini, M. C., & Ferraro, L. (2006). Experimental studies and theoretical aspects on A2A/D2 receptor interactions in a model of Parkinson's disease. Relevance for L-dopa induced dyskinesias. *Journal of the Neurological Sciences*, *248*(1–2), 16–22. https://doi.org/10.1016/j.jns.2006.05.019

Arias-Carrián, O., Stamelou, M., Murillo-Rodríguez, E., Menéndez-Gonzlez, M., & Pöppel, E. (2010, October 6). Dopaminergic reward system: A short integrative review. *International Archives of Medicine*, Vol. 3, p. 24. https://doi.org/10.1186/1755-7682-3-24

Athauda, D., & Foltynie, T. (2016, May 1). The glucagon-like peptide 1 (GLP) receptor as a therapeutic target in Parkinson's disease: Mechanisms of action. *Drug Discovery Today*, Vol. 21, pp. 802–818. https://doi.org/10.1016/j.drudis.2016.01.013

Athauda, D., MacLagan, K., Budnik, N., Zampedri, L., Hibbert, S., Skene, S. S., … Foltynie, T. (2018). What effects might exenatide have on non-motor symptoms in Parkinson's disease: A post Hoc analysis. *Journal of Parkinson's Disease*, *8*(2), 247–258. https://doi.org/10.3233/JPD-181329

Bayliss, J. A., Lemus, M. B., Santos, V. V., Deo, M., Davies, J. S., Kemp, B. E., … Andrews, Z. B. (2016). Metformin prevents nigrostriatal dopamine degeneration independent of AMPK activation in dopamine neurons. *PLoS ONE*, *11*(7), e0159381. https://doi.org/10.1371/journal.pone.0159381

Beggiato, S., Antonelli, T., Tomasini, M., Borelli, A., Agnati, L., Tanganelli, S., … Ferraro, L. (2014). Adenosine A2A-D2 Receptor-Receptor Interactions in Putative Heteromers in the Regulation of the Striato-Pallidal GABA Pathway: Possible Relevance for Parkinson's Disease and its Treatment. *Current Protein & Peptide Science*, *15*(7), 673–680. https://doi.org/10.2174/1389203715666140901103205

Bianchi, V. E., Herrera, P. F., & Laura, R. (2019). Effect of nutrition on neurodegenerative diseases. A systematic review. *Nutritional Neuroscience*. https://doi.org/10.1080/1028415X.2019.1681088

Bufkin, K. (2015). Multimodal Imaging Trials with Zebrafish Specimens. In *The Winthrop McNair Research Bulletin* (Vol. 1).

Burnstock, G. (2008, July). Purinergic signalling and disorders of the central nervous system. *Nature Reviews Drug Discovery*, Vol. 7, pp. 575–590. https://doi.org/10.1038/nrd2605

Burnstock, G., & Gentile, D. (2018, June 1). The involvement of purinergic signalling in obesity. *Purinergic Signalling*, Vol. 14, pp. 97–108. https://doi.org/10.1007/s11302-018-9605-8

Capiotti, K. M., Siebel, A. M., Kist, L. W., Bogo, M. R., Bonan, C. D., & Da Silva, R. S. (2016). Hyperglycemia alters E-NTPDases, ecto-5′-nucleotidase, and ectosolic and cytosolic adenosine deaminase activities and expression from encephala of adult zebrafish (Danio rerio). *Purinergic Signalling*, *12*(2), 211–220. https://doi.org/10.1007/s11302-015-9494-z

Carli, M., Kolachalam, S., Aringhieri, S., Rossi, M., Giovannini, L., Maggio, R., & Scarselli, M. (2017). Dopamine D2 Receptors Dimers: How can we Pharmacologically Target Them? *Current Neuropharmacology*, *16*(2), 222–230. https://doi.org/10.2174/1570159x15666170518151127

Chen, J. F., & Cunha, R. A. (2020). The belated US FDA approval of the adenosine A2A receptor antagonist istradefylline for treatment of Parkinson's disease. *Purinergic Signalling*. https://doi.org/10.1007/s11302-020-09694-2

Clark, P. J., Ghasem, P. R., Mika, A., Day, H. E., Herrera, J. J., Greenwood, B. N., & Fleshner, M. (2014). Wheel running alters patterns of uncontrollable stress-induced cfos mRNA expression in rat dorsal striatum direct and indirect pathways: A possible role for plasticity in adenosine receptors. *Behavioural Brain Research*, *272*, 252–263. https://doi.org/10.1016/j.bbr.2014.07.006

Costa, B., Ferreira, S., Póvoa, V., Cardoso, M. J., Vieira, S., Stroom, J., … Fior, R. (2020). Developments in zebrafish avatars as radiotherapy sensitivity reporters — towards personalized medicine. *EBioMedicine*, *51*. https://doi.org/10.1016/j.ebiom.2019.11.039

De Vogli, R., Kouvonen, A., & Gimeno, D. (2014). The influence of market deregulation on fast food consumption and body mass index: a cross-national time series analysis. *Bull World Health Organ*. https://doi.org/10.2471/BLT.13.120287

Dorsemans, A. C., Lefebvre d'hellencourt, C., Ait-Arsa, I., Jestin, E., Meilhac, O., & Diotel, N. (2017). Acute and chronic models of hyperglycemia in zebrafish: A method to assess the impact of hyperglycemia on neurogenesis and the biodistribution of radiolabeled molecules. *Journal of Visualized Experiments*, *2017*(124), 4–11. https://doi.org/10.3791/55203

Elbassuoni, E. A., & Ahmed, R. F. (2019). Mechanism of the neuroprotective effect of GLP-1 in a rat model of Parkinson's with pre-existing diabetes. *Neurochemistry International*, *131*, 104583. https://doi.org/10.1016/j.neuint.2019.104583

Fernández-Dueñas, V., Taura, J. J., Cottet, M., Gómez-Soler, M., López-Cano, M., Ledent, C., … Ciruela, F. (2015). Untangling dopamine-adenosine receptor-receptor assembly in experimental parkinsonism in rats. *DMM Disease Models and Mechanisms*, *8*(1), 57–63. https://doi.org/10.1242/dmm.018143

Ferré, S., & Ciruela, F. (2019). Functional and Neuroprotective Role of Striatal Adenosine A 2A Receptor Heterotetramers . *Journal of Caffeine and Adenosine Research*, *9*(3), 89–97. https://doi.org/10.1089/caff.2019.0008

Henderson, F., Johnston, H. R., Badrock, A. P., Jones, E. A., Forster, D., Nagaraju, R. T., … Hurlstone, A. (2019). Enhanced fatty acid scavenging and glycerophospholipid metabolism accompany melanocyte neoplasia progression in Zebrafish. *Cancer Research*, *79*(9), 2136–2151. https://doi.org/10.1158/0008-5472.CAN-18-2409

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., … Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), 498–503. https://doi.org/10.1038/nature12111

Kenny, P. J. (2011, February 24). Reward Mechanisms in Obesity: New Insights and Future Directions. *Neuron*, Vol. 69, pp. 664–679.

https://doi.org/10.1016/j.neuron.2011.02.016
Kurtys, E., Casteels, C., Real, C. C., Eisel, U. L. M., Verkuyl, J. M., Broersen, L. M., … de Vries, E. F. J. (2019). Therapeutic effects of dietary intervention on neuroinflammation and brain metabolism in a rat model of photothrombotic stroke. *CNS Neuroscience and Therapeutics*, *25*(1), 36–46. https://doi.org/10.1111/cns.12976
Kurtys, E., Eisel, U. L. M., Hageman, R. J. J., Verkuyl, J. M., Broersen, L. M., Dierckx, R. A. J. O., & de Vries, E. F. J. (2018). Anti-inflammatory effects of rice bran components. *Nutrition Reviews*, *76*(5), 372–379. https://doi.org/10.1093/nutrit/nuy011
Lavelle, A., & Hill, C. (2019, June 1). Gut Microbiome in Health and Disease: Emerging Diagnostic Opportunities. *Gastroenterology Clinics of North America*, Vol. 48, pp. 221–235. https://doi.org/10.1016/j.gtc.2019.02.003
Lu, M., Su, C., Qiao, C., Bian, Y., Ding, J., & Hu, G. (2016). Metformin prevents dopaminergic neuron death in MPTP/P-induced mouse model of Parkinson's disease via autophagy and mitochondrial ROS clearance. *International Journal of Neuropsychopharmacology*, *19*(9), 1–11. https://doi.org/10.1093/ijnp/pyw047
Luca, F., Perry, G. H., & Di Rienzo, A. (2010). Evolutionary Adaptations to Dietary Changes. *Annual Review of Nutrition*, *30*(1), 291–314. https://doi.org/10.1146/annurev-nutr-080508-141048
Lutz, T. A., & Woods, S. C. (2012). Overview of animal models of obesity. *Current Protocols in Pharmacology*, *CHAPTER*(SUPPL.58), Unit5.61. https://doi.org/10.1002/0471141755.ph0561s58
Macedo, I. C. de, Freitas, J. S. de, & Torres, I. L. da S. (2016). The Influence of Palatable Diets in Reward System Activation: A Mini Review. *Advances in Pharmacological Sciences*, *2016*. https://doi.org/10.1155/2016/7238679
Moreno, E., Chiarlone, A., Medrano, M., Puigdellívol, M., Bibic, L., Howell, L. A., … Guzmán, M. (2018). Singular Location and Signaling Profile of Adenosine A2A-Cannabinoid CB1 Receptor Heteromers in the Dorsal Striatum. *Neuropsychopharmacology*, *43*(5), 964–977. https://doi.org/10.1038/npp.2017.12
Nazario, L. R., da Silva, R. S., & Bonan, C. D. (2017, November 23). Targeting adenosine signaling in Parkinson's disease: From pharmacological to non-pharmacological approaches. *Frontiers in Neuroscience*, Vol. 11. https://doi.org/10.3389/fnins.2017.00658
Patil, S. P., Jain, P. D., Ghumatkar, P. J., Tambe, R., & Sathaye, S. (2014). Neuroprotective effect of metformin in MPTP-induced Parkinson's disease in mice. *Neuroscience*, *277*, 747–754. https://doi.org/10.1016/j.neuroscience.2014.07.046
Paudel, Y. N., Angelopoulou, E., Piperi, C., Shaikh, M. F., & Othman, I. (2020, February 1). Emerging neuroprotective effect of metformin in Parkinson's disease: A molecular crosstalk. *Pharmacological Research*, Vol. 152, p. 104593. https://doi.org/10.1016/j.phrs.2019.104593
Rahman Khan, F., & Sulaiman Alhewairini, S. (2019). Zebrafish (Danio rerio) as a Model Organism . In *Current Trends in Cancer Management*. https://doi.org/10.5772/intechopen.81517
Ribeiro, J. A., Cunha, R. A., Correia-de-Sa, P., & Sebastiao, A. M. (1996). Purinergic regulation of acetylcholine release. *Progress in Brain Research*, *109*, 231–241. https://doi.org/10.1016/s0079-6123(08)62107-x
Rotermund, C., Machetanz, G., & Fitzgerald, J. C. (2018, July 19). The therapeutic potential of metformin in neurodegenerative diseases. *Frontiers in Endocrinology*,

Vol. 9. https://doi.org/10.3389/fendo.2018.00400

Seidl, S. E., Santiago, J. A., Bilyk, H., & Potashkin, J. A. (2014). The emerging role of nutrition in Parkinson's disease. *Frontiers in Aging Neuroscience*, Vol. 6. https://doi.org/10.3389/fnagi.2014.00036

Shao, Y.-M., Ma, X., Paira, P., Tan, A., Herr, D. R., Lim, K. L., … Pastorin, G. (2018). Discovery of indolylpiperazinylpyrimidines with dual-target profiles at adenosine A2A and dopamine D2 receptors for Parkinson's disease treatment. *PLOS ONE*, *13*(1), e0188212. https://doi.org/10.1371/journal.pone.0188212

Soriano, A., Ventura, R., Molero, A., Hoen, R., Casado, V., Corte, A., … Royo, M. (2009). Adenosine A2A receptor-antagonist/dopamine D2 receptor-agonist bivalent ligands as pharmacological tools to detect A 2A-D2 receptor heteromers. *Journal of Medicinal Chemistry*, *52*(18), 5590–5602. https://doi.org/10.1021/jm900298c

Tóth, A., Antal, Z., Bereczki, D., & Sperlágh, B. (2019). Purinergic Signalling in Parkinson's Disease: A Multi-target System to Combat Neurodegeneration. *Neurochemical Research*, *44*(10), 2413–2422. https://doi.org/10.1007/s11064-019-02798-1

Zhang, L., Zhang, L., Li, L., & Hölscher, C. (2018). Neuroprotective effects of the novel GLP-1 long acting analogue semaglutide in the MPTP Parkinson's disease mouse model. *Neuropeptides*, *71*, 70–80. https://doi.org/10.1016/j.npep.2018.07.003

Zhou, X., Doorduin, J., Elsinga, P. H., Dierckx, R. A. J. O., de Vries, E. F. J., & Casteels, C. (2017). Altered adenosine 2A and dopamine D2 receptor availability in the 6-hydroxydopamine-treated rats with and without levodopa-induced dyskinesia. *NeuroImage*, *157*, 209–218. https://doi.org/10.1016/j.neuroimage.2017.05.066

CHAPTER 9

English Summary

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease and, with the increasing life expectancy of the population, the number of patients with this pathology is growing. Attempts are being made to find a cure or at least a better treatment, but so far, no intervention that can slow down the progression of the disease has been discovered. A better understanding of the basic mechanisms that underlie this disease is of great importance for the development of new drugs. One of the factors that can affect disease progression or onset is lifestyle. Lifestyle can influence different factors that are involved in PD, such as the purinergic system, the dopaminergic system, neuroinflammation and microbiota. In this thesis, these factors will be explored in different ways.

Chapter 2 provides an overview of the interaction of the purinergic and dopaminergic system in the context of PD. It also addresses the effect of adenosine receptor antagonists in combination with dopaminergic drugs like L-DOPA, the drug most frequently used to suppress the symptoms of this disease. Also, non-dopaminergic therapies to decrease the main adverse effects of long-term use of L-DOPA, the motor side effects, are discussed. Treatment with multiple targeted drugs, such as adenosine receptor antagonists and drugs targeting other neurotransmitter systems than the dopaminergic system, has received significant attention lately, since PD is associated with complex biological interactions. While pharmacological approaches to cure or ameliorate the symptoms of PD are still the leading strategy in this area, positive effects of emerging non-pharmacological approaches have been observed as well and inhibition of the function of adenosine appears to be involved in both strategies.

In **Chapter 3**, we investigated the interaction of purinergic and dopaminergic receptors in an animal model of PD. For this purpose, we investigated behavioural parameters, dopamine levels and expression levels of adenosine and dopamine receptors in the brain of adult zebrafish injected with 6-OHDA in the right telencephalon (8 μg/μL). The animals were evaluated 3, 7 and 28 days after injection of 6-OHDA. The locomotor parameter 'turn angle' and the anxiety parameter 'time spent in the superior zone' were decreased on day 28 and 7, respectively. Dopamine levels in the whole brain were not altered, but D_2 receptor expression was higher on

day 3, while expression levels of A_{2A1} receptors were increased on day 7, but had normalized again on day 28. Thus, after intra-encephalic injection of 6-OHDA, zebrafish presented behavioral changes, in particular in the turn angle and the time spent in the superior zone, despite the slight effect on the dopaminergic system. Further studies involving pharmacological and genetic manipulation are necessary to investigate the interaction of purinergic and dopaminergic neurotransmission in this PD model.

Considering the importance of zebrafish for studying different disease process, in **Chapter 4** we performed a translational project to investigate the feasibility of in vivo PET imaging in living healthy adult zebrafish and in a zebrafish model of inflammation. To optimize imaging conditions, the optimal dose of the anesthetic tricaine was determined and the distribution of the PET tracers ^{18}F-FDG and ^{18}F-NaF in control zebrafish was measured at various time points. We have shown that PET imaging in living zebrafish is feasible and that a concentration 0.1 g/L of tricaine for anesthesia and a distribution time of 30 min for ^{18}F-FDG and 150 min for ^{18}F-NaF provided optimal conditions. After the standardization, whole-body PET imaging with ^{18}F-FDG was able to reveal LPS-induced inflammation, as a significant difference in tracer uptake in the head region between control and LPS-injected animals was observed. In summary, this study demonstrated that in vivo PET imaging with ^{18}F-FDG and ^{18}F-NaF in living zebrafish is feasible and differences in uptake can be seen in an inflammation model.

The incidence of PD has been associated with lifestyle factors like diet. In the last part of this thesis, we therefore investigated the effect of diet in a rodent model for PD. In **Chapter 5**, however, we first analyzed the impact of a cafeteria diet (high caloric diet) on the reward system. Dopamine and its D_2 receptors are involved in the reward system. Consumption of a diet high in fat and sugar might lead to a change in D_2 receptors expression and dopamine turnover. The compulsive eating behavior that is seen in obese patients might be related to a decreased activation of the reward system or a low number of D_2 receptors. The main objective of the study described in chapter 5 was to investigate the effect of highly palatable food on the availability of dopamine D_2 receptors in rats. Male Wistar rats were divided in two groups: control diet (chow pellet, 14% fat) or cafeteria diet (high-fat pellet, 45% of fat; chocolate, cheese, and sugar water). Each animal underwent three ^{11}C-raclopride PET scans for assessment

of dopamine D_2 receptor availability: baseline (before the start of diet); after four weeks on the diet (day 28), and immediately after consuming highly palatable sweetened condensed milk (day 30). From day four onward, the body weight gain and caloric intake was significantly higher in the cafeteria diet group than in the control diet group. The animals in the standard diet group did not show any significant difference in ^{11}C-raclopride uptake in striatum between the baseline PET scan and the either of the follow-up scans. The cafeteria diet decreased the tracer binding on both follow-up time points, which is indicative of a reduction in the availability of the D_2 receptors. Our study suggests that D_2 receptors play a role in obesity and consumption of a high caloric diet. It also showed that the challenge with condensed milk was not able to change the dopaminergic response as observed by PET. More studies should be done to confirm if there are any changes in the levels of dopamine and related molecules.

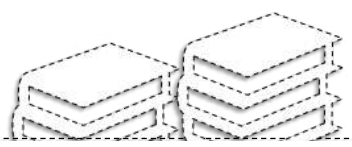
To elucidate the influence of a high fat diet on PD progression, we investigated the effect of a high-fat diet (HFD) on the availability of D_2 receptors and behavioral parameters in rats **(Chapter 6)**. Wistar rats were fed with either an HFD (60% fat) or a standard diet (STD; 10% fat) for three months. After two months of diet the animals were submitted to stereotactic administration of a low dose of 6-OHDA (2x 3µg) in the right striatum. ^{11}C-Raclopride PET scans were performed two days before and one month after the surgery. After three months, animals on the HFD had a higher bodyweight gain and caloric intake than those on the STD. The surgery caused a temporary decrease in bodyweight in both groups. No difference in the glucose tolerance test between the STD and HFD groups were found. ^{11}C-Raclopride PET showed a significantly decreased ipsilateral/contralateral tracer uptake ratio in the striatum in the HFD group one month after 6-OHDA injection, when compared to the STD and HFD groups before stereotactic surgery, however no significant difference was found between groups one month after 6-OHDA administration. This pattern was also found in the cylinder test, but no differences between groups and time points were found in other behavior analyses. Postmortem analysis of ^{11}C-raclopride uptake in the gut showed significantly lower tracer uptake in duodenum of animals in the HFD group than in the SD group. Concluding, the HFD aggravated the damage in the PD model in this study, as was demonstrated by a lower D_2 receptor availability and a reduced use of the contralateral paw. Both observations suggest a detrimental role of the HFD on the onset or progression of Parkinson's disease.

In **Chapter 7**, the impact of a high fat diet on inflammatory parameters in the PD model was investigated. In particular, the composition of the microbiota, development of neuroinflammation and clinical symptoms were evaluated in a validated rodent model (6-OHDA unilateral injection). The rats were fed with either a high-fat diet (HFD) or a standard diet (SD) for three months. After two months of diet, the rats were submitted to stereotactic surgery injecting a low dose of 6-OHDA. ^{11}C-PBR28 PET scans were performed before and one month after the 6-OHDA injection. Stool was collected before the start of the diet (baseline), after two months of the diet and one month after the 6-OHDA injection. Microbiome analysis was performed on stool samples. An increase in body weight, a decrease in the use of the ipsilateral paw, as assessed by the cylinder test, and increased uptake of the neuroinflammatory marker ^{11}C-PBR28 was shown in animals on a high fat diet one month after injection of 6-OHDA, as compared to the assessment before stereotactic surgery. No differences between time points were found in the SD group. The increased accumulation of ^{11}C-PBR28 in the HFD group after 6-OHDA administration was accompanied with alterations in the microbiota profile and no effect in the cytokine levels in plasma. This study suggests the documented impact of an HFD on neuroinflammation may be mediated by the gut microbiome in the 6-OHDA rat model of Parkinson's disease and suggest it is independent of peripheral inflammation.

Knowledge of the basic mechanisms underlying PD and their relation with changes in lifestyle can help scientists to better understand the factors that trigger the onset of the disease and aid the development of new treatments and diagnostic tools. My small contribution to this field is the possibility to investigate the interaction of two diseases (obesity and PD) in the same animal using the PET for longitudinal monitoring. These studies can help reveal the complicity of the interaction between behavior, microbiota, dopaminergic and purinergic response, and inflammation in the gut and the brain.

CHAPTER 10

Nederlandse Samenvatting



De ziekte van Parkinson (PD) is de op één na meest voorkomende neurodegeneratieve ziekte en met de stijgende levensverwachting van de bevolking groeit het aantal patiënten met deze pathologie. Er worden pogingen ondernomen om een remedie, of op zijn minst een betere behandeling te ontdekken, maar tot nu toe is er geen interventie gevonden die de progressie van de ziekte echt kan vertragen. Een beter begrip van de basale mechanismen die aan deze ziekte ten grondslag liggen is van groot belang voor de ontwikkeling van nieuwe geneesmiddelen. Een van de factoren, die het ontstaan en de progressie van deze ziekte kunnen beïnvloeden, is levensstijl. Verschillende factoren die bij PD betrokken zijn kunnen worden beïnvloed door levensstijl, waaronder het purinerge systeem, het dopaminerge systeem, neuro-inflammatie en de darmflora. In dit proefschrift worden deze factoren op verschillende manieren onderzocht.

Hoofdstuk 2 geeft een overzicht van de interactie tussen het purinerge en dopaminerge systeem in de context van PD. Het benoemt ook het effect van antagonisten van adenosinereceptoren in combinatie met dopaminerge geneesmiddelen zoals L-DOPA, het medicijn dat het meest wordt gebruikt om de symptomen van deze ziekte te onderdrukken. Ook worden niet-dopaminerge therapieën besproken om de belangrijkste nadelige effecten van langdurig gebruik van L-DOPA, namelijk de motorische bijwerkingen, te verminderen. Behandeling met meerdere gerichte geneesmiddelen, zoals antagonisten van adenosinereceptoren en geneesmiddelen die gericht zijn op andere neurotransmittersystemen dan het dopaminerge systeem, heeft de laatste tijd veel aandacht gekregen, omdat PD wordt geassocieerd met complexe biologische interacties. Hoewel farmacologische benaderingen om PD te genezen of symptomen te verminderen de belangrijkste strategieën op dit gebied zijn, zijn er ook opkomende positieve effecten van niet-farmacologische benaderingen aangetoond en lijkt remming van de adenosinefunctie bij beide strategieën een rol te kunnen spelen.

In **Hoofdstuk 3** hebben we de interactie tussen purinerge en dopaminerge receptoren onderzocht in een diermodel van PD. Voor dit doel hebben we gedragsparameters, dopamineniveaus en expressieniveaus van adenosine- en dopaminereceptoren in de hersenen van volwassen zebravissen, die geïnjecteerd waren met 6-OHDA in het rechter telencephalon (8 µg / µL), onderzocht. De dieren

werden 3, 7 en 28 dagen na injectie van 6-OHDA onderzocht. De bewegingsparameter ‘draaihoek’ en de angstparameter ‘tijd doorgebracht in de bovenste zone’ waren respectievelijk op dag 28 en 7 verlaagd. De dopamineniveaus in de totale hersenen waren niet veranderd, maar de D_2-receptorexpressie was hoger op dag 3, terwijl de expressieniveaus van A_{2A1}-receptoren was toegenomen op dag 7 en weer was genormaliseerd op dag 28. Zebravissen kunnen dus gedragsveranderingen vertonen na intra-encefale injectie van 6-OHDA, in het bijzonder in de draaihoek en de verblijfstijd in de bovenste zone, ondanks het geringe effect op het dopaminerge systeem. Aanvullende studies met farmacologische en genetische manipulatie zijn nodig om de interactie van purinerge en dopaminerge neurotransmissie te onderzoeken in dit PD-model in volwassen zebravissen.

Gezien het belang van zebravissen voor het bestuderen van verschillende ziekteprocessen, hebben we in **hoofdstuk 4** een project uitgevoerd om de haalbaarheid van in vivo beeldvorming met positron emissie tomografie (PET) bij levende, gezonde volwassen zebravissen en in een zebravis-ontstekingsmodel te onderzoeken. Om de beeldvormingscondities te optimaliseren, werd de optimale dosis van het anestheticum tricaïne bepaald en werd de distributie van de PET-tracers ^{18}F-FDG en ^{18}F-NaF in de controle-zebravis op verschillende tijdstippen gemeten. We hebben aangetoond dat PET bij levende zebravissen haalbaar is en dat een concentratie van 0,1 g/L tricaïne voor anesthesie en een distributietijd van 30 minuten voor ^{18}F-FDG en 150 minuten voor ^{18}F-NaF voor optimale omstandigheden zorgden. Na deze optimalisatie kon met ^{18}F-FDG PET een door LPS geïnduceerde immuunreactie in het brein worden gedetecteerd, aangezien significante verschillen in de opname van tracer tussen controle en met LPS geïnjecteerde dieren in de kop werden gemeten. Samenvattend toonde deze studie aan dat *in vivo* PET-beeldvorming met ^{18}F-FDG en ^{18}F-NaF in levende zebravissen haalbaar is en dat verschillen in opname te zien zijn in een ontstekingsmodel.

De incidentie van PD is in verband gebracht met leefstijlfactoren, zoals voeding. In het laatste deel van dit proefschrift hebben we daarom het effect van voeding in een knaagdiermodel voor PD onderzocht. In **Hoofdstuk 5** analyseerden we echter eerst de invloed van een cafetariadieet (calorierijk dieet) op het beloningssysteem. Dopamine en zijn D_2-receptoren zijn betrokken bij het beloningssysteem. Consumptie

van een dieet met veel vet en suiker kan leiden tot een afname van de expressie van D_2-receptoren en een verminderde dopamine-afgifte. Het dwangmatige eetgedrag, dat bij patiënten met obesitas wordt gezien, kan verband houden met een verminderde activering van het beloningssysteem of een laag aantal D_2-receptoren. Het hoofddoel van de studie beschreven in hoofdstuk 5 was om het effect van de inname van zeer smakelijk voedsel op de beschikbaarheid van dopamine D_2-receptoren bij ratten te onderzoeken. Mannelijke Wistar ratten werden in 2 groepen verdeeld: controle dieet (normale brokken met 14% vet) of cafetariadieet (hoog-vet brokken met 45% vet, chocolade, kaas en suikerwater). Elk dier onderging drie ^{11}C-raclopride PET-scans om de beschikbaarheid van dopamine D_2-receptoren te meten: basislijn (voor aanvang van het dieet); na 4 weken op het dieet (dag 28) en onmiddellijk na het consumeren van zeer smakelijke gezoete gecondenseerde melk (dag 30). Vanaf dag 4 was de gewichtstoename en calorie-inname in de cafetariadieet groep significant hoger dan in de controle dieetgroep. De dieren in de controle dieetgroep vertoonden geen significante verschillen in opname van ^{11}C-raclopride in het striatum tussen de basislijn PET-scan en de daaropvolgende scans. Het cafetariadieet verminderde de tracer-binding op beide evaluatietijdstippen, wat indicatief is voor een afname van de beschikbaarheid van de D_2-receptoren. Onze studie suggereert dat D_2-receptoren een rol spelen bij obesitas en consumptie van een calorierijk voedsel. Het toonde ook aan dat de impuls met gecondenseerde melk de dopaminerge respons, zoals waargenomen met PET, niet kon veranderen. Er moeten dus meer onderzoek worden gedaan om te laten zien wat de veranderingen zijn in de niveaus van dopamine en verwante moleculen.

Om de invloed van een vetrijk dieet bij PD op te helderen, onderzochten we het effect van een vetrijk dieet (HFD) op de beschikbaarheid van D_2-receptoren en gedragsparameters bij ratten (**Hoofdstuk 6**). Wistar ratten werden gedurende drie maanden gevoed met ofwel een HFD (60% vet) of een standaarddieet (STD; 10% vet). Na twee maanden op het dieet werden de dieren onderworpen aan een stereotactische toediening van een lage dosis 6-OHDA (2x 3 µg) in het rechter striatum. Twee dagen voor en één maand na de operatie, werden er ^{11}C-Raclopride PET-scans uitgevoerd. Na drie maanden hadden dieren op het HFD een grotere lichaamsgewichtstoename en calorie-inname dan die op het STD. De operatie voor het injecteren van 6-OHDA veroorzaakte een tijdelijke afname van het

lichaamsgewicht in beide groepen. Er werd geen verschil gevonden in de glucosetolerantietest tussen de STD- en HFD-groepen. ^{11}C-Raclopride PET vertoonde een significant verminderde ipsilaterale/contralaterale traceropname-ratio in het striatum in de HFD-groep 1 maand na 6-OHDA-injectie in vergelijking met de STD- en HFD-groep vóór stereotactische chirurgie, maar er werd geen significant verschil gevonden tussen de groepen 1 maand na 6-OHDA administratie. Dit patroon werd ook gevonden in de cilindertest, maar er werden geen verschillen tussen groepen en tijdstippen gevonden in andere gedragsanalyses. Postmortale analyse van de opname van ^{11}C-raclopride in de darmen toonde een significant lagere opname van de tracer in de twaalfvingerige darm van dieren in de HFD-groep dan in de SD-groep. Er kan dus geconcludeerd worden dat het HFD de schade in het PD-model in deze studie verergerde, zoals blijkt uit een lagere beschikbaarheid van D_2-receptor en een verminderd gebruik van de contralaterale poot. Beide waarnemingen suggereren een nadelige rol van het HFD bij het ontstaan en de progressie van de ziekte van Parkinson.

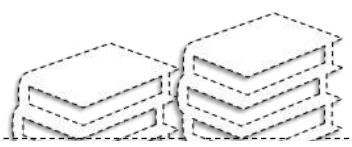
In **Hoofdstuk 7** werd de impact van een vetrijk dieet op inflammatoire parameters in het PD-model onderzocht. In het bijzonder werden de samenstelling van de darmflora, de ontwikkeling van neuro-inflammatie en de manifestaties van symptomen geëvalueerd in een knaagdiermodel voor PD (unilaterale 6-OHDA injectie). De ratten kregen gedurende drie maanden ofwel een vetrijk dieet (HFD), ofwel een standaarddieet (SD). Na twee maanden dieet werden de ratten onderworpen aan stereotactische operatie waarbij een lage dosis 6-OHDA werd geïnjecteerd. Voorafgaand en 1 maand na de 6-OHDA-injectie werden ^{11}C-PBR28 PET-scans uitgevoerd. De ontlasting werd verzameld vóór de start van het dieet (basislijn), na twee maanden op het dieet en 1 maand na de 6-OHDA-injectie. Darmflora-analyses werden uitgevoerd op de ontlastingsmonsters. Een toename van het lichaamsgewicht, een afname van het gebruik van de ipsilaterale poot, zoals gemeten in de cilindertest, en een verhoogde opname van de neuro-inflammatoire marker ^{11}C-PBR28 werden aangetoond bij dieren met een vetrijk dieet 1 maand na injectie van 6-OHDA, ten opzichte van direct vóór de stereotactische operatie. In de SD-groep werden geen verschillen tussen tijdspunten gevonden. De verhoogde opname van ^{11}C-PBR28 in de HFD-groep na toediening van 6-OHDA ging gepaard met veranderingen in het darmflora-profiel en niet in cytokinespiegels in plasma. Deze studie suggereert dat de

gedocumenteerde impact van een HFD op neuro-inflammatie kan worden gemedieerd door darmflora in het 6-OHDA-ratmodel van de ziekte van Parkinson en suggereert dat het onafhankelijk is van perifere ontsteking.

Het zoeken naar de beginselen die ten grondslag liggen aan PD en de relatie met veranderingen in levensstijl kan wetenschappers helpen om de factoren die het begin van de ziekte veroorzaken beter te begrijpen en de ontwikkeling van nieuwe behandelingen en diagnostische hulpmiddelen te ondersteunen. Mijn kleine bijdrage op dit gebied is de mogelijkheid creëren om de interactie van twee ziekten (obesitas en PD) in hetzelfde dier op longitudinale wijze te onderzoeken met behulp van de PET. Dit kan helpen de complexe interactie tussen gedrag, darmflora, dopaminerge respons en ontsteking in de darmen en de hersenen op te helderen.

CHAPTER 11

Resumo em Português



A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais prevalente e, com o aumento da expectativa de vida da população, o número de pacientes com essa patologia está crescendo. Tentativas para descobrir uma cura ou pelo menos um tratamento melhor estão sendo feitas, mas até o momento nenhuma intervenção que possa realmente retardar a progressão da doença foi descoberta. Uma melhor compreensão dos mecanismos básicos subjacentes a esta doença é de extrema importância para o desenvolvimento de novos medicamentos. Um dos fatores que podem afetar a progressão ou o aparecimento desta doença é o estilo de vida. O estilo de vida pode influenciar diferentes mecanismos envolvidos na DP, como: o sistema purinérgico, o sistema dopaminérgico, a neuroinflamação e a microbiota. Nesta tese, esses mecanismos serão explorados de diferentes maneiras.

O **capítulo 2** fornece uma visão geral da interação do sistema purinérgico e dopaminérgico no contexto da DP. Também aborda o efeito de antagonistas do receptor de adenosina em combinação com drogas dopaminérgicas como o L-DOPA, a droga mais frequentemente usada para suprimir os sintomas desta doença. Também são discutidas terapias não dopaminérgicas para diminuir os principais efeitos adversos do uso prolongado de L-DOPA, os efeitos colaterais motores. O tratamento com medicamentos que possuam múltiplos alvos, utilizando antagonistas dos receptores de adenosina e medicamentos com alvo em outros sistemas neurotransmissores além do sistema dopaminérgico, recebeu atenção significativa ultimamente, uma vez que a DP está associada a complexas interações biológicas. Embora as abordagens farmacológicas para curar ou melhorar as condições da DP sejam a principal estratégia nessa área, efeitos positivos emergentes de abordagens não farmacológicas também foram mostrados e a inibição da modulação da adenosina parece estar envolvida em ambas as estratégias.

No **capítulo 3**, investigamos a interação dos receptores purinérgicos e dopaminérgicos, em um modelo de DP no peixe-zebra adulto. Para isso, investigamos parâmetros comportamentais, níveis de dopamina e níveis de expressão de receptores de adenosina e dopamina no cérebro de peixes-zebra adultos injetados com 6-OHDA no telencéfalo direito (8 µg / µL). Os animais foram avaliados 3, 7 e 28 dias após a injeção (dpi) de 6-OHDA. O parâmetro locomotor "ângulo de giro" e o parâmetro de ansiedade "tempo gasto na zona superior" foram reduzidos nos dias 28

e 7, respectivamente. Os níveis de dopamina no cérebro inteiro não foram alterados, mas a expressão do receptor D_2 foi maior no dia 3, enquanto os níveis de expressão dos receptores A_{2A1} aumentaram em 7 dpi e normalizaram no 28° dia. O peixe-zebra é capaz de apresentar alterações comportamentais, mais específicas no ângulo de giro e no tempo gasto na zona superior após a injeção intraencefálica de 6-OHDA, apesar do leve efeito no sistema dopaminérgico. Estudos adicionais envolvendo manipulação farmacológica e genética são necessários para avaliar a capacidade do peixe-zebra adulto de contribuir para a investigação da inter-relação da neurotransmissão purinérgica e dopaminérgica neste modelo de DP.

Considerando a importância do peixe-zebra para o estudo de diferentes processos de doenças, no **capítulo 4**, realizamos um projeto translacional para investigar a viabilidade de implementação do peixe-zebra no campo da medicina nuclear. Testamos a viabilidade da imagem PET *in vivo* em peixe-zebra adultos saudáveis e em um modelo de inflamação em peixe-zebra. Para otimizar as condições de imagem, uma curva dose-resposta para o anestésico tricaína foi realizada e a distribuição dos radiofármacos PET ^{18}F-FDG e ^{18}F-NaF no peixe-zebra saudável foi determinada em diferentes tempos. Mostramos que a imagem PET no peixe-zebra vivo é possível e que uma concentração de 0,1 g / L de tricaína para anestesia e um tempo de distribuição de 30 minutos para ^{18}F-FDG e 150 minutos para ^{18}F-NaF foram as condições ideais. Após a padronização, a imagem PET de corpo inteiro com ^{18}F-FDG foi capaz de revelar inflamação induzida por LPS, pois foram observadas diferenças significativas na captação de marcadores entre animais controle e injetados com LPS na região da cabeça. Em resumo, este estudo demonstrou que a imagem PET *in vivo* em peixe-zebra é viável e reproduzível.

A incidência de DP tem sido associada a fatores de estilo de vida, como dieta. Na última parte desta tese, investigamos o efeito da dieta em um modelo em roedor para a DP. No **capítulo 5**, no entanto, analisamos primeiro as contribuições de uma dieta de cafeteria (dieta altamente calórica) no sistema de recompensa. A dopamina e seus receptores D_2 estão envolvidos no sistema de recompensa. O consumo de uma dieta rica em gordura e açúcar pode levar a uma diminuição na expressão dos receptores D_2 e no *turnover* da dopamina. O comportamento alimentar compulsivo observado em pacientes obesos pode estar relacionado à diminuição da ativação do

sistema de recompensa ou a um baixo número de receptores D_2. O principal objetivo do estudo descrito no capítulo 5 foi investigar o efeito da ingestão de alimentos altamente palatáveis na disponibilidade de receptores de dopamina D_2 em ratos. Ratos Wistar machos foram divididos em 2 grupos: dieta padrão (ração, 14% de gordura) ou dieta de cafeteria (ração com alto teor de gordura, 45% de gordura; chocolate, queijo e água com açúcar). Cada animal foi submetido a três PET *scans* com ^{11}C-raclopride para avaliação da disponibilidade do receptor de dopamina D_2: antes do início da dieta; após 4 semanas na dieta (dia 28) e imediatamente após consumir leite condensado altamente palatável (dia 30). A partir do dia quatro, o ganho de peso corporal e a ingestão calórica foram significativamente maiores no grupo dieta de cafeteria do que no grupo controle. Os animais do grupo da dieta padrão não mostraram diferenças significativas na captação de ^{11}C-raclopride no estriado entre o PET antes da dieta e as duas análises de acompanhamento. A dieta de cafeteria diminuiu a ligação do marcador em ambos os períodos de acompanhamento, o que é indicativo de uma redução na disponibilidade dos receptores D_2. Nosso estudo sugere que os receptores D_2 desempenham um papel na obesidade e no consumo de uma dieta rica em calorias. Mais importante, mostrou que o desafio com o leite condensado não foi capaz de alterar a resposta dopaminérgica conforme observado pelo PET. Mais estudos devem ser feitos para mostrar se há alguma alteração nos níveis de dopamina e moléculas relacionadas.

Para elucidar a influência da dieta hiperlipídica na DP, investigamos o efeito de uma dieta hiperlipídica (DH) na disponibilidade de receptores D_2 e parâmetros comportamentais em ratos (**capítulo 6**). Ratos Wistar foram alimentados com uma DH (60% de gordura) ou uma dieta padrão (10% de gordura) por três meses. Após dois meses de dieta, os animais foram submetidos à administração estereotáxica de baixa dose de 6-OHDA (2 x 3 µg) no estriado direito. PET *scans* utilizando ^{11}C-Raclopride foram realizadas dois dias antes e um mês após a cirurgia. Após três meses, os animais em DH apresentaram maior ganho de peso corporal e ingestão calórica do que aqueles em dieta padrão. A cirurgia causou uma diminuição temporária do peso corporal nos dois grupos. Não foi encontrada diferença no teste de tolerância à glicose entre os grupos DH e dieta padrão. O PET ^{11}C-raclopride demostrou uma taxa de captação ipsilateral / contralateral significativamente reduzida no estriado no grupo DH 1 mês após a injeção de 6-OHDA do que no grupo da dieta padrão e DH antes da

cirurgia estereotáxica, mas nenhuma diferença significativa foi encontrada entre os grupos 1 mês após administração de 6-OHDA. Esse padrão também foi encontrado no teste de cilindros, mas não foram encontradas diferenças entre os grupos e os tempos em outras análises de comportamento. A análise *post-mortem* do intestino mostrou captação de ^{11}C-raclopride significativamente menor no duodeno dos animais do grupo DH do que do grupo da dieta padrão. Concluindo, a DH agravou os danos no modelo de DP neste estudo, o que refletiu em uma menor disponibilidade do receptor D_2 e uso da pata contralateral. Ambas as observações sugerem um papel prejudicial da DH no início ou progressão da doença de Parkinson.

No **capítulo 7**, o impacto de uma dieta hiperlipídica nos parâmetros inflamatórios no modelo de DP foi investigado. Em particular, a composição da microbiota, o desenvolvimento da neuroinflamação e as manifestações clínicas foram avaliadas em um modelo de roedor validado (injeção unilateral de 6-OHDA). Os ratos foram alimentados com uma dieta hiperlipídica (DH) ou uma dieta padrão por três meses. Após dois meses de dieta, os ratos foram submetidos à cirurgia estereotáxica, injetando uma dose baixa de 6-OHDA. PET *scans* utilizando ^{11}C-PBR28 foram realizadas antes e 1 mês após a injeção de 6-OHDA. As fezes foram coletadas antes do início da dieta, após dois meses da dieta e após 1 mês da injeção de 6-OHDA. Análises de microbiomas foram realizadas em amostras de fezes. Um aumento no peso corporal, uma diminuição no uso da pata ipsilateral, conforme avaliada pelo teste do cilindro, e aumento da captação do marcador neuroinflamatório ^{11}C-PBR28 foram demonstrados em animais com dieta hiperlipídica após 1 mês da injeção de 6-OHDA, em comparação com os resultados antes da cirurgia estereotáxica. Não foram encontradas diferenças entre os tempos no grupo da dieta padrão. O aumento do acúmulo de ^{11}C-PBR28 no grupo DH após a administração de 6-OHDA foi acompanhado de alterações no perfil da microbiota e não nos níveis de citocinas no plasma. Este estudo sugere que o impacto da DH na neuroinflamação pode ser mediado pelo microbioma intestinal no modelo de rato 6-OHDA da doença de Parkinson e sugere que seja independente da inflamação periférica.

Em conclusão, a busca pelas vias básicas subjacentes à DP e a relação com as mudanças no estilo de vida podem ajudar os cientistas a entender melhor os fatores que desencadeiam o aparecimento da doença e auxiliar no desenvolvimento de novos

tratamentos e ferramentas de diagnóstico. Minha pequena contribuição para esse campo é a possibilidade de investigar a interação de duas doenças (obesidade e DP) no mesmo animal, utilizando o PET para monitoramento longitudinal. Isso pode ajudar a revelar a cumplicidade da interação entre comportamento, microbiota, resposta dopaminérgica e purinérgica e da inflamação no intestino e no cérebro.

List of Publications

Giacobbo BL, Doorduin J, Moraga-Amaro R, **Nazario LR**, Schildt A, Bromberg E, Dierckx RAJO, de Vries EFJ. Chronic harmine treatment has a delayed effect on mobility in control and socially defeated rats. Psychopharmacology (Berl). 2020 Feb 22. doi: 10.1007/s00213-020-05483-2.

Budel RG, da Silva DA, Moreira MP, Dalcin AJF, da Silva AF, **Nazario LR**, Majolo JH, Lopes LQS, Santos RCV, Antunes Soares FA, da Silva RS, Gomes P, Boeck CR. Toxicological evaluation of naringin-loaded nanocapsules in vitro and in vivo. Colloids Surf B Biointerfaces. 2019 Dec 23;188:110754. doi: 10.1016/j.colsurfb.2019.110754.

Haab Lutte A, Huppes Majolo J, **Reali Nazario L**, Da Silva RS. Early exposure to ethanol is able to affect the memory of adult zebrafish: Possible role of adenosine. Neurotoxicology. 2018 Dec;69:17-22. doi: 10.1016/j.neuro.2018.08.012. Epub 2018 Aug 26.

Lutte AH, **Nazario LR,** Majolo JH, Pereira TCB, Altenhofen S, Dadda ADS, Bogo MR, Da Silva RS. Persistent increase in ecto-5'-nucleotidase activity from encephala of adult zebrafish exposed to ethanol during early development. Neurotoxicol Teratol. 2018 Nov - Dec;70:60-66. doi: 10.1016/j.ntt.2018.10.004. Epub 2018 Oct 24.

Nazario LR, da Silva RS, Bonan CD. Targeting Adenosine Signaling in Parkinson's Disease: From Pharmacological to Non-pharmacological Approaches.Front Neurosci. 2017 Nov 23;11:658. doi: 10.3389/fnins.2017.00658. eCollection 2017. Review.

Nazario LR, Antonioli R Jr, Capiotti KM, Hallak JE, Zuardi AW, Crippa JA, Bonan CD, da Silva RS. Caffeine protects against memory loss induced by high and non-anxiolytic dose of cannabidiol in adult zebrafish (Danio rerio). Pharmacol Biochem Behav. 2015 Aug;135:210-6. doi: 10.1016/j.pbb.2015.06.008. Epub 2015 Jun 20.

Capiotti KM, Fazenda L, **Nazario LR**, Menezes FP, Kist LW, Bogo MR, Da Silva RS, Wyse AT, Bonan CD. Arginine exposure alters ectonucleotidase activities and

morphology of zebrafish larvae (Danio rerio). Int J Dev Neurosci. 2013 Feb;31(1):75-81. doi: 10.1016/j.ijdevneu.2012.09.002. Epub 2012 Sep 17.

Capiotti KM, Menezes FP, **Nazario LR,** Pohlmann JB, de Oliveira GM, Fazenda L, Bogo MR, Bonan CD, Da Silva RS. Early exposure to caffeine affects gene expression of adenosine receptors, DARPP-32 and BDNF without affecting sensibility and morphology of developing zebrafish (Danio rerio). Neurotoxicol Teratol. 2011 Nov-Dec;33(6):680-5. doi: 10.1016/j.ntt.2011.08.010. Epub 2011 Sep 3.

About the Author

Since childhood, I had a dream of being a researcher. I chose the Faculty of Pharmacy as the platform to fulfil my dream. Since then, my commitment and enthusiasm to this profession as a researcher keeps rising. Also, because the research can result in beneficial changes for the community.

In 2009, I started the Bachelor's in Pharmacy at Pontifical Catholic University of Rio Grande do Sul (PUCRS, Brazil). During undergraduate education, I spent more than 3 years at the Laboratory of Neurochemistry and Psychopharmacology (PUCRS) as a Scientific Initiation student. I was sponsored by the Brazilian Government (FAPERGS and CNPq Brazilian Organization) under the supervision of professors Dr. Carla Denise Bonan and Dr. Rosane Sousa da Silva. The laboratory utilizes zebrafish (Danio rerio) as the animal model and the main topic of study is neurodegenerative diseases.

In 2012, still as an undergraduate student, I was part of the Brazilian Program "Science Without Borders". I received a full scholarship to study at Hanyang University, South Korea, for six months. I also did an internship that was at the Immunology Laboratory of Seoul National University with Prof. Dr. Seung Hyeok Seok, researching about hyperpigmentation in zebrafish.

In 2013, after graduating in Pharmacy I applied to the Graduate Program in Cellular and Molecular Biology (PUCRS, Brazil), under the supervision of Dr. Prof. Rosane Souza da Silva and co-supervision of Dr. Prof. Cristina Maria Moriguchi Jeckel. The Master's project was entitled "Adaptation of Zebrafish to PET/CT: evaluation of images with ^{18}F-FDG and study of adenosine in the context of inflammation", which was sponsored by the Brazilian Government (FAPERGS). I defended my thesis successfully on June 2016. In 2015, I applied to the Abel Tasman Talent Program to initiate my double degree PhD between my home university (PUCRS, Brazil) and University Medical Center Groningen (UMCG, Netherlands). The subject of my thesis is the relation of Diabetes Mellitus (DM) Type 2 (lifestyle) and

Parkinson’s Disease (PD). My supervisors are: Dr. Prof. Rosane Souza da Silva, Dr. Prof. Cristina Maria Moriguchi Jeckel, Dr. Prof. Erik FJ de Vries, Dr. Janine Doorduin.

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DECLARATION

Groningen, 25 August 2020

The Board of the University of Groningen hereby declares that

LUIZA REALI NAZARIO

born in Porto Alegre (Brazil) on 20-12-1990,
gained a PhD in accordance with Article 7.18 of the Higher Education and Research Act (WHW)
and was awarded the degree of Doctor of the University of Groningen on 25-08-2020.

The University of Groningen and the Pontifical Catholic University of Rio Grande do Sul have jointly supervised the dissertation and awarded the double doctorate.

The thesis was entitled

PRE-CLINICAL INVESTIGATION OF BRAIN MECHANISMS ASSOCIATED WITH PARKINSON'S DISEASE: THE IMPACT OF DIET

Supervisors: Prof. E.F.J. de Vries
Prof. R. Souza da Silva

On behalf of the Board
of the University of Groningen,
The Registrar,

university of groningen

A. van den Bos, LLM