High fat diet-induced obesity causes a reduction in brain tyrosine hydroxylase levels and non-motor features in rats through metabolic dysfunction, neuroinflammation and oxidative stress

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ABSTRACT

Obesity is a health problem that has been associated with neuroinflammation, decreased cognitive functions and development of neurodegenerative diseases. Parkinson’s disease (PD) is a chronic neurodegenerative condition characterized by motor and non-motor abnormalities, increased brain inflammation, α-synuclein protein aggregation and dopaminergic neuron loss that is associated with decreased levels of tyrosine hydroxylase (TH) in the brain. Diet-induced obesity is a global epidemic and its role as a risk factor for PD is not clear. Herein, we showed that 25 weeks on a high-fat diet (HFD) promotes significant alterations in the nigrostriatal axis of Wistar rats. Obesity induced by HFD exposure caused a reduction in TH levels and increased TH phosphorylation at serine 40 in the ventral tegmental area. These effects were associated with insulin resistance, increased tumor necrosis factor-α levels, oxidative stress, astroglialosis and microglia activation. No difference was detected in the levels of α-synuclein. Obesity also induced impairment of locomotor activity, total mobility and anxiety-related behaviors that were identified in the open-field and light/dark tasks. There were no changes in motor coordination or memory. Together, these data suggest that the reduction of TH levels in the nigrostriatal axis occurs through an α-synuclein-independent pathway and can be attributed to brain inflammation, oxidative/nitrosative stress and metabolic disorders induced by obesity.

Introduction

Currently, it is estimated that obesity affects 650 million adult people worldwide, being considered a major causative factor of health complications including insulin resistance and type-2 diabetes [1,2]. Obesity has also been associated with age-related pathologies, including neurodegenerative diseases. Obesity is defined as a chronic inflammatory condition that involves increased levels of circulating pro-inflammatory cytokines, proteases and growth factors derived from adipose tissue [2,3]. Diet is the most important factor that contributes to obesity development [4].

All hypotheses relating to obesity with neurodegenerative diseases suggest an important role for neuroinflammation and subsequent insults to the blood–brain barrier [2,5,6]. It is known that adipose tissue plays a key role in the development of low-grade chronic inflammation due to the production of inflammatory mediators [7]. However, high levels of pro-inflammatory cytokines (such as tumor necrosis factor (TNF)-α and interleukins), growth factors and increased oxidative stress are also found in the brain of rats exposed to a high-fat diet (HFD) [3,8]. This accumulation of inflammatory mediators has also been described in many neurological complications associated with impairment of cognitive function [9]. In general, evidence suggests that comorbidities associated with obesity significantly contribute to cognitive deficits and accelerate the process of dementia, such as Alzheimer’s disease and Parkinson’s disease (PD) [10,11].

PD is a severe neurodegenerative disease characterized by the progressive loss of dopaminergic neurons in the substantia nigra (SN) pars compacta and reduction of dopamine synthesis and propagation in other regions of the nigrostriatal pathway, e.g. striatum (ST) and ventral tegmental area (VTA) [12]. Clinically, PD is identified by motor behavior irregularities detected as elevated resting tremor, bradykinesia,
muscle stiffness and postural instability associated to dopamine deficiency, as well as non-motor symptoms such as anxiety, cognitive impairment and depression. The non-motor aspects of the PD are very common, occurring years prior to motor symptoms [13]. The motor disturbances are correlated with degeneration of dopaminergic neurons and reduction of tyrosine hydroxylase (TH) levels, a rate-limiting enzyme for formation of L-DOPA and dopamine biosynthesis. On the other hand, the non-motor features are related to disturbances in non-dopaminergic regions [14].

Although intracellular α-synuclein (α-syn) inclusions (Lewy bodies) are a pathological hallmark of PD, the mechanisms responsible for neurodegeneration in this disease are still unclear [15]. Microglia-mediated neuroinflammation, oxidative stress, increased production of pro-inflammatory cytokines and inflammatory factors have attracted significant attention in the pathogenesis of PD [16–18]. Sporadic PD represents the majority of the cases of this neuropathology [19]. In line with this, epidemiological and clinical studies have indicated an important association between dietary intake, elevated body mass index, body fat deposition and metabolic disorders such as type-2 diabetes and the risk for the development of PD [20–22]. Experimental studies showed a reduction in TH expression and protein levels in the midbrain of obese mice [23,24]. Although the deleterious effects of obesity on the dopaminergic system seem to occur, the mechanisms involved in this process remain uncertain.

Therefore, the purpose of the present study was to investigate the effects of diet-induced obesity on parameters related to dopaminergic neurodegeneration and neuroinflammation. In this context, adult Wistar rats were exposed to diet-induced obesity through a 25-week controlled HFD. The levels of TH and α-syn, as well immunofluorescence of glial fibrillary acidic protein (GFAP, astrocytes marker), ionized calcium-binding adaptor molecule 1 (Iba1, marker of microglia) and phosphorylated TH were measured in the SN, VTA and ST of obese rats. In order to explain the relationship between obesity and neuronal damage, parameters of systemic and brain inflammation or oxidative/nitrosative stress were evaluated. Finally, we analyzed the effects of diet-induced obesity on total locomotion, anxiety-like behavior, memory and motor coordination determined by behavior tests. Together, our findings indicated that HFD induces reduction of TH levels while enhancing TH phosphorylation at Ser40 in the VTA, without affecting α-syn levels. We also detected increased levels of TNF-α and oxidative/nitrosative stress in the brain, as well as GFAP and Iba1 staining consistent with astrogliosis and microgliosis in SN and VTA. Impairment of locomotor activity, mobility and anxiogenic-like behavior also were identified in obese rats.

Materials and methods

Chemicals

Polyclonal IL-1β antibody, monoclonal TNF-α and monoclonal nitrotyrosine antibodies, and 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) nucleic acid staining were purchased from Sigma–Aldrich (St. Louis, USA). ELISA microplates were from Greiner Bio-One (Monroe, USA). Electrophoresis and immunoblot reagents were from Bio-Rad (Hercules, USA), GE Healthcare Brazilian Headquarter (São Paulo, Brazil) and Sigma–Aldrich. TH polyclonal antibody, TH phosphorylated at Ser40 antibody, α-synuclein monoclonal antibody, β-actin polyclonal antibody, GFAP polyclonal antibody, Iba1 polyclonal antibody, anti-rabbit Alexa 488 or 555 and anti-goat Alexa 555 antibodies, anti-rabbit immunoglobulin linked to peroxidase, anti-mouse immunoglobulin linked to peroxidase and biotin-labeled secondary antibody were from Cell Signalling Technology (MA, USA). The chemiluminescence substrate was obtained from West Pico detection kit from Thermo Scientific Pierce Protein Biology Products (Rockford, USA).

Animals and study design

Male Wistar rats (n = 10), 28 days old (90–110 g) from the Instituto de Ciências Básicas da Saúde Animal Care Facility-UFRGS were maintained in collective cages (four per cage) under temperature-controlled room (22 ± 2°C) with a 12 h light–dark cycle and tap water ad libitum. The animals were randomly distributed into two dietary groups: the control group (CTRL) that was placed on a standard diet (10% of energy from fat) or an HFD group that was exposed to a diet-induced obesity (43% of energy from fat) that was prepared in-house as described previously [25] for 25 weeks. The composition and caloric profile of the used diet are presented in Table 1. Food intake was assessed weekly and glucose intolerance and insulin resistance were evaluated at the

| Diet component (%) kcal | Standard diet | High-fat diet |
|-------------------------|---------------|---------------|
| Protein                 | 21            | 17            |
| Carbohydrate            | 69            | 40            |
| Fat*                    | 10            | 43            |
| Total, kcal/kg          | 3760          | 4660          |

* Standard diet: fat content derived from vegetable oils (soy oil).
* High-fat diet: fat content derived from soy oil and lard.
end of the dietary intervention (Table 2). The rats were subjected to behavior tests performed at different consecutive days, one week before euthanasia. At the day of euthanasia, the cerebrospinal fluid (CSF) and serum samples were collected and frozen for later analysis. SN, VTA and ST were removed using a rodent brain matrix (ASI-Instruments, Warren MI, USA), maintained in liquid nitrogen to determine the levels of specific proteins by immunoblotting and for oxidative stress analysis. Pro-inflammatory cytokines (TNF-α and IL-1β) were measured in the liver, adipose tissue, brain and plasma to evaluate the inflammatory profile. In a separate group of 12 animals (6 per group), the perfused whole brain was fixed with 4% paraformaldehyde and separated into slices to perform the detection of specific proteins by immunofluorescence. We used a total of 32 animals in this study. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996) and were carried out according to the determinations of the Brazilian Council for the Control of Animal Experimentation (CONCEA). The experimental protocol was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, (CEUA-UFRGS) register number 29493.

**IP-GTT, IP-ITT and insulin measurement**

In order to determine the effects of HFD on insulin resistance, intraperitoneal glucose tolerance test (IP-GTT) and intraperitoneal insulin tolerance test (IP-ITT) were performed 12 h fasting after 21 weeks of diet. To establish basal values of glucose blood samples were taken by lancing the tail vein. For IP-GTT blood samples were taken at 30, 60, 90 and 120 min after glucose administration (1 g/kg intraperitoneal, i.p.). For IP-ITT blood samples were taken at 15, 30, 60 and 90 min after human regular insulin administration (Novolin® R Novo Nordisk - 2IU/kg, i.p.). The incremental area under the curve was calculated relative to basal values. For insulin measurement, plasma and CSF samples of fasted rats were separated and kept at −80°C for later analysis by ELISA (Mercodia Ultrasensitive Rat Insulin ELISA kit). Homeostatic model assessment of insulin resistance (HOMA-IR), an equation that estimates insulin sensitivity was calculated according to the formula: fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5 [26].

**Behavior tests**

**Open field test**

Spontaneous locomotion, exploratory activity and anxiety profile were simultaneously evaluated in an open field test [27]. The animals were placed in the center of apparatus consisting of a black rectangular open field (50 cm²) and allowed to freely explore the arena for 10 min. In summary, the experiment consisted of two trials: training and test session separated by 24 h. Total distance traveled, distance traveled in the center zone, total time immobile, immobile episodes and number of rearings (standing on hind legs without paws pressed against the wall of the arena) were recorded for each animal using the ANY-Maze video-tracking software.

**Light/dark task**

In order to analyze the anxiety profile, the light/dark task was performed as previously described [28]. The light/dark task apparatus consisted of a rectangular acrylic box with two separated compartments. One compartment had black walls and a black floor (without illumination) with a size of 21× 35× 41 cm (height × length × width). The other had white walls and a white floor, with a size of 21 × 45 × 41 cm (height × length × width, illuminated by a 100-W lamp placed 45 cm above the center of the box). An 8 × 5 cm (height × length) opening joined both compartments. Each rat was placed in the light compartment facing away from the opening and allowed to explore the box for 5 min. The number of transitions between compartments, the time spent in the light compartment and risk assessment behavior (R.A.B, number of times the muzzle and two front paws extended into the light box from the dark box) were analyzed by ANY-Maze software.

**Object recognition**

The object recognition task was performed in the same apparatus of open field test and conducted as previously described [29]. During the sample sessions, rats were placed in the experimental apparatus in the presence of two identical objects (A; A′) to explore for 10 min. After that, rats were returned to their home cage for 24 h with food and water provided ad libitum. On the next day, the test session was carried out replacing one of the familiar objects by a novel object (B). The total exploration time (T) of each object was recorded.

| Table 2. Physiological measures of rats after HFD. |
|-----------------------------------------------|
| Measures           | Control     | HFD          |
| Body weight (g)    | 453.24 ± 25.06 | 541.70 ± 56.77* |
| Food intake (g)    | 169.52 ± 7.21   | 149.65 ± 9.16* |
| Energy intake (Kcal)| 641.89 ± 23.61  | 713.47 ± 25.58* |
| Fasting glucose (mg/dL)| 86.50 ± 11.67  | 107.33 ± 6.45** |
| Fasting insulin (mU/L)| 63.72 ± 64.74  | 310.40 ± 150.15** |

Note: Data are mean ± SD (n = 10/group). *p < 0.05 and **p < 0.01 vs CTRL group. Values of P were obtained by applying two-tailed Student’s t-test.
The discrimination ratio was calculated according to the formula: \((\text{Tnovel} - \text{Tfamiliar})/(\text{Tnovel} + \text{Tfamiliar})\).

**Rotarod test**

Motor coordination deficits were assessed by rotarod apparatus consisted of a striated rod providing a good grip (diameter: 3 cm), separated in four compartments (width: 8.5 cm) and located 27.2 cm above the floor grid. The animals were trained for 2 days (8 RPM for 120 s) until they demonstrated the ability to remain on the spindle for 60 s and then subjected to a baseline test trial on the accelerating spindles (4–40 RPM for 300 s) [30]. The time each rat remained on the drum was recorded.

**CSF collection**

Rats were anesthetized using isoflurane inhalation (Forane®, Abbott SA, Buenos Aires; 3% oxygen flow 0.5 L/min) via a calibrated vaporizer and were placed in a stereotaxic apparatus. The CSF was collected (100 μL) by directly puncturing the cisterna magna with an insulin syringe [31]. All samples were centrifuged (16,000 g at 4°C for 10 min) and stored at −80°C.

**TNF-α, IL-1β and nitrotyrosine levels**

To evaluate the inflammatory response induced by obesity TNF-α and IL-1β were measured in the liver, retroperitoneal white adipose tissue (WAT), ST and plasma by indirect ELISA [32]. For nitrosative damage in the peritoneal white adipose tissue (WAT), ST and plasma 137 mM, KCl 2.7 mM, Na2HPO4 10 mM, KH2PO4 1.8 mM, pH 7.4 and equal amounts of protein (40 μg/well) were fractionated and subjected to 12% or 15% SDS–PAGE and then transferred to a nitrocellulose membrane (Trans-blot SD semi-dry transferrcell, BioRad). Membranes were blocked with 5% skin milk and incubated overnight at 4°C with primary antibodies (anti-TH, anti-α-syn and anti-β-actin at 1:1000) and washed with TTBS three times. Anti-rabbit or anti-mouse IgG peroxidase-linked secondary antibody (1:2000) was incubated for 1 h at room temperature. Immunoreactivity was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent kit from Thermo Scientific (Luminol/Enhancer and Stable Peroxide Buffer) with an ImageQuant LAS 4000 imaging system (GE Healthcare, Pittsburgh, USA). Traced band area was normalized for background density and expressed as the product of band density and area that was quantified by Image J. Software [25].

**Sulphydryl groups quantification**

Oxidative status of thiol groups was assessed by the quantification of total reduced sulphydryl (SH) groups in SN, VTA, and ST samples [34]. For total SH content measurement, sample aliquot (60 μg per well) was diluted in PBS buffer pH 7.4 and 5,5-dithiobisnitrobenzoic acid (10 mM). After 60 min of incubation, the plate was read at 412 nm.

**Immunofluorescence staining**

Frozen serial coronal sections (20 μm) obtained from fixed brain tissue of 12 animals \((n = 6)\) were prepared as previously described [35]. The free-floating sections were incubated in 5% BSA for 2 h to block nonspecific binding. The sections were then incubated with rabbit polyclonal anti-TH (1:200), goat polyclonal anti-Iba1 (microglia marker; 1:500), rabbit polyclonal anti-GFAP (astrocyte marker; 1:500) in 0.1 M PBS buffer pH 7.4 containing 2% BSA. After 48 h of incubation at 4°C, the tissue sections were washed three times with PBS and resuspended in 50 μL of secondary antibodies anti-rabbit Alexa 488 or 555 and anti-goat Alexa 555 (1:500) for 1 h at room temperature. After washing, the slices were incubated with DAPI during 5 min for counterstaining nuclei. The negative controls were performed omitting the primary antibodies. The images were obtained using a Microscopy EVOS® FL Auto Imaging System.
(AMAFD1000 – Thermo Fisher Scientific; MA, USA), with magnification 400×, 200× and 100×. Immunofluorescence quantification of pSer40TH was performed using the software ImageJ measuring the pixels of images. In addition, a qualitative analysis were performed in the GFAP, IBA-1 and TH staining images.

Data analysis
Intergroup comparisons were performed by two-tailed Student’s t-test. For comparison of multiple means, one-way analysis of variance followed by Tukey’s post hoc test was applied. Correlation between total distance traveled × body weight and TH immunocontent of each animal was performed, using Pearson’s correlation. Statistical differences were considered significant when \( P < 0.05 \) calculated in GraphPad Prism software for Windows version 7.01.

Results
HFD exposure causes obesity and insulin resistance in rats
To evaluate the effects of HFD consumption, we measured body weight gain of rats every week. Differences in the body weight gain between groups starts from 13-week diet (Figure 1(A), \( P < 0.001, t (18) = 7.14 \)). Twenty-five weeks of HFD resulted in an increase of 19.2% in the total body weight compared to control group placed on a standard diet (Figure 1(A), \( P < 0.001, t (18) = 4.51 \)). This increased body weight was associated to a higher energy intake (kJ) arising from the diet (Figure 1(C), \( P < 0.05, t (18) = 6.50 \)) although the food intake in mass (grams) was lower (Figure 1(B), \( P < 0.05, t (18) = 5.39 \)). Moreover, changes in the body fat mass were found in the HFD-obese rats with an increase in the body fat percentage of retroperitoneal WAT (306 ± 191%, \( P < 0.001, t (18) = 6.71 \)), epididymal WAT (105 ± 22%, \( P < 0.001, t (18) = 5.02 \)), brown adipose tissue (AT) (161 ± 87%, \( P < 0.01, t (18) = 3.18 \)) and liver (32 ± 11%, \( P < 0.001, t (18) = 5.74 \), Figure 1(D,E)) when compared to CTRL group, most likely due to an increased fat deposition in the tissue (fatty liver), confirming the expected response of obesity induced by the HFD. However, there were no significant differences in muscle and brain mass between groups (Figure 1(D)).

The HFD exposure resulted in elevated fasting glucose (Figure 1(F), \( P < 0.001, t (18) = 4.94 \)) and insulin (Figure 1(G), \( P < 0.01, t (18) = 4.77 \)) levels, as well as HOMA-IR (Figure 1(H), \( P < 0.001, t (18) = 26.10 \)). On the other hand, we were unable to detect insulin in the rats CSF samples (data not shown). Hyperglycemia was observed in HFD-obese individuals 30 min after administration of glucose solution in the IP-GTT, remained significantly elevated up to 60 min and returned to baseline levels 120 min later. No changes were found in the blood glucose levels in CTRL group (Figure 1(I)). Similarly, glucose levels remained elevated in the HFD-obese rats after insulin administration at all time points evaluated in the IP-ITT.

Figure 1. Metabolic parameters of HFD-induced obesity, glucose intolerance and insulin resistance. (A) body weight gain per week and final body weight, (B) total food intake, (C) total energy intake, (D) tissue mass of retroperitoneal white adipose tissue (RETRO WAT), epididymal white adipose tissue (EPI WAT), brown adipose tissue (BROWN), gastrocnemius muscle (GASTRO), soleus muscle (SOLEUS), liver and whole brain (BRAIN), (E) tissue mass increase, (F) fasting glucose concentration (mg/dL), (G) fasting insulin concentration (pmol/L), (H) HOMA-IR, (I) intraperitoneal glucose tolerance test (IP-GTT) and area under the curve between 0 and 120 min (AUC IP-GTT), and (J) intraperitoneal insulin tolerance test (IP-ITT) and area under the curve between 0 and 90 min (AUC IP-ITT), of rats placed on 25 weeks of HFD. Data are mean ± SD (n = 10/group). *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \) vs control group (CTRL). Statistical differences for multiple means were determined by one-way ANOVA with Tukey’s post hoc test. For intergroup comparisons, values of \( P \) were obtained by applying two-tailed student’s t test (\( P < 0.05 \)).
(Figure 1(J)), pointing that diet markedly induced insulin receptor dysfunction, i.e. insulin resistance. Furthermore, the area under the curve of both tests was significantly increased in HFD-obese rats as compared to CTRL group (Figure 1(I,J); \(t(18) = 9.30\) and \(t(18) = 8.18\), respectively).

**Obesity leads to peripheral inflammation and neuroinflammation in rats**

To evaluate the inflammatory profile in rats after HFD exposure, we firstly analyzed pro-inflammatory cytokines levels in peripheral and brain tissues, as well as plasma. TNF-\(\alpha\) levels were significantly increased in the retroperitoneal WAT (\(P < 0.01, t(18) = 7.64\)), liver (\(P < 0.001, t(18) = 7.08\)), brain (striatum) (\(P < 0.05, t(18) = 3.48\)) and plasma (\(P < 0.05, t(18) = 2.62\)) of HFD-obese rats when compared to CTRL group (Figure 2(A)). As expected, in response to obesity, there was an increase in levels of circulating IL-1\(\beta\) in the liver (\(P < 0.01, t(18) = 10.29\)) and plasma (\(P < 0.01, t(18) = 4.21\)), although the same result was not observed in adipose tissue. Additionally, no differences were observed in IL-1\(\beta\) levels in brain tissue after 25 weeks of high-fat diet exposure (Figure 2(B)).

To investigate whether obesity promotes effects in the number and activity of glial cells, we carried out immunofluorescence analysis in the SN, VTA and ST slices obtained from brain sections of rats fed with HFD. We examined the status of astrogliosis and microglia activation with GFAP and Iba1 staining, respectively. Increased staining of GFAP and Iba1 was observed in the SN and VTA of HFD-obese rats (Figure 3(A,B)) but not in the ST (data not shown).

**TH protein content is decreased in the VTA of rats exposed to HFD**

To determine whether HFD-induced obesity affects the expression of biochemical parameters commonly associated to neuronal integrity and neurodegeneration in PD, TH and \(\alpha\)-syn protein levels were measured by immunoblotting in the SN, VTA and ST. TH protein content was markedly reduced in VTA of HFD-obese rats (\(P < 0.01, t(12) = 2.96\)) when compared to CTRL group, but no alterations were observed in SN (Figure 4(A,B)). No differences were found in the levels of \(\alpha\)-syn in both structures (Figure 4(E,F)). There were no alterations detected in any of these proteins in the ST (data not shown).

Additionally, we evaluated TH-positive cells in the SN, VTA and ST slices obtained from brain sections of rats exposed to HFD, using immunofluorescence analysis. Decreased staining of TH-positive cells in VTA slices of HFD-obese rats was detected (Figure 5(B)). Besides, morphology of TH-positive cells of VTA in HFD-obese rats was altered as well. No significant alterations were observed in the SN (Figure 5(A)) and ST (data not shown), regarding TH levels.

**TH phosphorylation at serine 40 is increased in the VTA of rats exposed to HFD**

Since TH activity is enhanced by phosphorylation of serine 40, we also performed IF analysis to evaluate the levels of phospho-TH at Ser40 (pSer40TH) in the VTA and SN. Levels of pSer40TH were not altered in the SN (Figure 6(A–C)), but in the VTA they were evidently increased in the HFD group (Figure 6(B–D); \(P < 0.01, t(10) = 19.69\)), indicating that TH activity in the VTA is enhanced in these animals.

**Rats feeding HFD present oxidative stress in VTA and SN**

Parameters of nitrosative and oxidative damage were quantified in the SN, VTA an ST samples from obese rats. The levels of nitrotyrosine were increased in the VTA (\(P < 0.01, t(18) = 5.24\)) and ST (\(P < 0.05, t(18) = 4.52\)) of HFD-obese rats (Figure 7(A)) but not in the
SN. Besides, HFD resulted in a decrease of free-thiol groups in SN ($P < 0.05$, $t$ (18) = 2.41) and ST ($P < 0.05$, $t$ (18) = 2.18) but no changes were detected in VTA samples (Figure 7(B)).

**HFD fed rats present behavioral alterations**

Behavioral tests were also performed in the CTRL group (black circles) and HFD group (grey squares). HFD-induced obesity produced a negative effect on the total distance traveled optimally seen in the first 3 min of open field training, without differences in an open field test (Figure 8(A,B)). Each minute of distance traveled in open field task is presented in Figure 8 ((D,E); $P < 0.05$, $t$ (18) = 5.62 and 3.56). No difference was observed in the distance traveled in the center zone between groups and therefore there was no characterization of anxiety behavior in this test (Figure 8(F)).

Corroborating with the results of decreased spontaneous locomotion, the number of immobile episodes...
and total immobile time was higher (Figure 8(C), \( P < 0.05, t (18) = 5.86 \) and \( t (18) = 3.39 \)) and the number of rearing (Figure 8(G,H), \( P < 0.05, t (18) = 5.07 \) and \( t (18) = 3.80 \)) was significantly lower in HFD-obese rats when compared to CTRL group. No difference was found in the correlation between total distance traveled in open field training and body weight, demonstrating that decreased total locomotion and mobility cannot be associated to increased body weight of the obese rats (Figure 8I). In contrast, there was a significant correlation between decreased total distance traveled in open field training with low levels of TH in the HFD-obese rats (Figure 8(J); \( P < 0.0212, r = 0.6291 \)). Furthermore, to test motor coordination, rats were trained on a rotarod during two consecutive days. We observed differences in time spent on rotating rod of HFD-obese rats compared to CTRL group during the first exposure of animals. However, both groups improved their ability to remain on a rotating rod as a function of the number of trials and, due to this, motor learning velocity was similar between groups in the test (Figure 9(A)).

Finally, the results found in light/dark task showed anxiogenic-like effect of high-fat diet characterized by a decreased number of transitions between compartments, time spent in the light compartment and risk assessment behavior of HFD-obese rats (Figure 9(D–F); \( P < 0.05 \) and \( P < 0.01 \), \( t (18) = 2.57 \), \( t (18) = 4.27 \) and \( 3.56 \)). In the object recognition task, there was no memory impairment observed in the HFD-obese rats (Figure 9(B,C)). In the test session, no significant difference was found in the time spent between familiar object and novel object (Figure 9(C)).

**Discussion**

Obesity-induced metabolic dysfunction is related to the increased incidence of dementia, morphological changes that could lead to neuronal death and impairment of cognitive functions. Because many of these
alterations are similar to those found in elderly people, obesity has been established in the literature as an accelerating factor of the aging process and thus could contribute to the onset of neurodegenerative diseases such as PD through events that still need to be clarified [3,11,12,14,36]. In this study, we contributed with some mechanisms that are underlying the cerebral and behavioral alterations induced by obesity. Obesity is considered a chronic inflammatory condition with a multifactorial etiology including diet, that is strongly related to metabolic disorders [1,4,7]. Herein, we used rats exposed to HFD [25] during 25 weeks as an experimental model of obesity. Rats fed with HFD developed obesity features – overweight, adiposity insulin resistance and peripheral inflammation.

It is well known that intraneuronal α-syn aggregation in the form of Lewy bodies, neurodegeneration (mainly dopaminergic) and TH loss represent the major pathological hallmarks of PD. Clinically, PD is characterized by non-motor (e.g. anxiety and depression) and motor symptoms (resting tremor, bradykinesia, rigidity and postural reflex disturbance) [15,19]. Herein, we provide evidence that diet-induced obesity promotes reduction of TH protein levels in the VTA, which was associated

**Figure 5.** Immunofluorescence detection of TH levels in the SN and VTA of rats placed on 25 weeks of HFD. Sections of the SN and VTA of animals placed on 25 weeks of HFD and control group were incubated with antibodies for TH and with DAPI for nuclear staining. Merged pictures are shown in the center. Bars represent 400 μm in upper panels and 100 μm in lower (insert) panels. Panels are representative from analysis of six different animals per group. The arrows indicate the TH-positive cells.
Figure 6. Immunofluorescence detection of pSer40TH levels in the SN and VTA of rats placed on 25 weeks of HFD. Sections of the SN and VTA of animals placed on 25 weeks of HFD and control group were incubated with antibodies for pSer40TH and with DAPI for nuclear staining. Merged pictures are shown in the center. Bars represent 400 μm in upper panels and 200 μm in lower (insert) panels. Panels are representative from analysis of six different animals per group. Quantitative analysis of pSer40TH immunofluorescence of SN (C) and VTA (D) is shown. The arrows indicate the pSer40TH-positive staining. Data are mean ± SD. Values of P were obtained by applying two-tailed Student’s t-test (**P < 0.01 vs control group (CTRL)).
to behavioral alterations, such as impairments in locomotor activity, mobility and anxiogenic-like behavior. Besides, the content of TH phosphorylated at Ser40 in the VTA was increased, which indicates that TH activity is enhanced. However, we did not observe motor deficits and modifications in α-syn levels in the obese rats.

In agreement to a previous study, our results show that HFD has negative effects on locomotion and spontaneous mobility of obese animals [23]. Despite most of the discussions relating obesity and behavioral disorders brings anxiety as an important factor in the development of this condition, our result showed that the anxiety-like behavior found in obese animals can occur as a consequence of the HFD. Our data agree with a recent study that presented an important relationship between metabolic syndrome and anxiety in animals exposed to a high-sucrose diet [37]. Importantly, we were unable to detect changes in motor coordination in the obese rats.

Figure 7. Effects of HFD in brain tyrosine nitration and free thiol content. (A) nitrotyrosine detection and (B) free thiol groups (sulfhydryl; SH) quantification in substantia nigra (SN), ventral tegmental area (VTA) and striatum (ST) of rats placed on 25 weeks of HFD. Data are mean ± SD (n = 10/group). *P < 0.05; **P < 0.01 vs control group (CTRL). Values of P were obtained by applying two-tailed Student’s t-test.

Figure 8. Analyses of the effect of high-fat diet over locomotor activity. (A) Total distance travelled, (B) distance travelled in the three first minutes (C) time immobile, (D) each minute of distance travelled in open field training, (E) each min of distance travelled in open field test (F) distance travelled in the center zone, (G) total immobile episodes in open field test of rats, (H) number of rearings in open field task, (I) linear correlation between total distance travelled in open field training and tyrosine hydroxylase (TH) immunodetection and (J) linear correlation between total distance travelled in open field training and body weight of rats placed on 25 weeks of HFD vs control group (CTRL). Data are mean ± SD (n = 10/group). *P < 0.05; **P < 0.01; ***P < 0.001 vs CTRL group. Statistical differences were determined by one-way ANOVA with Tukey’s post hoc test. Values of P were obtained by applying two-tailed Student’s t-test.
animals. During the two consecutive days of adaptation to the rotarod test, the obese rats fell more frequently from the apparatus than standard-diet group. However, both groups learned similarly to the displacement velocity required by the test, leading us to conclude that this adaptation time must have affected the result of the test taking into account the different protocols described to carry it out [30,38]. Other important point is that usually the motor alterations of PD occur only after almost 80% of dopaminergic neurons death, probably due to a compensatory mechanism during the disease development [39]. In addition, we did not observe changes in the memory in rats after exposure to HFD. We do not believe that more memory measurements would be necessary because object recognition is an effective test of hippocampal-independent memory. It is worth mentioning that previous studies demonstrated memory impairments in response to obesity [40], while in accordance with our data, other works did not find deficit in the object recognition test in rats after exposure to HFDs [41–43].

Studies aiming at identifying whether obesity represents a risk factor for the development of neurodegenerative diseases point to the diet-induced obesity as an aggravating condition to the dopaminergic neuronal loss and consequently decreased levels of nigral dopamine induced by neurotoxins [44,45]. Studies that are more recent have shown a linear relationship between diet-induced obesity and reduced levels of TH [23,24]. TH is the rate-limiting enzyme in catecholamine synthesis, considered the gold-standard marker of dopaminergic neurons in the central nervous system (CNS) [46]. In this way, together with our results, these data indicate that obesity can be acting on the dopaminergic neurons loss in VTA. Besides that, we found that decreased locomotor activity has a significant correlation with reduced TH levels, which indicates a remarkable connection between behavior changes and dopaminergic dysfunction.

TH phosphorylation is a major regulatory mechanism of catecholamine production, including dopamine. TH may be phosphorylated at different sites, including Ser8, Ser19, Ser31 and Ser40, via multiple protein kinases that display differential specificity for each site. The phosphorylation of Ser40 exerts the strongest effect on TH activation and is considered the major mechanism of TH activation [47]. Phosphorylation of TH takes place in response to cellular dopamine depletion, which, in turn, occurs in response to stimulation of dopamine release. Chronic stimulation of dopamine production and release often occur in conditions where dopaminergic cell loss is observed (such as PD), in order to compensate for the decrease in the number of dopamine-producing cells. A recent study, indeed, observed that pSer40TH levels were increased in the terminal field regions of dopaminergic neurons from the nigrostriatal axis in PD patients [48]. Our results are consistent with this scenario, as we observed a

**Figure 9.** Analyses of the effects of high-fat diet over motor coordination, memory and anxiety-like behavior. (A) Rotarod test after two days training, (B) total exploration time in sample session with identical objects and in test session with familiar and a novel object, (C) discrimination ratio of test session of object recognition test, (D) time spent in light compartment, (E) number of transition between light/dark compartments and (F) risk assessment behavior (R.A.B) in light/dark task of rats placed on 25 weeks of HFD vs control group (CTRL). Data are mean ± SD (n = 10/group). *P < 0.05; **P < 0.01; ***P < 0.001 vs CTRL group. Values of P were obtained by applying two-tailed Student’s t-test.
decrease in the content of TH-positive cells in the VTA with a concomitant increase in the levels of pSer40TH in this region. It is possible, in this context, that TH phosphorylation in the VTA is a response to decreased dopamine production due to loss of TH-positive cells.

Since α-syn changes were not observed, these results suggest that the reduction of TH in VTA occurs through a different pathway and it has a possible association with metabolic changes resulting from obesity such as glucose intolerance, insulin resistance and low-grade systemic inflammation. In fact, in addition to obesity, studies have shown an important relationship between dietary intake and neurodegenerative diseases. Caloric restriction provides a neuroprotective effect in PD through mechanisms involving metabolic mediators and attenuates Alzheimer’s disease pathology associated with improvement of age-related cognitive decline [49,50]. Changes in glucose metabolism and insulin concentrations appear to be closely related to damage of CNS, where chronic hyperinsulinemia and insulin resistance have been associated with memory impairment, decreased levels of neurotransmitters and the development of dementia [51]. For this reason, we strongly believe that the reduction in TH levels found in the nigrostriatal axis is associated with the metabolic disorders resulting from obesity.

There is also the possibility of an impairment of TH synthesis in response to increased inflammatory signaling in the brain. One of the earlier effects of obesity is the elevated inflammatory response, represented especially by high levels of cytokines such as TNF-α, which was increased in the circulation and in all tissues from obese animals. This continuous systemic inflammation resulted in a stimulation of the inflammatory activity in the brain with increased GFAP and Iba1 in SN and VTA, indicating an important participation of the inflammatory signaling in the impaired functioning of dopaminergic neurons in these regions. Other studies demonstrated that increased levels of pro-inflammatory cytokines and microglia activation are associated with cognitive deficits in obesity [3]. In addition, oxidative and nitrosative stress were also found increased in the brain of obese rats, which is in agreement with previously published data demonstrating that the brain inflammation and oxidative stress play a key role in neurodegenerative diseases [17,52].

In summary (Figure 10), we demonstrate that HFD-induced obesity and metabolic dysfunction provide
reduction of TH levels and enhanced TH phosphorylation probably via neuroinflammation and oxidative/nitrosative stress. Further investigations are required to uncover more in-depth mechanisms to explain the results found in our work and thus define the true role of obesity, insulin resistance and metabolic signaling in neurodegenerative diseases. New therapeutic approaches that aim to combat neuroinflammation appear to have important implications for preventing impairment of neuronal functioning and the development of neurodegenerative diseases such as PD in obese people especially those with genetically predisposed or exposed to environmental risks.

Acknowledgements

This work was supported by grants received from the following Brazilian public funds: Conselho Nacional de Desenvolvimento Científico e Tecnológico ( CNPq) #408435/2018-6, Fundação de Amparo à Pesquisa do Estado do RS (FAPERGS) #16/2551-0000499-4 and #17/2551-0000984-3, Propesq-UFRGS and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by grants received from the following Brazilian public funds: Conselho Nacional de Desenvolvimento Científico e Tecnológico ( CNPq) [grant number 408435/2018-6, Fundação de Amparo à Pesquisa do Estado do RS (FAPERGS) [grant numbers 16/2551-0000499-4 and 17/2551-0000984-3, Propesq-UFRGS and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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