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Glucagon-like peptide-2 reduces the obesity-associated inflammation in the brain

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ABSTRACT

Growing evidence suggests a link between obesity and neurodegeneration. The purpose of the present study was to explore the neuroprotective potential of glucagon-like peptide-2 (GLP-2) in the brain of high fat diet (HFD)-fed mice. Markers of inflammation and oxidative stress were analysed in the brains of obese mice chronically treated with [Gly^2]-GLP-2 (teduglutide), the stable analogue of the GLP-2, and they were compared to age-matched untreated obese and lean animals. Neurodegeneration was examined by TUNEL assay. HFD feeding increased the expression of pro-inflammatory mediators (NF-kB, IL-8, TNF-α, IL-1β and IL-6), glial fibrillary acidic protein (GFAP), index of gliosis and neurodegeneration, stress marker proteins (p-ERK, Hsp60 and i-NOS), amyloid-β precursor protein (APP). [Gly^2]-GLP-2 treatment significantly attenuated the HFD-induced increased expression of the various markers, as well as the higher levels of reactive oxygen species found in brains of untreated-HFD mice. Immunofluorescence confirmed that the increase of GFAP or APP in the brain cortex of HFD mice were less prominent in the [Gly^2]-GLP-2 treated group. TUNEL-positive cell number in brain sections of [Gly^2]-GLP-2-treated HFD-fed mice was significantly lesser in comparison with untreated-HFD animals and similar to STD fed mice. In conclusion, the results of the present study suggest that GLP-2 stable analogue improves the obesity-associated neuroinflammation and the central stress conditions, it reduces the neuronal apoptotic death, providing evidence for a neuroprotective role of the peptide.

1. Introduction

Glucagon-like peptide-2 (GLP-2), a 33-amino-acid peptide hormone, belongs to the so-called proglucagon-derived peptides, which originate from tissue-specific posttranslational processing by convertases of proglucagon (Amato et al., 2016). GLP-2 is synthesized in the gastrointestinal tract in the L-cells of the small intestine and colon, but is also produced in a discrete population of neurons in the brainstem and hypothalamus (Vrang and Larsen, 2010).

The biological activities of the peptide are mediated by a specific GLP-2 receptor (GLP-2R), which is a member of the G protein-coupled receptor superfamily. The GLP-2R expression is abundant in the gut (Drucker and Yusta, 2014; Janssen et al., 2013), but GLP-2R extra-intestinal presence, including the central nervous system (CNS), has been also reported (El-Jamal et al., 2014; Lovshin et al., 2001). Several studies have focused the attention on the intestinal physiology, revealing that GLP-2 promotes the growth of the intestinal epithelial cells (Drucker and Yusta, 2014), it improves mucosal blood flow and nutrient absorption (Guan et al., 2006), it enhances the epithelial barrier capacity (Benjamin et al., 2000) and it inhibits gastrointestinal motility (Amato et al., 2010; Cinci et al., 2011). In addition, recent evidences suggest a GLP-2 role in the control of glucose homeostasis (Baldassano et al., 2015; Baldassano et al., 2016a; Guan, 2014; Shi et al., 2013) and in the lipid metabolism (Baldassano et al., 2016b).

GLP-2 has been also shown to produce anti-inflammatory effects in rodent model of intestinal disease (El-Jamal et al., 2014; Ivory et al., 2008; Sigalet et al., 2007) and to suppress the lipopolysaccharide (LPS)-induced inflammation in murine peritoneal macrophages (Xie et al., 2014) and microglial BV-2 cells (Li et al., 2016), but the hypothetical anti-inflammatory effect in the CNS has not been explored yet.

Indeed, in contrast to the increasing number of studies describing the enteric functions of GLP-2, few investigations have been conducted to elucidate the roles of GLP-2 in the CNS (Vrang and Larsen, 2010). Multiple experimental approaches have localized the GLP-2R in areas of

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the rodent CNS including the hypothalamus, hippocampus, brainstem, cerebellum and cortex (Guan et al., 2012; Lovshin et al., 2004; Tang-Christensen et al., 2000). Activation of GLP-2R in the hypothalamus may be responsible for the inhibitory effects of central GLP-2 administration on food intake (Tang-Christensen et al., 2000) and for the increase in glucose tolerance and insulin sensitivity (Shi et al., 2013).

Metabolic diseases have been reported to contribute to cognitive decline (Azizi and Mirshafiey, 2012). In particular high fat diet (HFD) can lead to obesity, insulin resistance, fatty liver disease, dementia including Alzheimer's disease (AD) (Ghareeb et al., 2011). Previously, we demonstrated that in HFD mice the obesity is associated with peripheral and central insulin resistance, cerebral inflammation, increase in oxidative stress and amyloid-β precursor protein (APP), amyloid-β peptide (Aβ), BACE1 and GSK3β expression (Nuzzo et al., 2015), enzymes involved in APP processing and formation of amyloid plaques and neurofibrillary tangles, respectively.

In vitro studies have suggested that GLP-2 is able to defend neurons from excitotoxic damage (Vrang and Larsen, 2010) and to reduce glutamate-induced cell death in cultured hippocampal cells (Lovshin et al., 2004). In addition, GLP-2 has been reported to play a role in protecting the circuitry of the enteric nervous system (Sigalet et al., 2010) and to exert beneficial influence against obesity-related insulin resistance (Baldassano et al., 2015; Baldassano et al., 2016a), condition which could lead to neurodegeneration (Nuzzo et al., 2015). Thus, we hypothesized that the peptide can exert anti-inflammatory and neuroprotective effects also in the brain of obese HFD mice. In this view, obese mice were chronically treated for four weeks with the stable analogue of the GLP-2, [Gly^2]-GLP-2 (teduglutide), and markers of inflammation, oxidative stress and neurodegeneration conditions were analysed in comparison with age-matched untreated obese and lean animals.

2. Materials and methods

2.1. Animals

The animal procedures for the care and use of laboratory animals were in conformity with the Italian D.L. no. 116 of 27 January 1992, subsequent variations, and recommendations of the European Economic Community (86/609/ECC). The studies were authorized by Ministero della Sanita` (Rome, Italy). Four-week old male C57BL/6 J (B6) mice, purchased from Harlan Laboratories (San Pietro al Natisone Udine, Italy) were housed under standard conditions of light (12 h light:12 h darkness cycle) and temperature (22–24 °C), with free access to water and food. After acclimatization (1 week), the animals were weighed and randomly divided in two groups and assigned either to a standard diet (STD) (code 4RF25, Mucedola, Milan, Italy) or to high-fat diet (HFD) consisting of 23% protein, 38% carbohydrates, and 34% fat with 60% energy derived from fat (code PF4051/D, Mucedola). After 8 weeks on HFD, the HFD group was further randomly subdivided into further sub-groups, which were differently treated once a day for 4 weeks: the first one was injected intraperitoneally (i.p) with 100 µl of Gly2-GLP-2 (5 µg) (treated-HFD) and the second one was injected i.p with 100 µl of PBS (vehicle) (untreated-HFD). The dose, the route and length of treatment were selected on the basis of previous studies describing the effects of the peptide (Baldassano et al., 2016a; Hadjiyanni et al., 2009; Iakoubov et al., 2009). Nevertheless, to verify the efficacy of the dose and period of treatment, gut mucosal growth was analysed (Fig. 1s). At the end of the study period, metabolic parameters were analysed, then the animals were weighed and killed by cervical dislocation. The brains of age-matched animals were immediately explanted, weighed and processed for subsequent analysis.

2.2. Biochemical analyses

Glucose was measured using a commercial glucometer (GlucoMen LX meter, Menarini, Italy) in blood collected from the tail vein. Plasma insulin was quantified by a mouse ELISA kit (Alpco diagnostics, Salem, NH USA). Triglycerides and cholesterol were measured using a Cobas 6000 Analyzer (Roche Diagnostics Ltd., Rotkreuz Switzerland) in blood collected by cardiac puncture.

2.3. Peptide

Synthetic Gly^2-GLP-2 was provided by Caslo Laboratory (Lyngby, Denmark). Purity (≥ 95%) and correctness of structure were confirmed by mass, sequence, and HPLC analysis.

2.4. Total protein extraction and Western blotting

Brains of mice were homogenized in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride PMSF, 1 mM DTT, 0.1% SEMS) with protease inhibitors (Amersham Life Science, Munich, Germany) and phosphatase inhibitor cocktail II and III (Sigma-Aldrich, Milan, Italy). To remove insoluble material, tissue lysates were sonicated and centrifuged (14.000 rpm, at 4 °C, for 30 min). Proteins (30 µg) were resolved by 10% SEMS-PAGE gel and transferred onto nitrocellulose filters for Western blotting using antibodies described in Table 1, which reports also the secondary antibodies. Band intensities were analysed with a gel documentation system (Bio-Rad Laboratories, Milan, Italy), expression was normalized with β-actin expression. The protein levels were expressed as intensity relative to the control.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Concentrations of Interleukin-1β (IL-1β) and Interleukin-6 (IL-6) were measured in the brain homogenates (20 mg of tissue sample) using mouse ELISA kits (Cloud-Clone Corp, Wuhan, Hubei) according to the manufacturers' protocols.

2.6. Immunofluorescence analysis

For tissue preparation, the brains removed from STD, untreated-HFD and Gly^2-GLP-2-treated HFD mice were fixed in 4% formalin for 24 h, then were sectioned in half, embedded in paraffin and spliced in coronal section (5 µm) using a microtome. Brain sections including the cerebral cortex were mounted on slides and deparaffinised in xylene solution. Then, the slides were hydrated in a series of graded ethanol (96%, 85%, 70%, 50%) for 5 min each. After washing in water and PBS the slides were incubated with 3% BSA/PBS for 1 h. Next, the sections were incubated with the primary antibody against APP (1:25; Santa Cruz Biotechnology) or against GFAP (1:50; Cell Signaling Technology) at 4 °C overnight. After washing in PBS, the slides were incubated with anti-rabbit Cy3-conjugate and anti-mouse Cy3-conjugate secondary antibodies (1:500; Cell Signaling Technology). Nuclear staining was performed using Hoechst 33258 (5 µg/ml) for 20 min. The samples were analysed by using a DHL fluorescent microscope (Leica Microsystems, Heidelberg, Germany) at a magnification of 20× by experimenters blinded to treatment conditions. GFAP positive fluorescence intensity was measured using Leica QFluoro program. Brain sections incubated only with secondary antibody were used as negative control. Data represents the mean fluorescence intensity integrated above the baseline. The baseline subtraction avoids systematic errors from underlying auto-fluorescence.

2.7. TUNEL assay

Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL)-positive apoptotic nuclei were detected in brain paraffin sections using an in situ cell death detection kit (Promega Madison, WI USA) according to manufacturer's instructions. The

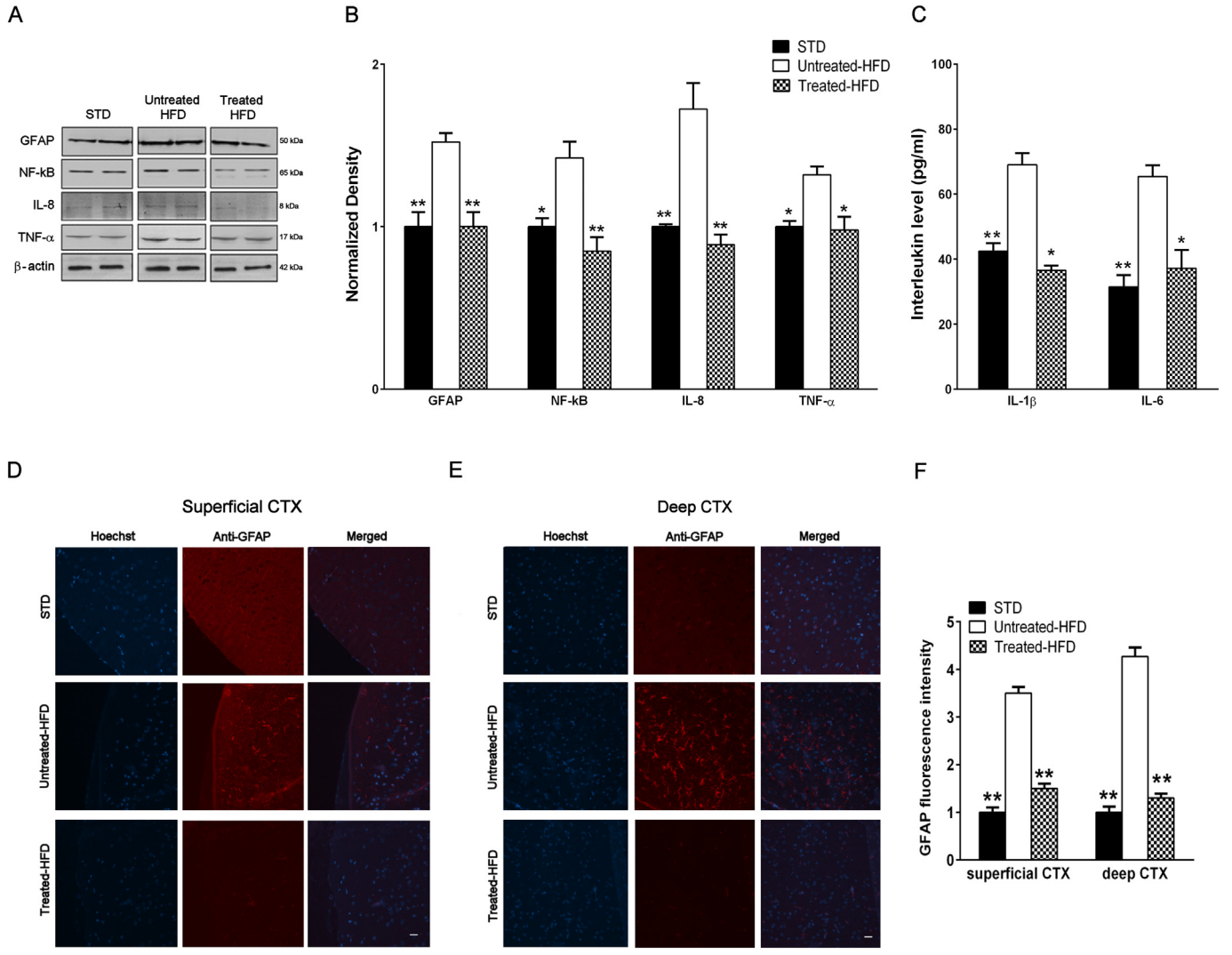

Fig. 1. [Gly^2]-GLP-2 (5 μg) decreases neuroinflammation and modulates astrocyte activation in the brain of HFD-fed mice. A) Western blot showing GFAP, NF-kB, IL-8, TNF-α extracted from brain lysates of standard diet-fed animals (STD), untreated-HFD and Gly^2-GLP-2-treated HFD mice. β-Actin was used as loading control. B) Densitometric analysis of immunoreactivity. The optical intensities of the bands were normalized to STD whose values were: GFAP1028 ± 46,1; NF-kB 1444,5 ± 9,2; IL-8 2231,5 ± 28,9; TNF-α 3061 ± 44,1. Data are means ± SEM. n = 6 per group. * $p < 0.05$, ** $p < 0.02$ versus untreated-HFD group. C) Levels of IL-1β and IL-6 quantified by ELISA in brain homogenates from STD- untreated-HFD and Gly^2-GLP-2-treated HFD mice. Data are means ± SEM. n = 6 per group. * $p < 0.05$, ** $p < 0.02$ versus untreated-HFD group. D-E) Staining by immunofluorescence of glial fibrillary protein (GFAP) in superficial (primary somatosensory area) or deep (ventral retrosplenial area) cerebral cortex sections of STD- untreated-HFD and Gly^2-GLP-2-treated-HFD mice. Nuclei were stained with Hoechst 33528. Scale bars: 20 μm. F) Histogram showing quantitative analysis of GFAP in superficial or deep cortex sections of STD-, untreated-HFD and Gly^2-GLP-2-treated-HFD-mice. Data are means ± SEM. n = 5 per group. ** $p < 0.02$ versus untreated-HFD group.

number of apoptotic cells was counted by two of us blind to treatment conditions in cerebral cortex selected fields to calculate the ratio of apoptotic cell per brain area.

2.8. Analysis of reactive oxygen species (ROS) generation

ROS generation was assessed by fluorimeter analysis as previously described (Heidari et al., 2016). 0.3 mg of tissue from STD, HFD and Gly^2-GLP-2-treated HFD brains were weighed and suspended on 100 μl of PBS1X. Then, they were incubated with 1 mM dichlorofluorescein diacetate (DCFH-DA) for 10 min at room temperature in the dark. The conversion of non-fluorescent DCFH-DA to the highly fluorescent compound 20,70-dichlorofluorescein (DCF) by esterase activity can be used to monitor the presence of peroxides due to the oxidative burst in the brains. Therefore, the emitted fluorescence is directly proportional to the concentration of ROS. The brain tissues were analysed by fluorimeter (Microplate reader WallacVictor 2–1420 Multilabel Counter; PerkinElmer, Inc.). The excitation filter was set at 485 nm and the emission filter was set at 530 nm.

2.9. Statistical analysis

The results are presented as mean ± SEM. Statistical evaluation was performed by ANOVA, followed by followed by Bonferroni post hoc test using Prism 6.0, GraphPad (San Diego, CA, USA). Results with a p-value < 0.05 were considered statistically significant.

3. Results

3.1. [Gly^2]-GLP-2 and metabolic parameters

The mean body weight of the untreated HFD group was significantly higher than of mean value of STD-fed animals, whereas it was not significantly different from [Gly^2]-GLP-2-treated mice (Table 2). In

Table 1
Codes and Sources of the Antibodies for western-blot analysis.

Antigen	Host	Code	Dilution	Source
anti-APP	Rabbit-polyclonal	sc-9129	1:1000	Santa Cruz Biotechnology
anti-BACE1	Rabbit-monoclonal	5606	1:1000	Cell Signaling Technology
anti-PSN1	Rabbit-monoclonal	3622	1:1000	Cell Signaling Technology
anti ERK 1/2	Mouse-monoclonal	sc-514,302	1:1000	Santa Cruz Biotechnology
anti p-ERK	Mouse-monoclonal	sc-7383	1:500	Santa Cruz Biotechnology
anti-Hsp 60	Rabbit-polyoclonal	4870	1:500	Cell Signaling Technology
anti-i-NOS	Rabbit -polyoclonal	2977	1:500	Cell Signaling Technology
anti-GFAP	Mouse-monoclonal	3670	1:1000	Cell Signaling Technology
anti-NF-kB	Goat-polyoclonal	sc-372	1:1000	Santa Cruz Biotechnology
anti-IL-8	Rabbit-polyoclonal	ab18672	1:500	Abcam
anti-TNF-α	Rabbit-monoclonal	6945S	1:500	Cell Signaling Technology
anti-GLP-2R	Goat-polyoclonal	sc-46,997	1:200	Santa Cruz Biotechnology
anti-β-Actin	Rabbit-polyoclonal	A2228	1:500	Sigma
anti-Mouse IgG, HRP conjugate	Goat	401,253	1:10000	Calbiochem
anti-Rabbit-IgG, HRP conjugate	Goat	7074	1:10000	Cell Signaling Technology
anti-Goat IgG, HRP conjugate	Rabbit	Ab6741	1:10000	Abcam

Abbreviations: anti-APP, anti-amyloid precursor protein; anti-BACE 1, anti-β-secretase 1; anti-PSN1, anti-Presenilin 1; anti-ERK 1/2, anti-map kinase 1/2; anti-p-ERK, anti-phospho-map kinase; anti-Hsp 60,anti-heat shock protein 60; anti-i-NOS, anti-inducible nitric oxide synthase; anti- GFAP, anti-glial fibrillary acidic protein; anti-NF-kB, anti-nuclear factor kappa B; anti-IL-8, anti-interleukin 8; anti-TNFα, anti-tumor necrosis factor alpha.

addition, the untreated HFD group had significantly higher fasting blood glucose levels and increased plasma insulin, triglyceride and cholesterol concentrations compared to control group (Table 2), suggesting the presence of glucose and lipid metabolism impairment. However, as previously shown (Baldassano et al., 2016a), [Gly^2]-GLP-2 treatment improved the glucose metabolic parameters, but did not the lipid parameters (Table 2).

3.2. [Gly^2]-GLP-2 decreases neuroinflammation in the brain of HFD mice

To evaluate whether [Gly^2]-GLP-2 treatment was able to improve the HFD-induced neuroinflammation conditions, expression of some pro-inflammatory mediators and GFAP was evaluated. Western blot analysis revealed that HFD feeding increased the expression of GFAP (+ 52 ± 5.3%), NF-kB (+42 ± 10%), IL-8 (+72 ± 16%), TNF-α (+32 ± 5%) compared with STD-fed mice. However, [Gly^2]-GLP-2 treatment (5 μg) significantly attenuated this HFD-induced increased expression (Fig.1A and 1B). Moreover, the levels of IL-1β and IL-6 detected by ELISA in brains of HFD-fed mice were significantly higher respect to STD mice or [Gly^2]-GLP-2-treated HFD mice, suggesting an attenuation of the HFD-induced neuroinflammation (Fig. 1C).

3.3. [Gly^2]-GLP-2 modulates astrocyte activation in the brain of HFD mice

To further assess the presence of injury, immunofluorescence was performed to analyze the presence of GFAP, as an index of gliosis and neurodegeneration, in brain sections. Immunofluorescence staining revealed a 3.5/4 fold increase in GFAP immunoreactivity only in superficial (primary somatosensory area) and deep (ventral retrosplenial area) cerebral cortex sections of HFD-fed mice related to STD controls. In the [Gly^2]-GLP-2 treated group the GFAP immunoreactivity was less prominent (about +1.5 fold increase in comparison with STD) (Fig. 1D, E and F). No positive area was detected in other brain regions (hippocampus, thalamus, hypothalamus).

3.4. [Gly^2]-GLP-2 reduces stress in the brain of HFD mice

Protein markers of stress such as p-ERK, Hsp60 and i-NOS were analysed by Western blotting to verify the potential of [Gly^2]-GLP-2 to decrease the stress conditions. We found that all tested proteins were overexpressed in untreated-HFD mice compared to STD fed mice, suggesting that a condition of stress was present. However, the expression of p-ERK/ERK, i-NOS and Hsp60 was significantly reduced in brains of [Gly^2]-GLP-2-treated HFD mice (Fig. 2A and B). Furthermore, the ROS generation was detected by DCFH-DA fluorimetric assay in brain lysates from STD-, untreated-HFD and [Gly^2]-GLP-2-treated HFD mice. The analysis showed higher levels of ROS in untreated-HFD (+88 ± 10.4%) than in STD animals. [Gly^2]-GLP-2 treatment reduced significantly the HFD-induced ROS increase (+15 ± 3.5%) (Fig. 2C), suggesting the peptide ability to reduce the oxidative stress.

3.5. Effects of [Gly^2]-GLP-2 on neurodegeneration

To evaluate the presence of fragmented DNA, index of apoptotic cell death, TUNEL assay was used. We found that TUNEL-positive cell number in superficial and deep cerebral cortex areas of HFD-fed mice was significantly higher in comparison with STD-fed animals, providing evidence that apoptotic cell death occurred in obese mice. The [Gly^2]-GLP-2 treatment significantly reduced the presence of neurons with fragmented DNA in brain cortex sections, suggesting decrease of apoptotic cellular death in treated animals (Fig. 3A and B).

Brain extracts from HFD mice revealed a significant increase in expression of APP (+ 80 ± 18%,) compared to STD mice (Fig. 4A and B). However, no significant change was observed for BACE1 or PSN1 (Fig. 4A and B), two enzymes required for processing of APP to produce Aβ. [Gly^2]-GLP-2 treatment significantly reduced the HFD-induced increase in the expression of APP (+35 ± 11%) and it did not modify BACE1 and PSN1expression levels (Fig. 4A and B). Additionally, we examined the presence of APP by immunofluorescence using superficial

Table 2
Basal metabolic parameters of STD - untreated and [Gly^2]-GLP-2-treated-HFD mice.

	Body weight (g)	Fasting blood glucose (mg/dl)	Fasting plasma insulin (ng/ml)	Triglyerides (mg/dl)	Cholesterol (mg/dl)
STD	25.3 ± 0.8	105 ± 3.7	0.32 ± 0.03	79 ± 2.0	92 ± 3.6
Untreated HFD	32.1 ± 2.5*	165 ± 9.2*	1.10 ± 0.25*	100 ± 12	166 ± 11*
Treated HFD	31.8 ± 0.7	135 ± 3.4$^{\#}$	0.5 ± 0.04$^{\#}$	112 ± 6.2	142 ± 10

Data are mean values ± SEM of at least n = 6 mice/group. * $p < 0.05$ versus STD group; # $p < 0.05$ versus. untreated HFD group.

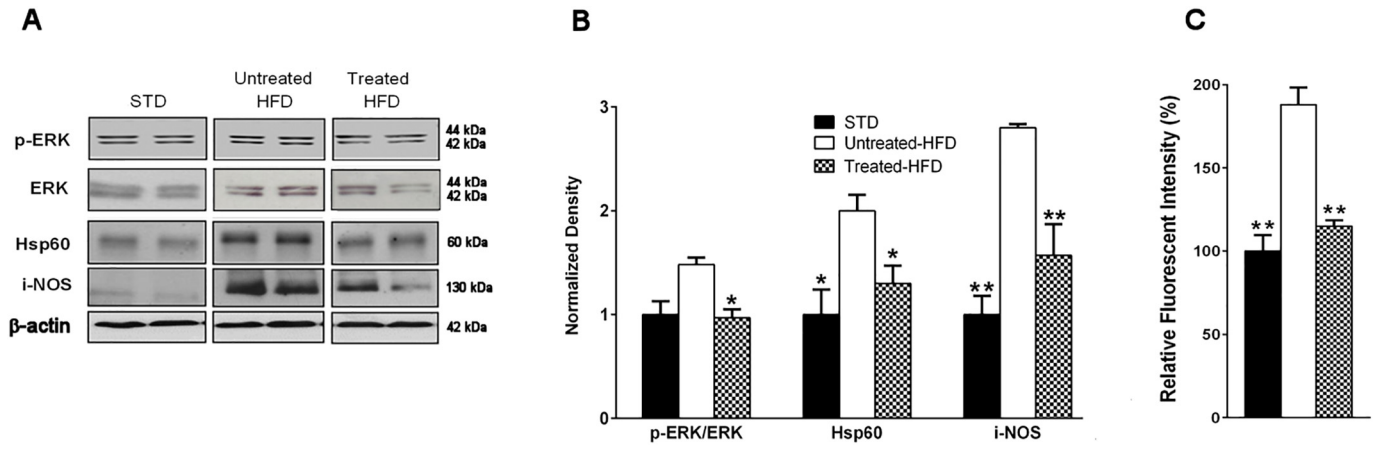

Fig. 2. [Gly^2]-GLP-2 (5 μg) reduces stress in the brain of HFD mice. A) Western blot showing p-ERK/ERK, Hsp60, i-NOS extracted from brain lysates of standard diet-fed animals (STD), untreated-HFD and Gly2-GLP-2-treated HFD mice. β-Actin was used as loading control. B) Densitometric analysis of immunoreactivity. The optical intensities of the bands were normalized to STD whose values were: p-ERK 9751,5 ± 35,1; ERK 10525 ± 51,6; Hsp60 7815 ± 49,5; i-NOS 206 ± 28,3. Data are means ± SEM. n = 6 per group. *p < 0.05, **p < 0.02 versus untreated-HFD group. C) ROS production detected by DCF-DA fluorescence in brain lysates from standard diet-fed animals (STD), untreated-HFD and Gly^2-GLP-2-treated-HFD mice. The intensity was normalized to STD. Data are means ± SEM. n = 6 per group. **p < 0.02 versus untreated-HFD group.

and deep cortex sections. We observed increased immunoreactivity of anti-APP in both examined cerebral cortex sections of untreated-HFD mice compared to STD fed mice or [Gly^2]-GLP-2-treated HFD mice (Fig. 4C and D).

3.6. [Gly^2]-GLP-2 and GLP-2R expression

We investigated also whether GLP-2R was expressed in mouse brain tissue. Western blotting experiments showed that the receptor was present in cerebral homogenate (Fig. 5A). In addition, diverse brain areas showed a differential expression, according to the literature (Guan et al., 2012; Lovshin et al., 2004; Tang-Christensen et al., 2000) (Fig. 5B). However, the expression of GLP-2R in STD-, untreated-HFD and treated-HFD mice was not significantly different (Fig.5A and C).

4. Discussion

The present study provides the first experimental evidence that treatment with Gly^2-GLP-2 improves the obesity-associated neuroinflammation and the central stress conditions. It reduces the neuronal apoptotic death, suggesting a neuroprotective action of the peptide. These effects occurred independently of changes in body weight.

The obesity and high-fat diets are major risk factors for the development of type 2 diabetes, cardiovascular disease, dementia, cognitive decline, and potentially AD (Muhammad et al., 2009). HFD-fed mice rapidly increase body weight and they are currently used to characterize metabolic changes associated to obesity, including pathological signs of AD (Kim et al., 2016; Nuzzo et al., 2015). In fact, mice fed a HFD show in the cortex increased Aβ and other markers of AD, formation of plaques and impaired spatial learning (Kim et al., 2016; Nuzzo et al., 2015; Valladolid-Acebes et al., 2011).

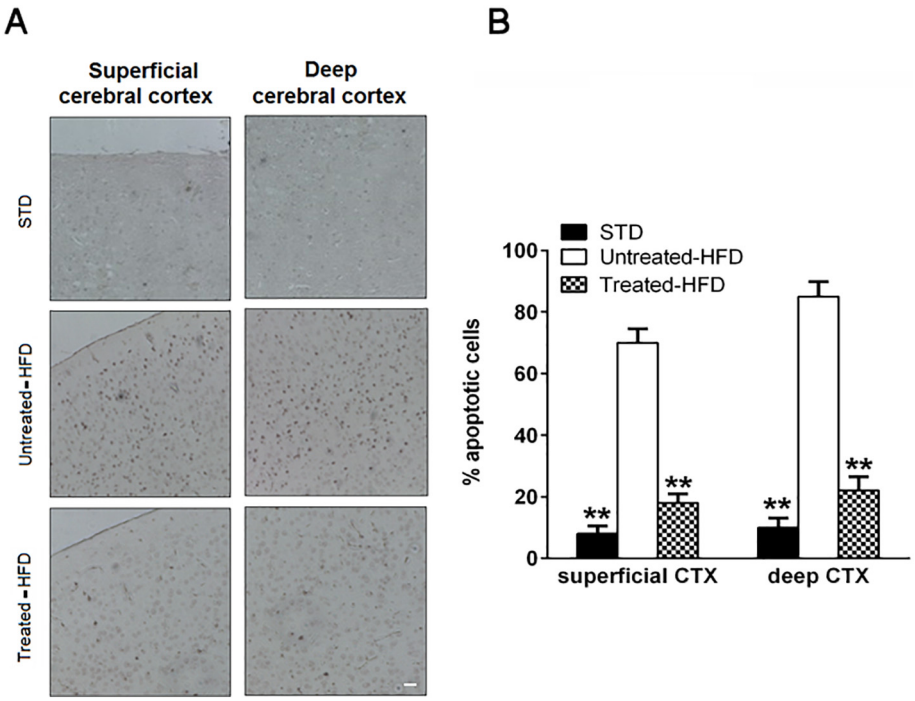

Fig. 3. [Gly^2]-GLP-2 (5 μg) reduces neuronal apoptosis in the brain cortex of HFD mice. A) Micrographs showing Tunel-positive cells (brown) of superficial (primary somatosensory area) or deep (ventral retrosplenial area) cortex sections from standard diet-fed animals (STD), untreated-HFD and Gly^2-GLP-2-treated-HFD-mice. Scale bar: 20 μm. B) Histogram showing the number of Tunel positive nuclei/100 nuclei in superficial or deep cortex sections of STD-, untreated-HFD and Gly^2-GLP-2- treated-HFD-mice. Data are means ± SEM. n = 5 per group. **p < 0.02 untreated-HFD group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

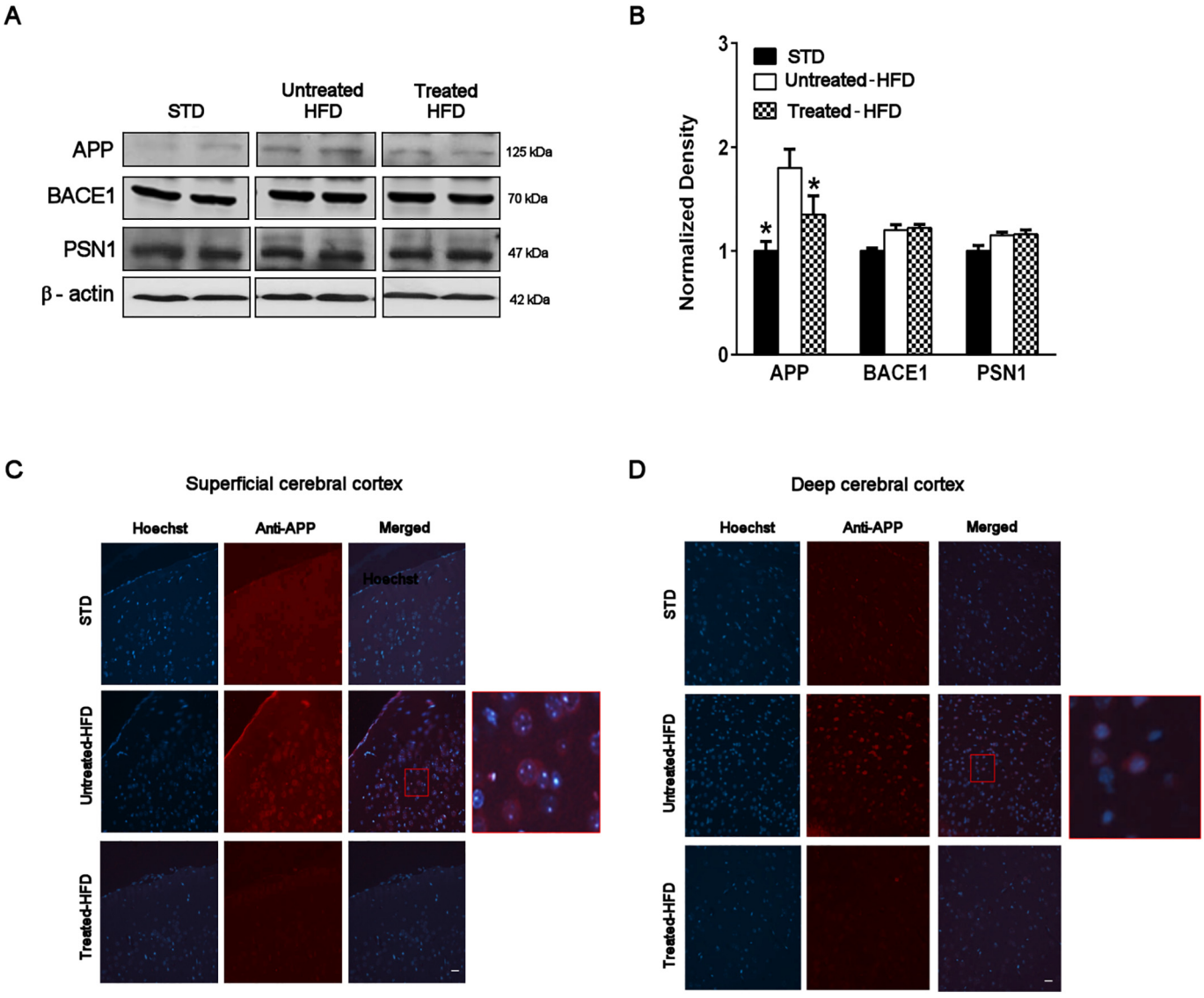

Fig. 4. Effects of [Gly^2]-GLP-2 (5 μg) on expression of different proteins. A) Western blot showing amyloid precursor protein (APP), BACE1 and PSN1 extracted from brain lysates of standard diet-fed animals (STD), untreated-HFD and Gly^2-GLP-2 treated-HFD mice. β-Actin was used as loading control. B) Densitometric analysis of immunoreactivity. The optical intensities of the bands were normalized to STD whose values were: APP 352,1 ± 70,1; BACE1 4065,5 ± 239,7; PSN1 3878,5 ± 593,3. Data are means ± SEM. n = 6 per group. * $p < 0.05$ versus untreated-HFD group. C-D) Staining by immunofluorescence of APP in superficial (primary somatosensory area) and deep (ventral retrosplenial area) cerebral cortex from STD-, untreated-HFD and Gly^2-GLP-2- treated-HFD mice. High magnification of the squared area is shown. Nuclei were stained with Hoechst 33528. Scale bars: 20 μm.

GLP-2 is known to exhibit anti-inflammatory activity in colitis models and protective effects in inflammatory bowel disease (Ivory et al., 2008; Sigalet et al., 2007) as well as to reduce the gene expression of pro-inflammatory cytokines in the intestinal mucosa (Moore et al., 2010). Because GLP-2 positively influences glucose metabolism in obesity conditions (Baldassano et al., 2015; Baldassano et al., 2016a) and it exerts beneficial effects on inflammation, this study verified whether the peptide is able to ameliorate the obesity-associated central inflammation and neurodegeneration.

Inflammation is increasingly recognized as a key contributor to the pathogenesis of neurodegenerative disease, such as Parkinson disease and AD (Ferreira et al., 2014; Tai et al., 2013). Moreover, the presence of inflammatory markers in AD brain, including elevated levels of cytokines and gliosis in damaged regions has been extensively reported (Perry et al., 2010). Generally, cytokines increase activity and expression of secretases, contributing to Aβ deposition and pathogenic changes in AD (Glass et al., 2010). Therefore, we performed in the brain of mice fed STD or HFD expression analysis of some pro-inflammatory cytokines (TNF-α, IL-1β and Il-6) associated with obesity, T2DM and neurodegeneration (Sastre et al., 2006). Specifically, TNF-α is a multifunctional cytokine secreted by microglial cells in response to different insults playing an important role in cell survival, apoptosis and inflammation (Ferreira et al., 2014). Moreover, TNF-α and IL-6 are have been reported to be involved in neuroinflammation (Alam et al., 2016) and IL-1β levels are enhanced in cerebrospinal fluid and brain parenchyma of both humans and rodents immediately after brain injury (Shaftel et al., 2008). IL-8, a microglia-derived chemokine playing a crucial role in the recruitment of neutrophils and T cells into CNS (Cross and Woodroofe, 1999) has been associated not only with tick-borne encephalitis but also in the pathogenesis of AD (Alsadany et al., 2013; Galimberti et al., 2003). NF-kB, which can be activated by TNF-α, is crucial in regulating inflammation because it transactivates inflammatory genes by binding to specific sequences in the promoters (Khodabandehloo et al., 2016).

Our results demonstrate that HFD feeding had a significant effect on induction of CNS inflammation. Although 8 weeks of HFD could be a

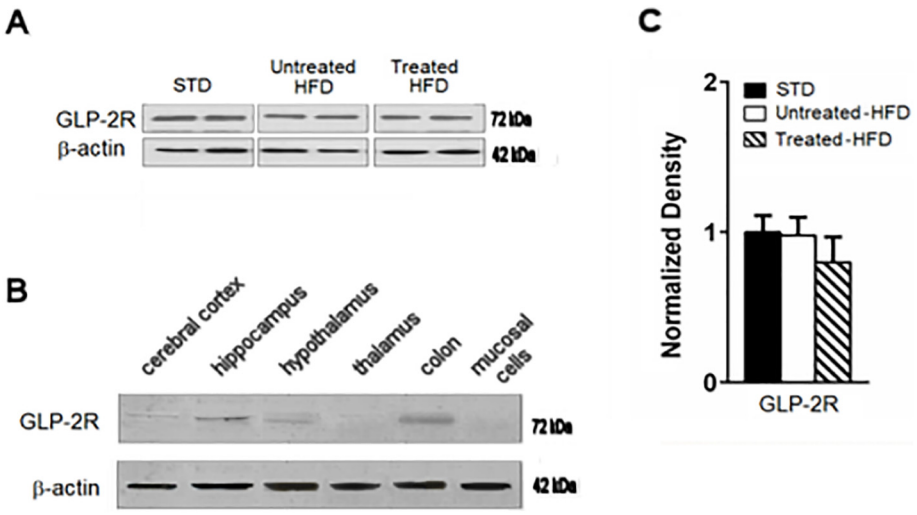

Fig. 5. Expression of GLP-2 receptor. A) Western blot showing GLP-2R in brain lysates of standard diet-fed animals (STD), untreated-HFD and Gly2-GLP-2-treated HFD mice. β-Actin was used as loading control. B) GLP-2R protein expression in different brain regions from STD animals, in mouse colon (positive control) and intestine mucosal cells (negative controls), obtained by gently scraping the tissue. β-Actin was used as loading control. C) Densitometric analysis of immunoreactivity. The intensity of the band was normalized to STD whose value was 438 ± 28,2. Data are means ± SEM. n = 6 per group.

short period to induce brain inflammation, other studies demonstrated that pro-inflammatory cytokines, such as TNF-α and IL-1β are increased in mouse brains after 2 months of HFD (Kim et al., 2016) and once released from different cerebral regions may activate apoptotic signals (De Souza et al., 2005; Jeon et al., 2012). Reactivity of astrocytes and microglia has been observed as early as 3 days after the start of HFD (Thaler et al., 2012) and this response is caused by dietary factors rather than by increases in body weight itself (Gao et al., 2014). In addition Baufeld et al. (2016) found that GFAP-positive astrocytes are significantly increased after 8 weeks of HFD. Anyway, NF-kB, IL-8, TNF-α, IL-1β and IL-6 were significantly reduced in brain tissue from Gly^2-GLP-2-treated HFD-fed animals, in comparison to untreated-HFD mice, suggesting that the peptide can minimize the HFD-induced deleterious inflammatory effects. Considering that NF-kB coordinates the gene expression for pro-inflammatory enzymes and cytokines, including i-NOS, TNF-α, IL-1β and IL-6 (Dai et al., 2011), our findings suggest that the anti-inflammatory activity of the peptide could be mediated at least in part by reduced expression of the transcription factor. The hypothesis of a GLP-2 central anti-inflammatory action further was supported by the observation that Gly^2-GLP-2 inhibited the increased expression of GFAP stimulated by HFD. In fact, in agreement with the results of Baufeld et al. (2016), we found a significant difference in GFAP expression between STD- and HFD-fed mice suggesting that HFD feeding increases gliosis and glia activation and induces a central neural inflammatory state. Such a condition markedly improved in the brain of peptide-treated mice, at least in superficial and deep areas of the cortex.

Oxidative stress plays an important role in different aspects of acute and chronic inflammation. Signaling pathways leading to NF-kB activation are under control of the cell redox state and are modulated by oxidants (Gloire et al., 2006). In according to previous studies (Moroz et al., 2008; Nuzzo et al., 2015), we found that HFD feeding increases the expression of protein markers of stress, such as phosphorylated ERK, Hsp60, i-NOS, as well as the ROS production. These observations confirm a close association between neuroinflammation and stress condition in the brain of obese mice. Furthermore, it is interesting to note that chronic treatment with Gly^2-GLP-2 resulted in a decrease in stress marker proteins as well as in intracellular ROS genesis suggesting that the peptide is also able to affect the cell redox state in the brain of HFD-fed mice and consequently the redox-sensitive signal transduction pathways, such as that modulating the NF-kB activity. This latter finding is consistent with the antioxidant activities of GLP-2 previously demonstrated in rodents (Arda-Pirincci and Bolkent, 2011) and with the GLP-2 ability of inhibiting ERK phosphorylation in inflamed cultured cells (Xie et al., 2014; Li et al., 2016) and it suggests that the peptide might reduce the negative consequences of inflammation on the cerebral functions.

It is well known that oxidative stress induced by increased intracellular ROS production is an important mediator of apoptotic cell death in diverse cell systems (Kannan and Jain, 2000). Consequently, TUNEL assay was performed in different brain areas in order to detect apoptotic cells. The increase in the cell number with fragmented DNA, index of an impairment of cell survival, which was present in the cortical superficial and deep areas of the HFD untreated-animals, was not observed in the [Gly^2]-GLP-2-treated HFD mice, suggesting a preventive role of the peptide in inducing apoptosis. This result appears particularly interesting in consideration that the neurodegenerative process leading to AD, especially at the beginning, predominantly affects the cerebral cortex (Jeong, 2017).

Because activation of pro-inflammatory cytokines may lead to accumulation and/or to cytotoxic effects of APP (Sastre et al., 2006), we analysed the expression of this protein. We found that APP was up-regulated in the brain of HDF mice and that the peptide treatment was able to reduce the HFD-induced increased expression. Increase of APP expression may be correlated with obesity condition and increased pro-inflammatory cytokine expression (de la Monte and Tong, 2014; Nuzzo et al., 2014). However, the observation that BACE1 or PSN1 expression was not modified in HFD brain in comparison with STD mice suggests that these enzymes are likely not involved in APP processing yet. It is interesting to note that immunofluorescence revealed also a reduced presence of APP in the cortex of [Gly^2]-GLP-2-treated HFD mice, which instead, was increased in untreated HFD animals. Our results are consistent with a recent study reporting that GLP-2 protects and improves memory function in mice injected with intracerebroventricular LPS, which induces neuroinflammation and pro-inflammatory cytokine expression increase (Iwai et al., 2015). On the other hand, [Gly^2]-GLP-2 chronic treatment improves also glucose dysmetabolism in HFD mice and because impairment of insulin signaling induced by HFD is linked to neurodegeneration (Nuzzo et al., 2015), [Gly^2]-GLP-2 could exerts neuroprotection through its metabolic actions on glucose homeostasis. However, further studies are necessary to clarify this point.

Indeed, we are not currently able to establish to what extent the effects of peptide are mediated by GLP-2 receptors. Although it is still unknown if GLP-2 can cross the blood-brain barrier, it is likely to hypothesize that the peptide is transported into the brain where it exerts its action, as other members of the same family, including GLP-1 (Dogrukol-Ak et al., 2004). Therefore, peripheral administered Gly^2-GLP-2 would have access to the brain. On the other hand, our experiments pointed out that GLP-2R is present in mouse brain different regions, as reported in the literature (Guan et al., 2012; Lovshin et al., 2004; Tang-Christensen et al., 2000). However, contrarily to previous

observations obtained in mouse gut (Baldassano et al., 2013; Rotondo et al., 2011), GLP-2R expression did not show any significant difference among STD-, untreated HFD- and treated HFD-animals. GLP-2R is coupled with Gs protein and increases intra-cellular cAMP levels in the intestinal mucosa and hippocampal cells (Lovshin et al., 2004; Walsh et al., 2003). Activation of cAMP signaling was demonstrated to inhibit inflammation (Grandoch et al., 2010; Wang et al., 2012). Consequently, GLP-2 could improve the inflammatory conditions via the cAMP pathway.

5. Conclusions

The findings of the present study support the hypothesis that GLP-2 may exert an anti-inflammatory and anti-oxidant action and consequently GLP-2 may be protective against the neuronal death in brains of obese mice. Further studies need to elucidate the mechanisms responsible for peptide action and confirm the potential therapeutic usefulness.

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