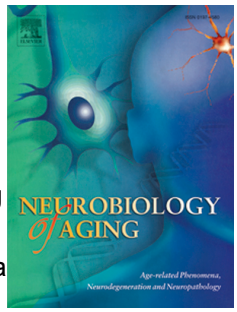

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Dipeptidyl peptidase-4 inhibitors and sulfonylureas prevent the progressive impairment of the nigrostriatal dopaminergic system induced by diabetes during aging

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ABSTRACT

The nigrostriatal dopaminergic system (NDS) controls motor activity and its impairment during type 2 diabetes (T2D) progression could increase Parkinson's disease risk in diabetics. If so, whether glycemia regulation prevents this impairment needs to be addressed. We investigated whether T2D impairs the NDS and whether dipeptidyl peptidase-4 inhibition (DPP-4i; a clinical strategy against T2D but also neuroprotective in animal models) prevents this effect, in middle-aged mice. Neither T2D (induced by 12 months of high-fat diet) nor aging (14 months) changed striatal dopamine content assessed by HPLC. However, T2D reduced basal and amphetamine-stimulated striatal extracellular dopamine, assessed by microdialysis. Both the DPP-4i linagliptin and the sulfonylurea glimepiride (an antidiabetic comparator unrelated to DPP-4i) counteracted these effects. The functional T2D-induced effects did not correlate with NDS neuronal/glial alterations. However, aging itself affected striatal neurons/glia and the glia effects were counteracted mainly by DPP-4i. These findings show NDS functional pathophysiology in T2D and suggest the preventive use of two unrelated anti-T2D drugs. Moreover, DPP-4i counteracted striatal age-related glial alterations suggesting striatal rejuvenation properties.

Keywords

dipeptidyl peptidase-4 inhibitors, dopaminergic system, obesity, oligodendrocytes, diabetes

1. Introduction

Recent data suggest that type 2 diabetes (T2D) is implicated in the pathogenesis of motor system disorders, including Parkinson's disease (PD) (Biosa et al., 2018; Cereda et al., 2011; Hu et al., 2007; Xu et al., 2011; Yue et al., 2016). Additionally, a recent study has shown that, when present in PD patients, T2D induces a more aggressive PD phenotype (Pagano et al., 2018). The close interplay between the nigrostriatal dopaminergic system (NDS) and metabolic control has also been recently shown in humans (Ter Horst et al., 2018). It must be underlined that the evidence in support of an association between PD and T2D is not conclusive, with studies also indicating no association (Cereda et al., 2011; Savica et al., 2012; Simon et al., 2007) or even an inverse association (Miyake et al., 2010; Powers et al., 2006). The pathophysiological mechanisms behind increased risk of PD in T2D patients are still largely undetermined. Possible causes include mitochondrial dysfunction, impaired insulin signaling, and metabolic inflammation (Santiago and Potashkin, 2014). Moreover, hyperglycemia induced by streptozotocin in rats (a model of T1D) preferentially induces degeneration of the NDS (Renaud et al., 2018).

Obesity is the number one risk factor for developing T2D and, not surprisingly, animal studies have investigated the role of obesity and obesity-induced T2D on the NDS. These studies have shown that insulin resistance and prediabetes induced by only 3 months of high-fat diet (HFD) in young rodents attenuate dopamine (DA) release and clearance (Morris et al., 2011), and reduces DA content in *striatum* (Nguyen et al., 2017). Other studies in young rodents employing shorter HFD-feeding (sometimes even in absence of hyperglycemia) have confirmed the deleterious role of obesity on the nigrostriatal pathway (Barry et al., 2018; Cone et al., 2013; Fritz et al., 2018; Jang et al., 2017; Speed et al., 2011). However, like T2D (CDC, 2017), PD is mainly a disease prevalent in people over the age of 60 (Collier et al., 2017). Thus, it is important to determine if and how overt T2D induced by a long-term intake

of an obesogenic diet during aging can impair the NDS when a head-to-head comparison is made with age-matched controls in which such a function is also reduced. Indeed it has been shown that aging-related changes in the DA system approach the biological threshold for parkinsonism, a so called “pre-parkinsonian state” (Collier et al., 2017).

Another essential question to address is whether the treatment of T2D could prevent the impairment of NDS and, by doing so, could reduce the risk/incidence of PD in T2D. Indeed studies have shown that several antidiabetic drugs can counteract neurodegenerative processes (also in non-diabetics) leading to a significant improvement in different clinical settings (Patrone et al., 2014). Perhaps the most interesting discovery in relation to PD in T2D patients is a recent study showing that the use of glitazones (anti-diabetic drugs specifically targeting insulin resistance) is associated with a decreased risk of PD incidence in populations with diabetes (Brakedal et al., 2017).

Dipeptidyl-peptidase 4 inhibitors (DPP-4i, also named gliptins) are oral antidiabetic drugs used to treat T2D. DPP-4i mediate their anti-diabetic effects primarily by inhibiting the degradation of endogenous glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), resulting in prolongation of postprandial insulin secretion and insulin-sensitizing effects (Deacon and Holst, 2013). Recent research has shown that DPP-4i can also reduce stroke-induced brain damage in animal models in presence or absence of diabetes [reviewed in (Darsalia et al., 2019; Darsalia et al., 2017)]. Furthermore, several reports have shown that gliptins mediate positive pleiotropic effects in animal models of Alzheimer’s disease (AD) [reviewed in (Chalichem et al., 2017)] and in diabetic patients with AD (Isik et al., 2017). Interestingly, recent studies have also shown that DPP-4 inhibition protects the NDS system in PD model (Nassar et al., 2015) and, importantly, reduces PD incidence in the clinical setting (Svenningsson et al., 2016). GLP-1 and GIP are regarded as main DPP-4 substrates and drugs targeting the GLP-1R can counteract PD in animal models

[reviewed in (Athauda et al., 2017b; Holscher, 2018)] as well as reduce the severity of motor symptoms in non-diabetic PD patients (Athauda et al., 2017a). However, DPP-4 cleaves additional substrates and we have recently shown that the DPP4i linagliptin induces neuroprotection independently from blood glucose regulation (Darsalia et al., 2013) and GLP-1R (Chiazza et al., 2018; Darsalia et al., 2016). Therefore, the molecular mechanisms underlying gliptin-mediated effects in the brain are still mostly undetermined.

The aim of this study was to determine whether obesity-induced T2D in middle-aged mice damages the NDS functionally and structurally, and whether linagliptin prevents these effects. To address the potential specificity of the effects mediated by linagliptin, we performed a head-to-head comparison to the sulphonylurea glimepiride (Khunti et al., 2018), which induces direct insulin secretion and glycaemia regulation bypassing the GLP-1/GIP system.

2. Materials and methods

2.1. Animal models and experimental design

Seventy-three, male C57/BL6j mice (Charles River Laboratories, Germany) were used in three studies. Mice were randomly assigned to experimental groups. They were housed in controlled conditions, in 12-hour light/dark cycle with free access to food and water. All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures were in accordance with the ethical standards of the Karolinska Instituet and Pronexus AB, where the studies were conducted. The ethical approval numbers are: S7-13 (Karolinska Institutet) and N96/13, N274/13, N27/14 (Pronexus AB).

Study 1. To determine the effect of T2D and/or aging on striatal tissue DA by HPLC and basal and amphetamine-stimulated striatal extracellular DA by microdialysis, we used 8-

months-old mice (n=5) and 14-months-old middle-aged mice (n=6) fed with normal chow i.e. standard diet (SD-y and SD-m, respectively), and 14-months-old middle-aged mice fed with high-fat diet (ssniff E15126-34, 54% calories from fat, Germany) for 12 months (HFD-m) (n=7). Additionally, to study the potential effect of antidiabetic treatments on DA levels, middle-aged HFD-fed mice received either linagliptin (in food leading to an average dose of 5-7 mg/kg b.w. per day; HFD-m-Lina) (n=7) or glimepiride (in food leading to an average dose of 2-4 mg/kg b.w. per day; HFD-m-Gli) (n=6) for the last 3 months before killing. The experimental design is shown in Fig.1S A.

Study 2. To determine potential T2D and/or aging-induced neural alterations in the brain areas of the dopaminergic system (*substantia nigra pars compacta* and *corpus striatum*), we used 2-months-old mice (SD-y) (n=7) and 14-months-old middle-aged mice (SD-m) (n=6) fed with SD, and 14-months-old middle-aged mice fed with HFD 4-12 months (HFD-m) (n=6). The experimental design is presented in Fig.1S B.

Study 3. To determine potential effect of anti-T2D treatments on neural alterations induced by either T2D or aging in the NDS, we used 14-months-old mice fed with HFD for 12 months that received either linagliptin (in food, leading to an average dose of 5-7 mg/kg b.w. per day; HFD-m-Lina) (n=9) or glimepiride (in food, leading to an average dose of 2-4 mg/kg b.w. per day; HFD-m Gli) (n=7) for the last 3 months before killing. Control mice received HFD for 12 months (HFD-m) (n=9). The experimental design is depicted in Fig.1S C.

2.2. Body weight, glycemic level, DPP-4i activity and GLP-1 levels

Blood glucose levels after overnight fasting and body weight were measured in all animals. In order to verify the bioactivity of linagliptin, plasma DPP-4 activity and total active GLP-1 levels were determined in the plasma (blood collected in the fed state) by enzyme

immunoassay (EIA) and by ELISA, respectively (Meso Scale discovery, Gaithersburg, MD, USA).

2.3. Microdialysis and HPLC

Microdialysis experiments were carried out on awake, freely moving mice following the protocol described elsewhere (Kehr, 1999; Kehr, 2006). DA was separated and measured by ion-exchange narrow bore column liquid chromatography with electrochemical detection as described elsewhere (Kehr, 2006). Detailed microdialysis and HPLC protocols are provided in Supplementary material.

2.4. Immunohistochemistry (IHC)

In study 2 and 3, mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with saline followed by 4% paraformaldehyde (PFA). After explant, brains were post-fixed overnight in 4% PFA and then placed in 20% sucrose solution for 3 days. Afterwards, brains were cut using sliding microtome (Leica, Germany). Briefly: brains were attached to the specimen platform using OCT and frozen with dry ice. During cutting, freezing was maintained by powdered dry ice in the specimen tray. The brains were cut at 40 micrometre-thick sections and collected in a cryoprotective solution for storage at -20 °C. The primary antibodies used for IHC (Table1) and detailed protocols are provided in Supplementary material.

2.5. Quantitative microscopy

The DARPP-32-, GAD67-, GFAP-, Iba-1-, PV-, and TH-positive cells were quantified using a computerized stereology toolbox equipped with Visiopharm v. 4.2.1.0 software for digital image analysis (NewCast, Denmark), connected to Olympus BX51 epifluorescent/light

microscope (Olympus, Japan). Olig2-, GPR17-, PCNA- and GSTπ-positive cells were quantified using an inverted fluorescence microscope (200M; Zeiss, Milan, Italy) connected to a PC computer equipped with Axiovision software (Zeiss). In *striatum*, positive cells were counted on three coronal sections *per* animal (1.50, 0.00 and -1.00 mm distance to Bregma). Quantification of TH+ cells in *substantia nigra pars compacta* was done on three sagittal sections *per* animal (1.40, 2.10 and 2.60 mm distance to Bregma). The cell density per 1 mm^2 for all the IHC markers was determined. Mean volume (in μm^3) of Iba-1+ and PV+ cells was measured, using nucleator technique (Gundersen et al., 1988), by Visiopharm software. Experiments were performed by persons blinded to group assignment and outcome assessment.

2.6. Statistical analysis

In study 1, data from microdialysis experiments were first checked for sphericity to determine the appropriate statistical approach. Data did not pass the sphericity test, thus the mixed-effects model (REML) with Geisser-Greenhouse correction was used to study overall diet and treatment effects on amphetamine-induced DA release and followed by the Tukey's multiple comparison test to determine the differences between experimental groups on amphetamine-induced DA release. Additionally, to show the effect of T2D and drug treatment on evoked DA release, the incremental area under the curve was computed for each sample (mouse) and the groups were analyzed for outliers using ROUT method (Motulsky and Brown, 2006). One outlier was identified (HFD-m group) and excluded from further analyses. The differences between the groups were analyzed using Welch's ANOVA test followed by unpaired *t*-test with Welch's correction where differences between following pairs of experimental groups were compared: SD-y vs. SD-m; SD-m vs. HFD-m; HFD-m vs. HFD-m-Lina; HFD-m vs. HFD-m-Gli; HFD-m-Lina vs. HFD-m-Gli. Data from HPLC experiments were analyzed

using ordinary One-way ANOVA. In study 2 and 3, to analyze cell density and mean cell volume, one-way ANOVA followed by either Tukey's or Dunnett's multiple comparisons tests was performed (see Figure captions). More information regarding choice of statistical tests used in the studies is available in Supplementary material.

All data were analyzed using Graphpad Prism 8 and are presented as line or bar graphs showing means ± SEM. Differences between the groups were considered significant when *p* values were less than 0.05 (*p<0.05; **p<0.01, ***p<0.001, ****p<0.0001).

3. Results

Twelve months of high-fat diet induce obesity and glucose intolerance (Fig. 2S A-B). Linagliptin and glimepiride reduce hyperglycemia but have no effect on body weight (Fig. 3S C-D). In linagliptin-treated mice (HFD-m-Lina), plasma DPP-4 activity and GLP-1 levels were significantly decreased (Fig. 3S A) and increased (Fig. 3S B), respectively, as expected based on the drug mechanism of action. See Supplementary material for more detailed information.

3.1. Diabetes impairs basal and amphetamine-stimulated DA release. This effect is counteracted by linagliptin and glimepiride

To determine the effects of HFD on DA release after amphetamine challenge, we measured extracellular DA levels using microdialysis. Results in Figures 1 A1 and A2 show that the overall treatment (diet and drugs) effect on DA release was not statistically significant ($p \leq 0.1002$), however there was a statistically significant ($p=0.0015$) interaction between treatment and DA release over the course of amphetamine challenge, which imply that the effect of DA over time differed between the groups. Follow-up analyses, to determine differences between the groups using the Tukey's multiple comparisons test, showed a

statistically significant reduction of DA release in middle-aged diabetic mice (HFD-m) (Fig.1 A3). This effect was completely abolished by both linagliptin and glimepiride treatments (HFD-m-Lina and HFD-m-Gli, respectively). No age-related changes were observed in non-diabetic mice (SD-y vs. SD-m) (Fig.1 A3). Data were further analyzed by computing the incremental area under the curve (evoked DA release) after amphetamine challenge and by analyzing the differences between selected pairs (groups). Similarly, this analysis confirmed the prior observation of abolished DA release in middle-aged diabetic mice ($p=0.03$) and of the reversing effect of linagliptin ($p=0.02$) and glimepiride ($p=0.02$) treatments, without detecting any significant effect of aging in SD-fed mice (Fig.1 B). We also compared extracellular DA levels before amphetamine challenge (mean DA for times -40, -20 and 0 min) and the results showed a significant lowering effect in HFD-m mice (see the square in Fig.1 A1).

3.2. Diabetes, linagliptin and glimepiride do not affect total intra-striatal DA levels

To further investigate whether reduced extracellular DA release was caused by overall decrease of DA content in *striatum*, we performed HPLC analyses of striatal tissue lysates. Results show that neither diabetes nor aging had any significant effect on the overall content of DA in this brain region (Fig.1 C). The levels of this neurotransmitter in *striatum* were also unaffected by linagliptin or glimepiride treatments (Fig.1 C).

3.3. Neither diabetes nor aging affect dopaminergic neurons in *substantia nigra*, as well as medium spiny neurons and GAD67-positive interneurons in *striatum*

We quantified neurons in *substantia nigra pars compacta* (SNpc) and *striatum*. The results show that aging or diabetes had no effect either on dopaminergic neurons in SNpc and in DARPP-32+medium spiny neurons or GAD67+ interneurons in *striatum*. The results of the

quantitative analysis and representative pictures are explained in detail on page 7 of the Supplementary material and shown in Fig. 4S. Additionally, the results showed no neuronal injury in *striatum* of obese/T2D mice compared to control, based on Hsp70/72 assessment (Fig. 5S).

3.4. Aging, but not diabetes, affects parvalbumin-positive interneurons in *striatum*. This effect is not counteracted by linagliptin or glimepiride

Results of the quantification of subpopulation of GABAergic interneurons show age-induced decrease in density of the parvalbumin (PV)+ interneurons in *striatum* (Fig.2 A, E; 70.78±6.29 vs. 52.48±2.49 in SD-y and SD-m group, respectively; p=0.0213 and 70.78±6.29 vs. 48.75±2.4 in SD-y and HFD-m group, respectively; p=0.0038). This aging effect was also observed for the mean cell body volume of PV+ interneurons (Fig.2 B; 910.7±27.49 vs. 816.1±19.08 in SD-y and SD-m group, respectively; p=0.0274 and 910.7±27.49 vs. 796.1±23.87 in SD-y and HFD-m group, respectively; p=0.006). We did not record any further effect induced by diabetes on both parameters (Fig.2 A-B and E). Linagliptin treatment had effect neither on PV+ cell density nor mean cell volume. After glimepiride treatment, a further decrease in volume of PV+ interneurons was observed in *striatum* of middle-aged mice (796.6±23.98 vs. 696.1±18.03 in HFD-m and HFD-m-Gli group, respectively; p=0.013) (Fig.2 D and F).

3.5. Aging, but not diabetes, increases neuroinflammation in *striatum*. Linagliptin, but not glimepiride, partially counteracts this effect

To assess potential T2D -induced neuroinflammation in *striatum*, we quantified Iba-1+ positive microglia and reactive GFAP+ astrocytes. Results show an aging-induced increase in the density of Iba-1+ microglia (Fig.3 A, E; 122.7±17.53 vs. 371.3±35.70 in SD-y and SD-m

groups, respectively; p<0.0001 and 122.7±17.53 vs. 325.1±14.89 in SD-y and HFD-m groups, respectively; p<0.0001). We also observed an age-induced effect on the mean cell body volume of these cells (Fig.3 B, E; 174.1±12.65 vs. 274.3±12.79 in SD-y and SD-m groups, respectively; p<0.0001, and 174.1±12.65 vs. 244.2±8.95 in SD-y and HFD-m groups, respectively; p=0.0002). No additional effect of diabetes was observed (Fig.3 A-B, E).

Chronic DPP-4 inhibition by linagliptin did not affect the density of microglia (Fig.3 C, F), but decreased the cell body volume of Iba-1+ cells in the *striatum* of diabetic mice (207.8±6.08 vs. 186.8±3.79 in HFD-m and HFD-m-Lina group, respectively; p=0.0126), while glimepiride did not (HFD-m vs. HFD-m-Gli, p=0.18) (Fig.3 D, F).

Similar changes as for microglia were observed for striatal GFAP+ astrocytes, with a significant age-induced increase in the density of GFAP+ cells (78.25±6.25 vs. 166.6±12.52 in SD-y and SD-m groups, respectively; p=0.0002, and 78.25±6.25 vs. 146.5±11.23 in SD-y and HFD-m groups, respectively; p=0.0004) (Fig.4 A, C), which was inhibited by chronic linagliptin (239.4±19.06 vs. 184.5±7.66 in HFD-m and HFD-m-Lina group, respectively; p=0.033), but not glimepiride treatment (p=0.21) (Fig.4 B, D).

3.6. Aging, but not type 2 diabetes, impairs oligodendrocytes maturation. Linagliptin partially counteracts this effect

To investigate the potential effects of T2D on the oligodendrocyte lineage, we first quantified the number of cells expressing the transcription factor Olig2, i.e. cells at any stage of oligodendrocyte maturation (Barateiro and Fernandes, 2014), in *striatum*. Aging significantly reduced the density of Olig2+ cells, with no additional effect induced by diabetes (Fig.5 A, C) (number of Olig2+ cells/mm^2: 351.21±9.17 vs 214.94±9.19 vs. 236.98±17.45 in SD-y, SD-m, and HFD-m, respectively; p<0.0001), thus indicating an overall depletion of cells in the oligodendrocyte lineage. Additionally, aging reduced by half the fraction of Olig2+ cells

co-expressing the marker of proliferation PCNA over the total Olig2+ cell population in *striatum*, once again with no additional effect of diabetes (16.41±1.45 vs 8.23±1.35 in SD-y and SD-m, respectively, p=0.0065; 7.68±1.28 in HFD-m, p=0.0038 vs SD-y; Fig.5 B-C). Interestingly, despite a lack of effect on the total number of Olig2+ cells (Fig.5 D, F) (HFD-m vs. HFD-m-Lina group, p=0.095), linagliptin significantly enhanced the fraction of PCNA+/Olig2+ cells (6.62±0.89 vs 9.29±0.66 in HFD-m and HFD-m-Lina, respectively, p=0.0476; Fig.5 E-F), suggesting that DPP-4 inhibition can partially restore the proliferation of this cell lineage. Glimepiride promoted a partial recovery of the total number of Olig2+ cells (Fig.5 D, F) (193.23±8.78 vs 277.40±10.85 in HFD-m and HFD-m-Gli group, respectively; p<0.0001), but only a tendency albeit not statistically significant to increase in the percentage of proliferating cells (p=0.4253; Fig.5 E-F) was observed.

We next evaluated the density of immature oligodendrocytes expressing the GPR17 receptor and of more differentiated oligodendrocytes in *striatum* expressing the GSTπ marker. Quantification of GPR17+ cells (i.e. oligodendrocytes precursor cells up to the stage of immature oligodendrocytes) (Fumagalli et al., 2011) show that the density of these cells was significantly decreased in the *striatum* of middle-aged mice (20.8±0.83 vs 9.71±1.04 in SD-y and SD-m, respectively, p=0.0002) (Fig.6 A-B). Diabetes seemed to, at least partially, inhibit this effect (14.85±1.80 in HFD-m, p=0.036 vs SD-m). Similarly, quantification of the density of GSTπ+ cells, i.e. more mature cells of the oligodendrocyte lineage, shows a significant decrease with age (134.9±9.77 vs 72.44±13.71 in SD-y and SD-m, respectively, p=0.0136; Fig.6 E-F). No effect of diabetes was observed (HFD-m vs. SD-m, p=0.7048). Interestingly, linagliptin induced an increase in both GPR17+ (19.17±2.73 vs 33.9±2.17 in HFD-m, and HFD-m-Lina, respectively, p=0.0017; Fig.6 C-D) and in mature GSTπ+ oligodendrocytes (74.81±9.17 vs 112.7±13.04 in HFD-m and HFD-m-Lina, respectively, p=0.0478; Fig.6 G-

H). Glimepiride induced a non-statistically significant trend towards increase, but only in the latter cell population (p=0.3740).

4. Discussion

We show that 12 months of HFD did not affect striatal DA content but reduced extracellular DA release in *striatum* under basal conditions and, more importantly, after amphetamine challenge. This functional effect was not associated with neuronal/glial alterations in *substantia nigra* or *striatum*. We also show that linagliptin, but also the glycemic comparator glimepiride (not acting via DPP-4 inhibition), could normalize the T2D-induced effect on both basal and amphetamine-induced extracellular DA levels. Finally, along the characterization of potential structural alterations induced by T2D in *striatum* we show that aging, irrespectively from T2D, induced neuronal and glial alterations. These aging-dependent effects did not correlate with functional changes in basal or amphetamine-induced extracellular DA levels and could be partially counteracted by both drugs, with a more potent and overall effect induced by linagliptin.

To our knowledge this is the first study showing that obesity/T2D dramatically impairs the function of the NDS in the middle-aged mouse. These findings might be clinically relevant, since T2D has been associated to motor dysfunction and PD (see Introduction). Moreover, motor dysfunction disorders are strongly associated with aging (Bennett et al., 1996; Collier et al., 2017) and so is T2D (CDC, 2017). Nevertheless, literature addressing PD and T2D modeling during aging is very limited, possibly reflecting difficulties in obtaining aged animals. Previous studies have shown that obesogenic diets administered to young rodents from only 2 weeks (Barry et al., 2018) till up-to 4 months (Cone et al., 2013; Fritz et al., 2018; Jang et al., 2017; Morris et al., 2011; Nguyen et al., 2017; Speed et al., 2011) can already negatively affect the NDS. These observations could not be extrapolated to overt T2D

since: 1) the mice employed in these studies were mainly insulin-resistant and "early diabetics/prediabetics"; 2) aging itself decreases striatal DA content and can impair the NDS (Collier et al., 2007). Thus, a head-to-head comparison between middle-aged SD and HFD-fed mice was performed in our study. The molecular mechanisms (see Introduction) at the basis of the recorded effects were out of the scope of this study but they obviously represent the next step of this work. It will also be important to determine whether the effects recorded after amphetamine challenge are specific to the amphetamine mechanism of evoked DA release (Sulzer et al., 2016), implicating a critical role of VMAT2 and possible presynaptic silencing of DA vesicles (Pereira et al., 2016). The fact that there was no significant difference in striatal DA content between control and HFD-treated mice suggests that chronic HFD led to aberrant exocytosis of vesicular DA, as observed by blunted response of extracellular DA to amphetamine challenge. The mechanisms behind the effects of chronic HFD on impaired DA signaling in the mouse *striatum* are not known at present. It will also be important to understand whether impaired DA release relies on the direct impairment of DA neurons or also local striatal interneurons (reviewed in (Berke, 2018)) are involved in the reported impairment. The answer to these questions will be necessary to develop strategies aimed at reducing the risk of T2D patients to develop motor disorders.

In the second part of the study, we showed that both linagliptin and glimepiride could counteract the identified functional effect of T2D on the NDS. Neuroprotective effects induced by GLP-1R agonists on the NDS system, independently from glycemia regulation, have been shown in the past decade (Bertilsson et al., 2008; Li et al., 2009; Zhang et al., 2019) and reviewed in (Athauda et al., 2017b; Holscher, 2018). This made us hypothesizing that linagliptin (but not glimepiride) could protect the NDS independently by glycemia regulation and likely via GLP-1. Our findings disprove our hypothesis about the specific restorative effects of DPP-4i on the impairment of the dopaminergic system by T2D,

irrespectively of GLP-1 and/or their anti-diabetic properties. However, they are not less interesting since they suggest the possibility that different anti-diabetic treatments targeting glycemia regulation could be efficacious to counteract T2D-induced motor dysfunction, despite their differential mechanisms of action. The peripheral effects mediated by gliptins are mainly due to the inhibition of the degradation of GLP-1, leading to an increased insulin-to-glucagon ratio and consequent reduction of HbA1c (Deacon and Holst, 2013). The additional effects of DPP-4i in the brain are mostly undetermined, although they may be related to the inhibition of other peptides (Avogaro and Fadini, 2018) (Chiazza et al., 2018). Recent studies have also shown that DPP-4i are neuroprotective (Darsalia et al., 2015) and can improve brain function via attenuating mitochondrial dysfunction, insulin resistance, inflammation, and apoptosis (Gault et al., 2015; Pintana et al., 2013; Sa-Nguanmoo et al., 2017). Sulfonylureas such as glimepiride are a frequently prescribed anti-T2D medication due to their low cost and effectiveness, despite potential hypoglycemic risks (Costello and Shivkumar, 2018). These drugs bind to sulfonylurea receptors to allow insulin secretion from pancreatic beta cells (Khunti et al., 2018). Our hypothesis relied on the neurotrophic rather than the glycemic properties of DPP-4i, thus the results showing the efficacy of glimepiride to normalize DA release were unexpected. It would be interesting to determine if linagliptin or glimepiride could increase the DA release in non-diabetic mice and, therefore, the lack of these two groups could be viewed as a weakness of this study. However, since healthy animals do not exhibit DA release impairments, such experiment would have a limited clinical value. Moreover, since both drugs showed similar effects on DA release, one could speculate that this effect is related to insulin action or glycaemia regulation, which would not take place in non-diabetic mice linagliptin-treated and could lead to hypoglycemia after glimepiride treatment, thus complicating the interpretation of the results. It would be plausible to think that the common effects mediated by the anti-T2D drugs on the NDS,

showed in our study, could be mediated by increased peripheral insulin levels, due to the lack of reported additional shared mechanisms of action between gliptins and sulfonylureas in the brain. The normalization of fasting blood glucose obtained by both drugs indicate that insulin was up-regulated by the treatments. However, since the efficacy of glimepiride on the NDS system was unexpected, we did not measure peripheral insulin levels after fasting (the blood was collected in fed state to quantify the incretin/GLP-1 effect). This represents a limitation of this study and, therefore, more studies thoroughly investigating the efficacy of both drugs on insulin after fasting and to also improve insulin sensitivity directly in the *striatum* are warranted.

A variety of brain cell types have been shown to become compromised during aging, thus contributing to disease progression (Palmer and Ousman, 2018). In an initial effort to determine whether T2D led to major cellular alteration in the NDS, we quantified relevant neuronal cell types related to this system, i.e. the dopaminergic neurons in *substantia nigra*, DARPP-32 positive cells (the major cell type in *striatum*), GAD67+ interneurons in *striatum* (GAD 67 is the rate limiting enzyme for the formation of GABA) and striatal PV+ interneurons. Around 95% of the latter cell population produce the glial cell line-derived neurotrophic factor which is required for the survival of dopaminergic neurons in response to injury (Hidalgo-Figueroa et al., 2012). T2D had no effect on any of these neuronal cell types. However, we showed an effect of aging in decreasing PV+ interneurons number and cell body volume. Although speculative, these data could suggest decreased striatal neuroplasticity during aging, in line with a recent study showing atrophy of PV+ interneurons in *striatum* of Huntington's disease patients (Reiner et al., 2013). Both linagliptin and glimepiride showed no effect on these cells.

When investigating Iba-1+ microglia and GFAP+ astroglia in *striatum*, our data show no effect induced by T2D. However, we showed that aging induced a dramatic increase in

microglial cells and in their activation (as measured by increased cellular volume) and in GFAP+ astrocytes. In Study 1 and 2 we did not observe the same number of GFAP+ cells in middle-aged mice fed with HFD, possibly due to different batches of antibodies and/or fixation procedures and this could represent a weakness of the study. However, each study had its own control group to compensate such differences. Regardless, linagliptin could normalize the effect of aging on both GFAP and Iba-1+ cells, while glimepiride induced only a trend in the same direction on Iba-1+cells. The current view is that aging can promote the development of a mild, albeit chronic, inflammatory state in CNS reflected in the activation of both microglia and astroglia (Palmer and Ousman, 2018). This increased basal inflammation may be a risk factor for the development of motor and cognitive impairment, depression and age-related neurodegenerative disorders such as AD, PD, and ALS (Ransohoff, 2016; Spittau, 2017). Our study fully supports this view. Although speculative, our data also suggest that DPP-4i can exert anti-aging effects by decreasing such a mild inflammatory state in *striatum*. Whether these effects can be reflected in improved motor function, it is an interesting hypothesis to be challenged by the use of specific animal models where motor function is impaired by aging.

Concerning oligodendrocytes, our data in the aged *striatum* show not only an overall reduction in Olig2+ cells but, even more relevant, in their proliferation rate. This could reflect a reduced ability of oligodendrocyte precursor cells (OPCs) to promptly react to harmful events in an attempt to replace dead or malfunctioning oligodendrocytes and promote regeneration (Neumann and Kazanis, 2016). *In vitro* evidence also suggests that under specific conditions Olig2+ cells can generate new neurons (Boccazzi et al., 2016; Nunes et al., 2003), although *in vivo* data remain controversial (Guo et al., 2010; Kang et al., 2010). Additionally, OPCs and more mature oligodendrocytes are also known to foster neuronal communication, and to exert neuroprotective trophic effects. Our data show an aging-induced

decrease in the density of GPR17+ cells, namely OPCs/immature oligodendrocytes whose terminal differentiation is triggered by GPR17 down-regulation (Fumagalli et al., 2017). The concomitant reduction in the number of GSTπ^+ mature oligodendrocytes in the aged brain suggests defective OPC maturation, and confirms an overall depletion of cells of this lineage, likely as a consequence of the increased neuroinflammation observed in our and other studies (Palmer and Ousman, 2018). Loss of myelin-producing cells has been observed in aging and age-related pathologies, such as AD and PD, and represents one of the long-term functional consequences of stroke (Shen et al., 2008). Treatment with linagliptin exerted an overall protective role against age-induced effects on cells in the oligodendrocyte lineage. Based on the number of signaling pathways activated by DPP-4i (see above), we can speculate anti-aging properties for this class of drugs which could be exploited in neurodegenerative disorders.

The paradoxical increase in the density of GPR17$^+$ cells observed in T2D with respect to SD-m mice could be explained by considering that one of the many transcription factors controlling receptor expression is FOXO1 (Ren et al., 2012). Although direct evidence is lacking, hyperglycemia and/or decreased insulin signaling/insulin resistance in T2D could promote aberrantly persistent GPR17 expression in oligodendrocytes by maintaining high levels of active FOXO1, thus preventing GPR17 down-regulation and blocking cells at immature stages. Additionally, a recent study has demonstrated that whole-body metabolism is controlled by GPR17 expressed by oligodendrocytes, and that GPR17-deficient mice show decreased body weight in respect to WT animals after up to 26 week HFD feeding (Ou et al., 2019). Although the role of GPR17 in promoting food intake is still a matter of debate, it is tempting to speculate a contribution of altered GPR17 expression in promoting the pathological consequences of T2D on body weight and metabolism. High-fat diet is likely to promote food intake by disrupting hypothalamic insulin and leptin signalling by inducing

hypothalamic inflammation and lipotoxicity also in concert with advanced glycation end products as direct result of chronic hyperglycaemia. Nevertheless, in parallel with all its beneficial effects (see above), linagliptin also promotes a full recovery of the number of $GPR17^{+}$ cells to values similar to SD-y animals, suggesting that this receptor contributes to the drug's rejuvenation activities.

In conclusion, in a preclinical setting of clinical relevance we showed that T2D/obesity decreases extracellular DA in *striatum* and severely impairs extracellular striatal DA release after amphetamine challenge. This suggests a detrimental role of T2D in the regulation of motor function. Whether the reported effects could represent an early pathophysiological mechanism induced by T2D on the NDS is a relevant hypothesis to be tested in future studies. Importantly, we also show that glycemic regulation, similarly achieved by both linagliptin and glimepiride could normalize the impairment of DA release induced by T2D. Although additional studies need to support this data, these results suggest that increased insulin production *per se* and/or consequent decreased glucotoxicity in T2D patients may be beneficial to prevent and/or delay motor disorders involving the NDS. Finally, we identified neuronal and glial alterations in *striatum* that were induced by aging but were not associated with T2D or with striatal DA changes. The glial effects could be counteracted by linagliptin and, to a lesser extent, also by glimepiride. Our data support the accepted view in the field that aging has a detrimental role on neuroplasticity, on myelin and nerve fibers, and that it increases neuroinflammation. Importantly, our results suggest that DPP-4i can normalize the identified glial alterations and, by doing so, they could exert rejuvenation properties. Whether these effects are mediated by incretins remains to be studied. These effects could be exploited for the treatment of motor disorders related with aging.

Disclosure

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FIGURE CAPTIONS

Fig.1 Effects of high-fat diet, linagliptin and glimepiride on basal and amphetamine-stimulated dopamine levels in *striatum* and on total intra-striatal DA levels. Dopamine (DA) release after amphetamine challenge (initiated at time 0) in *striatum* of young-adult and middle-aged mice (SD-y and SD-m, respectively), and middle-aged mice fed with high-fat diet (HFD-m), untreated or receiving either linagliptin (HFD-m-Lina) or glimepiride (HFD-m-Gli) for 3 months (**A**). Extracellular DA levels before amphetamine challenge (**A1** square). Results from the Mixed-effects model (REML) (**A2**) and Tukey's multiple comparisons tests (**A3**). Incremental area under the curve of DA release after amphetamine challenge (**B**). Total striatal tissue levels of dopamine by HPLC analysis in the five experimental groups (**C**). Welch ANOVA test followed by unpaired *t* test with Welch's correction where differences between following pairs of experimental groups were compared: SD-y vs. SD-m; SD-m vs.

HFD-m; HFD-m vs. HFD-m-Lina; HFD-m vs. HFD-m-Gli; HFD-m-Lina vs. HFD-m-Gli. ns – not significant. Histograms show means ± SEM, *p<0.05, **p<0.01, ****p<0.0001, n=4-7

Fig.2 Effects of diabetes, aging and anti-diabetic treatments on parvalbumin (PV)+ interneurons in *striatum*. Density (**A**), mean volume (**B**) and representative microphotographs (**E**) of PV+ interneurons in the *striatum* of SD-y, SD-m, and HFD-m mice. Density (**C**), mean volume (**D**) and representative microphotographs (**F**) of PV+ interneurons in *striatum* of HFD-m mice and HFD-m mice that received either linagliptin (HFD-m-Lina) or glimepiride (HFD-m-Gli) for 3 months. One-way ANOVA followed either by Tukey's (A-B) or Dunnett's (C-D) multiple comparisons test. Histograms show means ± SEM, *p<0.05, **p<0.01, n=5-7

Fig.3 Effects of diabetes, aging and anti-diabetic treatments on Iba-1+ microglia in *striatum*. Density (**A**) and mean volume (**B**) of Iba-1+ microglial cells, and representative microphotographs of the staining (**E**) in *striatum* of SD-y, SD-m, and HFD-m mice. Density (**C**) and mean volume (**D**) of Iba-1+ microglial cells, and representative microphotographs of the staining (**F**) in *striatum* of HFD-m mice and HFD-m mice that received either linagliptin (HFD-m-Lina) or glimepiride (HFD-m-Gli) for 3 months. One-way ANOVA followed either by Tukey's (A-B) or Dunnett's (C-D) multiple comparisons test. Histograms show means ± SEM, *p<0.05, ***p<0.001, ****p<0.0001, n=6-7

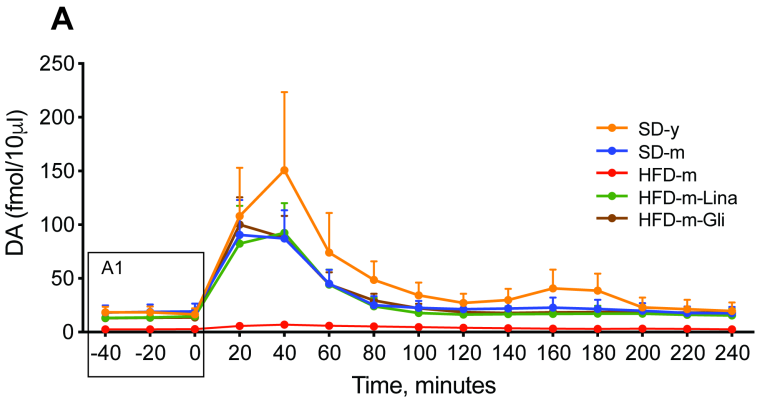
Fig.4 Effects of diabetes, aging and anti-diabetic treatments on GFAP+ astrocytes in *striatum*. Density of GFAP+ astrocytes (**A**) and representative microphotographs of the staining (**C**) in *striatum* of SD-y, SD-m, and HFD-m mice. Density of GFAP+ astrocytes (**B**) and representative microphotographs of the staining (**D**) in *striatum* of HFD-m mice and HFD-m mice treated either with linagliptin (HFD-m-Lina) or glimepiride (HFD-m-Gli) for 3 months. One-way ANOVA followed either by Tukey's (A) or Dunnett's (B) multiple comparisons test. Histograms show means ± SEM, *p<0.05, ***p<0.001, n=6-7

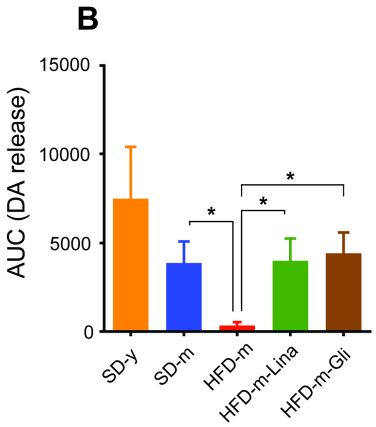
Fig.5 Effects of diabetes, aging and anti-diabetic treatments on the fraction of proliferating oligodendrocytes in *striatum*. Density of Olig2+ cells (**A**), percent of double stained PCNA/Olig-2+ cells over total number of $Olig2^{+}$ cells (**B**) and representative microphotographs of the staining (**C**) in *striatum* of SD-y, SD-m, and HFD-m mice. Density of Olig2+ cells (**D**), percent of double stained PCNA/Olig-2+ cells over total number of

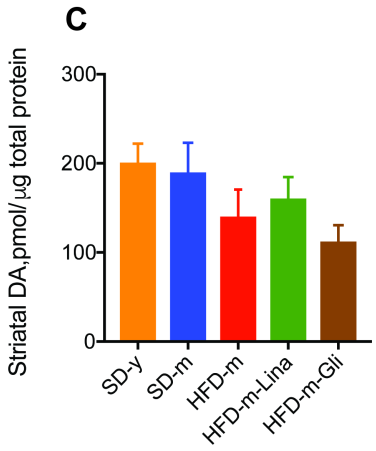
Olig2^{+} cells (**E**) and representative microphotographs of the staining (**F**) in *striatum* of HFD-m mice and HFD-m mice treated either with linagliptin (HFD-m-Lina) or glimepiride (HFD-m-Gli) for 3 months. One-way ANOVA followed either by Tukey's (A-B) or Dunnett's (D-E) multiple comparisons test. Histograms show means ± SEM, *p<0.05, **p<0.01, ****p<0.0001, n=5-9

Fig.6 Effects of diabetes, aging and anti-diabetic treatments on GPR17+ and GST-π+ mature oligodendrocytes in *striatum*. Density of GPR17+ (**A**) and GST-π+ cells, and representative microphotographs (**B** and **F**, respectively) in *striatum* of SD-y, SD-m and HFD-m mice. Density of GPR17+ (**C**) and GST-π+ (**G**) cells, and representative microphotographs (**D** and **H**, respectively) in *striatum* of HFD-m mice and HFD-m mice treated either with linagliptin (HFD-m-Lina) or glimepiride (HFD-m-Gli) for 3 months. One-way ANOVA followed by either Tukey's (A, E) or Dunnett's (C, G) multiple comparisons test. Histograms show means ± SEM, *p<0.05, **p<0.01, ***p<0.001, n=5-9

Authors' contributions: GL performed IHC studies and stereology analyses, acquired and processed images, wrote the manuscript. GM performed oligodendroglia studies, acquired and processed images, edited the manuscript. JK coordinated the functional DA studies and edited the manuscript. TY performed the DA functional studies. EC&AD performed part of the IHC studies and edited the manuscript. HP performed/wrote the statistical analysis of the DA functional studies. JS and TK coordinated ELISA studies, contributed to discussion, and edited the manuscript. TN and MPA provided expertise and resources and edited the manuscript. SC conceived the oligodendrocytes studies, contributed to discussion and edited the manuscript. VD designed, conceived and performed *in vivo* studies, and wrote the manuscript. CP conceived, designed, and coordinated the research plan, wrote the manuscript.





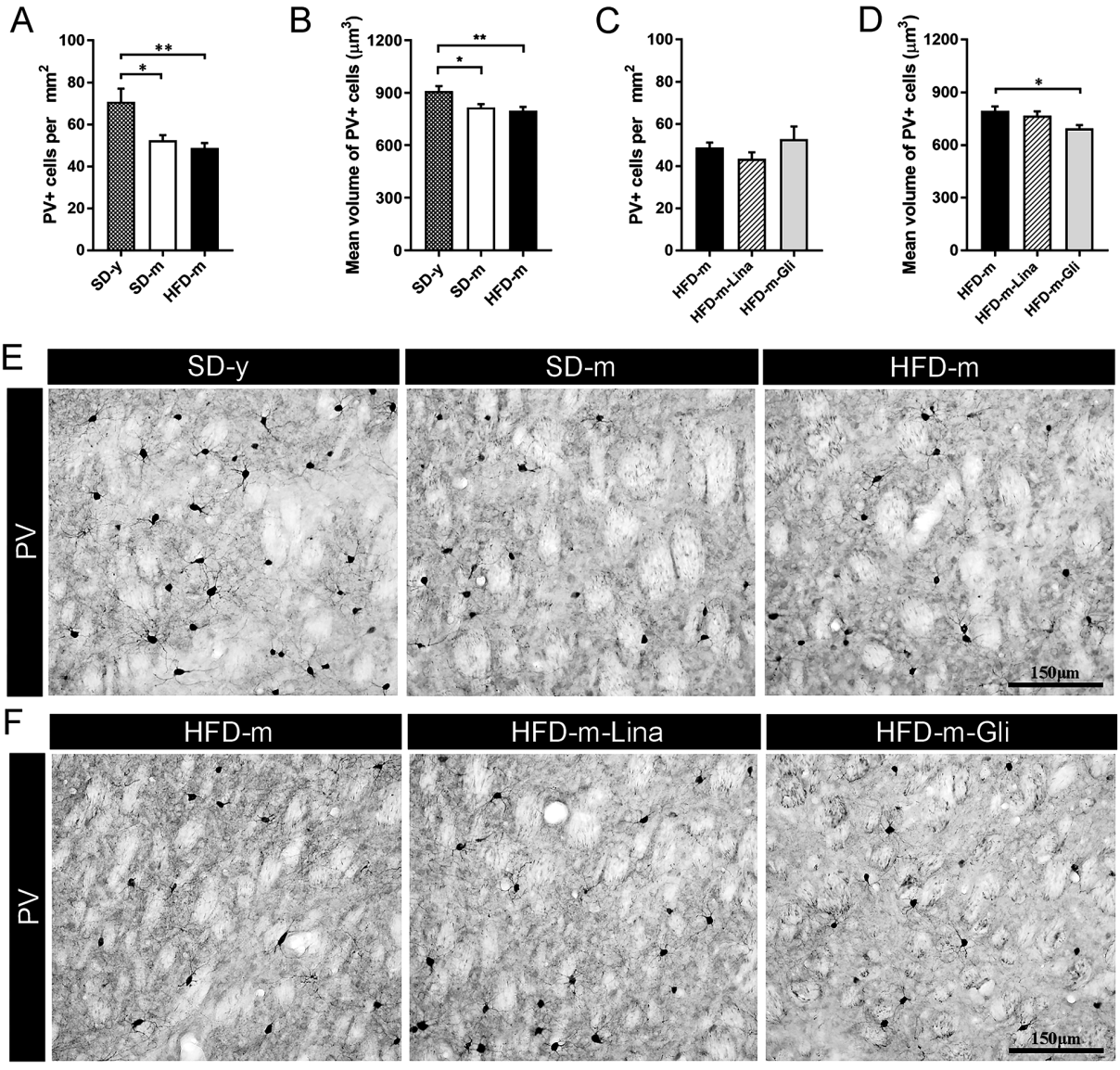


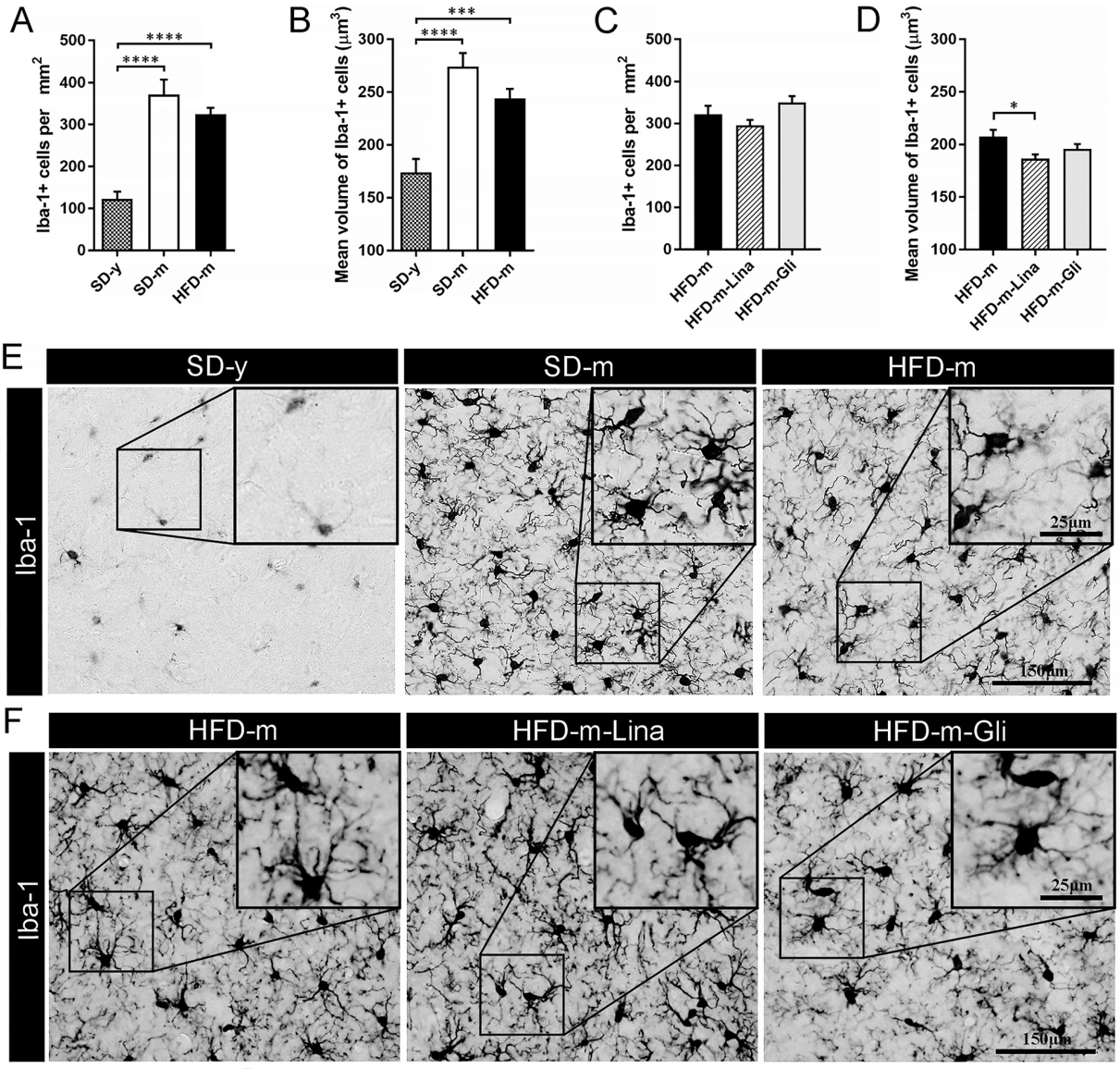
A2

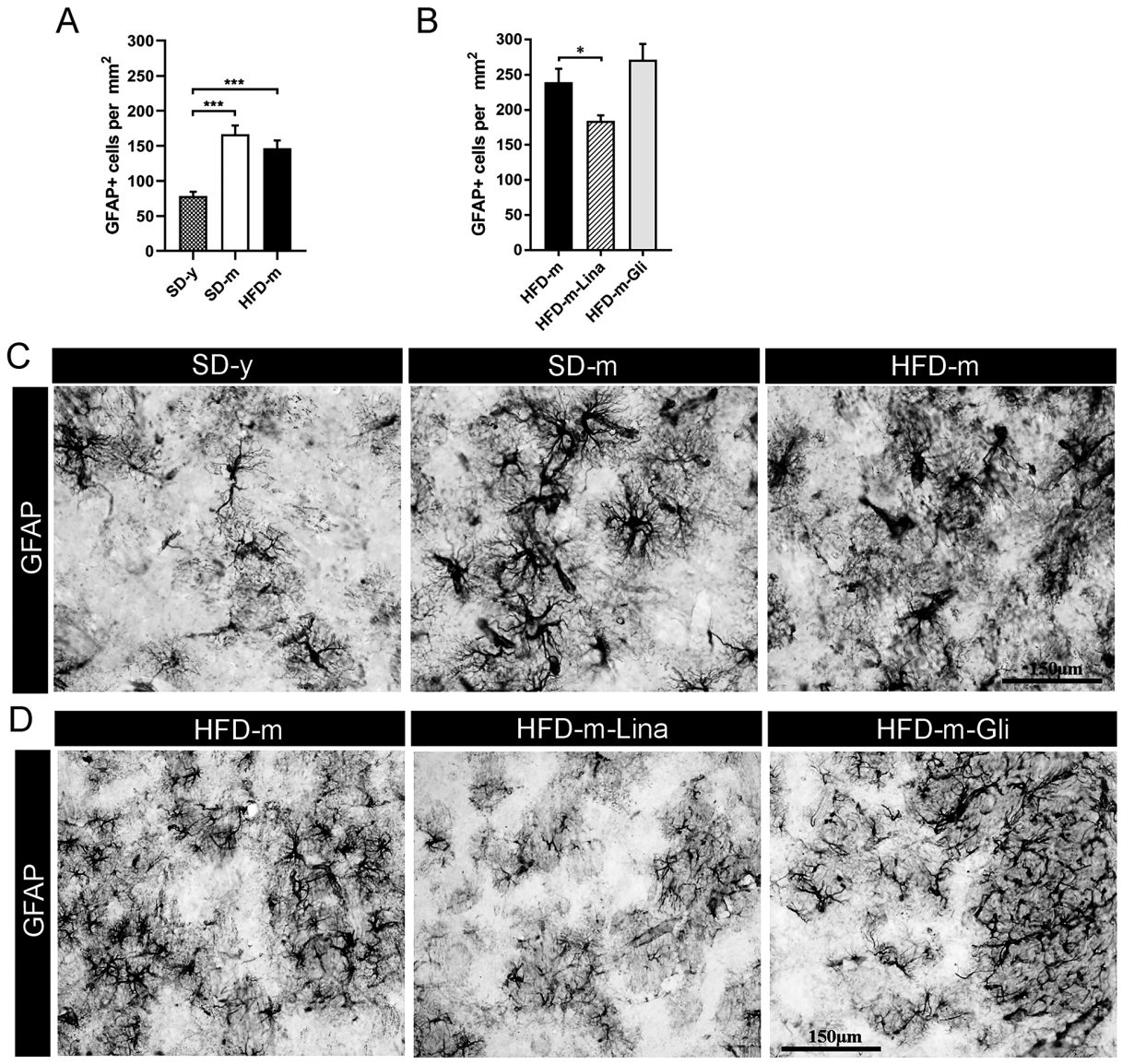
Mixed-effects model (REML)	Summary	P Value
DA release over time (-40 - 240 minutes)	****	<0,0001
Effect of treatment (diet/drugs) on DA release	ns	0,1002
Interaction of time and treatment	**	0,0015

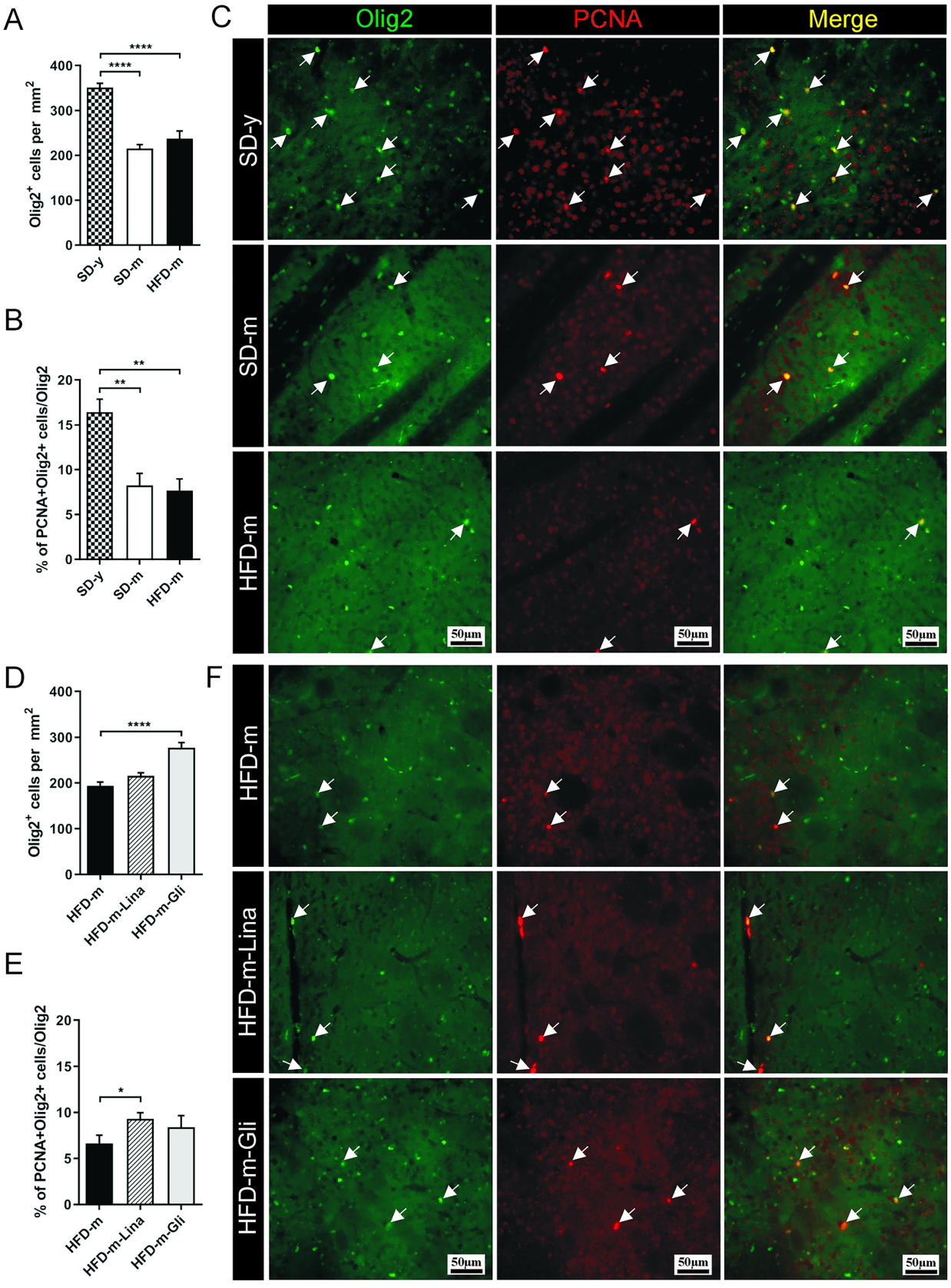
A3

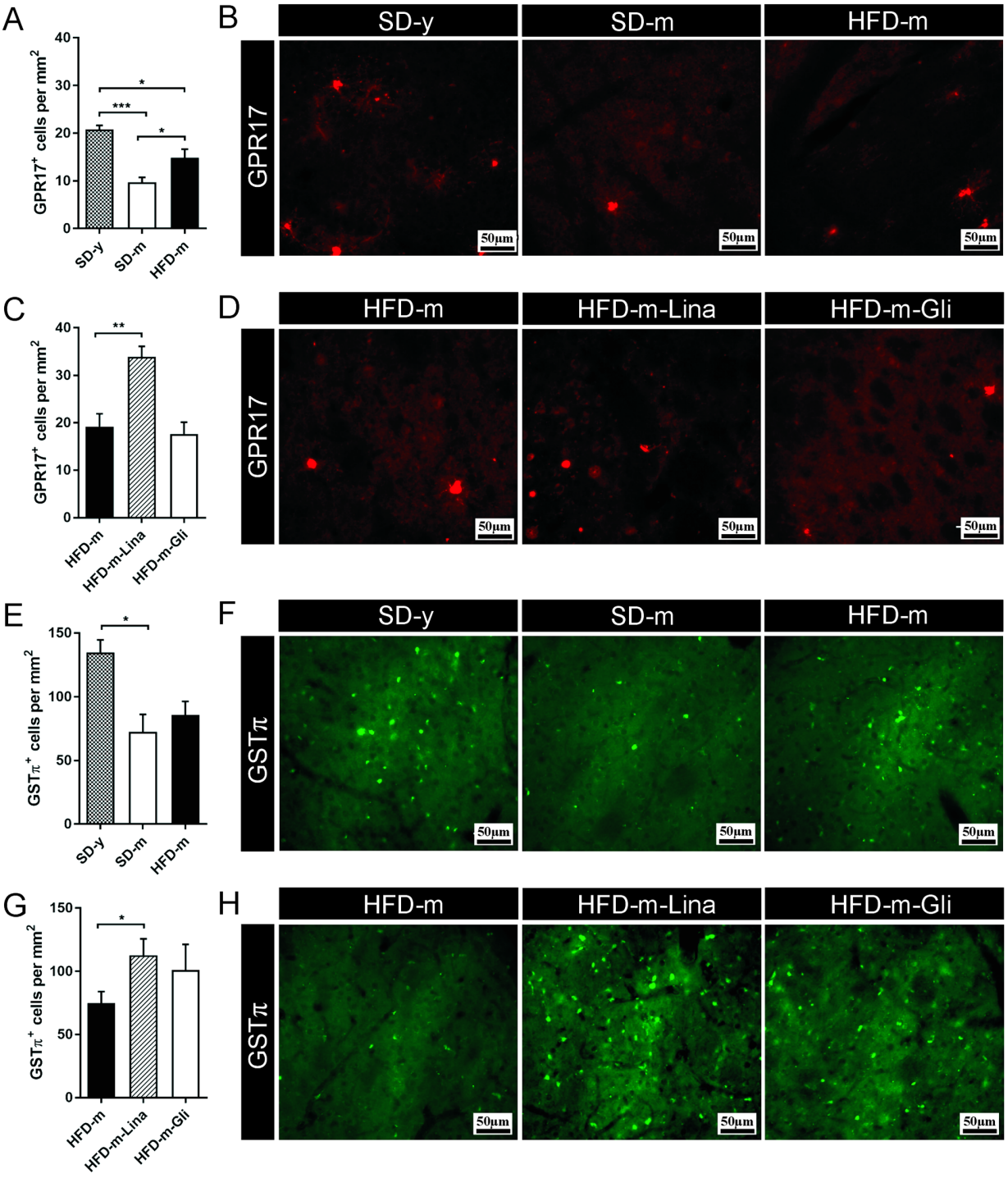
Tukey's multiple comparisons test	Summary	Adjusted P Value
SD-y vs. SD-m	ns	0,5133
SD-m vs. HFD-m	****	<0,0001
HFD-m vs. HFD-m-Lina	****	<0,0001
HFD-m vs. HFD-m-Gli	****	<0,0001
HFD-m-Lina vs. HFD-m-Gli	ns	0,9935


A
PV+ cells per mm2
100
80
60
40
20
0
**
*
SD-y
SD-m
HFD-m
B
Mean volume of PV+ cells (μm3)
1200
900
600
300
0
**
*
SD-y
SD-m
HFD-m
C
PV+ cells per mm2
100
80
60
40
20
0
HFD-m
HFD-m-Lina
HFD-m-Gli
D
Mean volume of PV+ cells (μm3)
1200
900
600
300
0
*
HFD-m
HFD-m-Lina
HFD-m-Gli
E
SD-y
SD-m
HFD-m
PV
150μm
F
HFD-m
HFD-m-Lina
HFD-m-Gli
PV
150μm


A
Iba-1+ cells per mm2
500
400
300
200
100
0
****
****
SD-y
SD-m
HFD-m
B
Mean volume of Iba-1+ cells (μm3)
300
250
200
150
100
***
****
SD-y
SD-m
HFD-m
C
Iba-1+ cells per mm2
500
400
300
200
100
0
HFD-m
HFD-m-Lina
HFD-m-Gli
D
Mean volume of Iba-1+ cells (μm3)
300
250
200
150
100
*
HFD-m
HFD-m-Lina
HFD-m-Gli
E
SD-y
SD-m
HFD-m
Iba-1
25μm
150μm
F
HFD-m
HFD-m-Lina
HFD-m-Gli
Iba-1
25μm
150μm


A
GFAP+ cells per mm2
300
250
200
150
100
50
0
***
***
SD-y
SD-m
HFD-m
B
GFAP+ cells per mm2
300
250
200
150
100
50
0
*
HFD-m
HFD-m-Lina
HFD-m-Gli
C
SD-y
SD-m
HFD-m
GFAP
150μm
D
HFD-m
HFD-m-Lina
HFD-m-Gli
GFAP
150μm


A
Olig2+ cells per mm2
400
300
200
100
0
****
****
SD-y
SD-m
HFD-m
B
% of PCNA+Olig2+ cells/Olig2
20
15
10
5
0
**
**
SD-y
SD-m
HFD-m
C
Olig2
PCNA
Merge
SD-y
SD-m
HFD-m
50μm
50μm
50μm
D
Olig2+ cells per mm2
400
300
200
100
0
****
HFD-m
HFD-m-Lina
HFD-m-Gli
E
% of PCNA+Olig2+ cells/Olig2
20
15
10
5
0
*
HFD-m
HFD-m-Lina
HFD-m-Gli
F
HFD-m
HFD-m-Lina
HFD-m-Gli
50μm
50μm
50μm


A
B
SD-y
SD-m
HFD-m
GPR17
GPR17+ cells per mm2
SD-y
SD-m
HFD-m
50μm
C
D
HFD-m
HFD-m-Lina
HFD-m-Gli
GPR17
GPR17+ cells per mm2
HFD-m
HFD-m-Lina
HFD-m-Gli
50μm
E
F
SD-y
SD-m
HFD-m
GSTπ
GSTπ+ cells per mm2
SD-y
SD-m
HFD-m
50μm
G
H
HFD-m
HFD-m-Lina
HFD-m-Gli
GSTπ
GSTπ+ cells per mm2
HFD-m
HFD-m-Lina
HFD-m-Gli
50μm

Highlights

- Type 2 diabetes (T2D) impairs the release of dopamine *in striatum* during aging
- The DPP-4 inhibitor linagliptin and sulfonylurea glimepiride prevent this effect
- Aging but not T2D induces neuronal and glial alterations in *striatum*
- The effects on glia are partially but more selectively reduced by DPP-4 inhibition