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ORIGINAL ARTICLE

Daidzein Pro-cognitive Effects Coincided with Changes of Brain Neurotensin1 Receptor and Interleukin-10 Expression Levels in Obese Hamsters

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Abstract

At present, concerns are pointing to "tasteful" high-fat diets as a cause of conditioning physical-social states that through alterations of some key emotional- and nutritional-related limbic circuits such as hypothalamic and amygdalar areas lead to obesity states. Feeding and energetic homeostatic molecular mechanisms are part of a complex neuronal circuit accounting for this metabolic disorder. In an attempt to exclude conventional drugs for treating obesity, daidzein, a natural glycosidic isoflavone, which mimics estrogenic neuroprotective properties against increased body weight, is beginning to be preferred. In this study, evident anxiolytic-like behaviors were detected following treatment of high-fat diet hamsters with daidzein as shown by extremely evident ($p < 0.001$) exploration tendencies in novel object recognition test and a notably greater amount of time spent ($p < 0.01$) in open arms of elevated plus maze. Moreover, the isoflavone promoted a protective role against neurodegeneration processes as shown by few, if any, amino cupric silver granules in amygdalar, hypothalamic and hippocampal neuronal fields when compared with obese hamsters. Interestingly, elevated expression levels of the anorexic neuropeptide receptor neurotensin1 in the above limbic areas of obese hamsters were extremely reduced by daidzein, especially during recovery of cognitive events. Contextually, such effects were strongly paralleled by increased levels of the anti-neuroinflammatory cytokine, interleukin-10. Our results corroborate a neuroprotective ability of this natural glycosidic isoflavone, which through its interaction with the receptor neurotensin1 and interleukin-10 pathways is correlated not only to improved feeding states, and subsequently obesity conditions, but above all to cognitive performances.

Keywords Obesity · Phytoestrogen · Anxiolytic · Mnemonic behaviors · Neurotensin receptor · Interleukins

Abbreviations

ACS	Amino cupric silver
AMY	Amydala
CTRL	Control
CHOD-PAP	Cholesterol oxidase–peroxidase 4-aminoantipyrine
DI	Discrimination index
DZ	Daidzein
EPM	Elevated plus maze
GOD-POD	Glucose oxidase–peroxidase
GPO-PAP	Glycerol phosphate oxidase- peroxidase 4-aminoantipyrine
HFD	High-fat diet
HIP	Hippocampus
HTH	Hypothalamus
IL-10	Interleukin-10
NOR	Novel object recognition test
NTR1	Neurotensin1 receptor

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Introduction

Obesity is a major growing global metabolic dysfunction, tightly linked to a continuous consumption of highly enriched food products (Chau et al. 2013; Kumar and Kelly 2017). Despite the balanced quantity of food sources to support the daily energy expenditure processes, they, in most cases, are not of a healthy nature and thus lead to a marked development of fat storage (Piaggi et al. 2018). "Diet-induced obesity" rats exhibit widely impaired working memory plus learning performances (Spencer et al. 2017; Wang et al. 2015) and consequently revealed a greater predisposition to neurological disorders, such as Alzheimer's disease (Hill et al. 2019). The metabolic disorders that account for increased body weight are often associated to not only cardiovascular diseases and metabolic syndromes (Weiss et al. 2009) but also to neuropsychiatric syndromes, which feature among others anxiety and panic states (De Noronha et al. 2017; Sanderlin et al. 2017). These disturbances are mainly related to key non-functioning encephalic centers that through the interaction of complex neuronal signaling systems along with psychological plus social alterations are able to substantially modify energy homeostasis events, feeding, and stressful responses (Gómez-Pinilla 2008). Conversely, healthy and controlled habits tend to improve motor, social, and mnemonic behavioral activities (Carlson et al. 2019; Kanoski and Davidson 2011).

Due to the necessity of avoiding the use of drugs with numerous collateral alterations, a greater amount of attention has been focused on "safer" natural drugs from vegetable extracts such as polyphenols, which are excellent non-pharmacological therapeutic agents able to improve living conditions in obese patients. The presence of phytochemical compounds in the matrix of plant foods promoting antioxidant effects accounts for their neuroprotective actions (Kumar and Khanum 2012). Studies have widely shown that phytoestrogens are responsible for diminished food intake and subsequently the development of mild anorexic states via the suppression of hypothalamus (HTH) orexigenic elements (Andreoli et al. 2016). Even rats that were exposed to a high fat diet (HFD) displayed notably improved health conditions after treatment with the soybean isoflavone daidzein (DZ) as indicated by substantial reduced body weight and adipose tissue (Rivera et al. 2013). It appears that treatment with DZ and other isoflavones like genistein tend to promote cross-talking properties with its major neuroreceptor target, i.e., neurotensin (NT)ergic pathway (Subedi et al. 2017) as suggested by its ability to promote opioid-independent analgesic actions in many cerebral pain pathways featuring metabolic and inflammatory disorders (Feng et al. 2015).

NT, a tridecapeptide widely distributed in the brain, exerts a wide range of biological effects such as hypothermia plus antinociception in schizophrenia and Parkinson's disease (White et al. 2012). The actions of this molecule are mediated through three NT receptors, namely, NTR1, 2 plus 3/sortilin (Li et al. 2016), which are densely localized in limbic areas (amygdala, AMY; hippocampus, HIP; and HTH) involved with controlling emotional states plus cognitive and feeding performances. Of the different receptors, NTR1 has shown to mediate anorectic effects (Cui et al. 2005) due to its expression being notably reduced in animal models lacking leptin receptor, and so tend to manifest obesity states–linked dysfunctions (Levitas-Djerbi et al. 2015). More importantly, administration of agonists/antagonists of NTR1 has also been shown to improve learning and overall cognitive processes (Tirado-Santiago et al. 2006; Xiao et al. 2014).

In view of the above indications, it was our intention to evaluate neurobehavioral effects of DZ on motor performances, anxiety plus mnemonic capabilities in HFD hamsters (*Mesocricetus auratus*). For this study, elevated plus maze (EPM) and novel object recognition (NOR) tests were adopted since they are retained useful non-stressful tools for evaluating anxiety- and cognitive-related metabolic impairments. Neurobehavioral protective role of DZ was also correlated to the expression of its major NTR site (NTR1) and the principal anti-inflammatory factor, interleukin-10 (IL-10), which ameliorates HFD-linked mnemonic deficits (Lauridsen et al. 2017). IL-10 is also known to inhibit HFD-induced weight gain plus reduce insulin resistance to glucose intolerance during physical activity (Dorneles et al. 2016). Moreover, the anti-inflammatory factor has shown to provide beneficial effects to brain neuronal and glial elements exposed to stressful situations (Jung et al. 2019; Pan et al. 2013). Results of these studies may be a good starting point for using DZ as a preferential therapeutic alternative on treating patients exhibiting neurodegenerative and cognitive disturbances associated with HFD-related metabolic disorders.

Materials and Methods

Animals and Treatments

Syrian golden hamsters (7 weeks old; Charles River, Como-Italy), with free access to food and water, were allowed to adapt to their new conditions: room temperature (20–22 °C), relative humidity at 50–60%, 14-h light/10-h dark cycle (lights on 06:00 a.m.). Hamsters were then divided into 4 treatment groups: HFD baseline group = hamsters fed with HFD (60% of energy from fat, Envigo Laboratories, Udine,

Italy; $n=6$) for 12 consecutive weeks. Some hamsters received the same diet for 15 days ($n=6$) and 30 days ($n=6$) more; control (CTRL) group was fed for this same period with a standard diet ($n=15$). The other 2 groups received the same diet but at the end of the 12th week, HFD ($n=20$) and CTRLs ($n=18$) received DZ (200 mg DZ/kg diet; 98% DZ, from Santa Cruz Biotechnology). In this case, HFD groups were treated with such an isoflavone for 15 days ($n=10$) and 30 days ($n=10$) plus CTRLs 15 days ($n=9$) and 30 days ($n=9$) for the entire behavioral sessions according to other indications (Zeng et al. 2010). In order to avoid any type of attraction interferences, food chow containing phytoestrogen was crushed and conglutinated to produce closely resembled pellets for appearance and hardness. Animal maintenance and all experimental procedures were approved by Italian University Minister plus Department of Biology, Ecology & Earth Science (University of Calabria, Italy), and were carried out in accordance with the Guide for Care and Use of Laboratory Animals issued by National Institute of Health directive no. 26 (4-03-2014). Efforts were made to minimize animal suffering and reduce the number of experiments.

Behavioral Analysis

Home Cage

At the end of the 12th week of HFD and for the entire period of DZ treatment, body weight was determined weekly while motor performances were monitored daily for 15 min at different intervals (11:00 a.m., 2:00 p.m., 5:00 p.m.) for all groups according to indications deriving from our previous studies (Fazzari et al. 2018; Alò et al. 2019). The different behavioral activities were recorded and evaluated in the home cage during all time intervals (Fazzari et al. 2018) using a high-resolution Waterproof Action Camera (DBPOWER- SJ4000 SPORTS HD DV). Data were analyzed with a specific software EthoLog (version 2.2.5; Visual Basic, São Paulo, Brazil), according to previous behavioral studies (Zizza et al. 2017, 2018).

EPM

This apparatus allowed us to evaluate anxiety-like responses in HFD hamsters. It is a wooden maze consisting of two open arms (50×10 cm) and two enclosed arms of the same size, arranged so that identical arms were opposite to each other. The arms emerged from a central platform (10×10 cm), and the entire apparatus was raised 50 cm above the floor on four metal legs. White lights illuminated the arena, and the animals were released into the center of the platform facing one of the open arms and allowed to explore it for 5 min. Entry into an arm was defined as the process of entering the arm using all four legs. Time spent in open and closed arms and number of entries were recorded using the same camera as above. The specific software program Etholog 2.2.5 was used to determine time spent by the animal in each arm or in the platform according to indications of previous studies (Alò et al. 2017). Between each trial, the apparatus was cleaned with 70% ethanol to prevent odor stimuli that could interfere with behavioral performances.

NOR test

HFD hamsters ± DZ were subjected to a novel object recognition (NOR) test to define mnemonic abilities to explore novel experiences with respect to CTRL (Fazzari et al. 2018). Behavioral tests were executed in an open-field arena with transparent plexiglass walls for our rodent model ($50\times50\times30$ cm). The objects consisted of hard plastic blocks differing in size, shape, and color (van Goethem et al. 2012). Object number 1 was a white cube containing a yellow spot on the top, a red spot on the front- and back-side, while the lateral sides were white. Object number 2 was instead a larger triangular-shaped structure with the front- and back- sides containing a red circle. They were heavy enough so animals could not move them. During NOR test, the rodents exhibited natural exploratory maneuvers preferentially toward novel objects rather than familiar ones according to the previously outlined phases plus modifications (Avolio et al. 2019; Müller et al. 2015):

Habituation: Animals, after being handled for 2 min, were placed three times for two consecutive days (10 min period/session) into the empty arena without objects for exploration purposes plus reduction of stress and thus avoiding neophobia responses.

Training: This phase was carried out immediately after the third habituation session, in which hamsters were placed in the open-field arena with its head positioned opposite to the two identical objects and allowed to explore them for 5 min.

Test: One hour after training session, all the animals underwent a testing session (5 min interval), in which preference for familiar versus novel object (encountered for the first time) was assessed. For our study, position of the objects was randomized for each session in order to avoid spatial biases during exploration. Between each trial, objects were removed and cleaned along with the arena using 70% ethanol to prevent odor stimuli that could interfere with behavioral performances. All observations were recorded and analyzed as above.

For this study, exploration was defined as a "direct contact" of either the animal's mouth or nose at a distance < 2 cm with the objects. Any other movement around the object like climbing over or sitting was not considered an exploratory

behavior (Müller et al. 2015). Recognition ability was evaluated as a discrimination index (DI) according to the following relationships:

Index values may range from 1 (exploration of novel object) to − 1 (exploration of familiar object). A positive DI value referred to good mnemonic responses, whereas a DI value equivalent to 0 or negative value indicated poor or lack of cognition (Müller et al. 2015).

Protein Extraction and Western Blotting Analysis

After the last behavioral session, the above hamster groups were sacrificed, brains of HFD (baseline, $n = 4$), HFD 15d ($n = 6$), HFD 30 days ($n = 6$), HFD + DZ 15 days ($n = 8$), HFD + DZ 30 days ($n = 7$), and CTRL ($n = 8$) were removed. AMY, HIP, and HTH were dissected out and stored at − 80 °C. Frozen tissues were disrupted by ice-cold homogenization buffer (20 mM Tris–HCl, pH 7.6, 15 mM Triton X-100, 10% glycerol, 2 mM EDTA) containing a cocktail of protease inhibitors for 10 min at 4 °C. After 1 h incubation on ice, the supernatants were collected by centrifugation at 4 °C (13,000 rpm, 20 min). Protein content was determined by Bio-Rad protein assay (Bio-Rad Laboratories). Tissue extracts (40 µg) were added to 8 µl loading buffer and denatured for 7 min at 95 °C. Pre-stained protein molecular weight markers (Thermo Scientific) and tissue extracts were separated by SDS-PAGE using 10% polyacrylamide gel at 100 V for 1.5–2 h. Proteins were transferred to a nitrocellulose membrane and blocked with either 5% bovine serum albumin or non-fat milk. Nitrocellulose membranes were incubated overnight at 4 °C using the following antibodies: rabbit polyclonal anti-NTR1 (H-130; sc-15311, 1:500, Santa Cruz Biotechnology) rat monoclonal anti-IL-10 (JES5-2A5; ab33471, 1:500, Abcam), and mouse monoclonal anti-beta Actin (mAbcam 8226; ab8226, 1:1000, Abcam). Blots were incubated with appropriate horseradish peroxidase (HRP) conjugated goat anti-rabbit (P044801-2, Dako), goat anti-rat (GTX77339; GeneTex), or goat anti-mouse (P044701-2, Dako) secondary antibody and developed using enhanced chemiluminescence detection system (Bio-Rad Laboratories). Optical density (O.D.) was assessed with the NIH ImageJ software. Protein expression was normalized to the beta Actin protein.

Blood Samples Analyses

At the same time, blood samples were collected from the above hamsters with heparinized tubes and immediately centrifuged at 2000 rpm for 10 min. The serum was then decanted and stored at 4 °C. Serum levels of the following molecular parameters: triglycerides, total cholesterol, and glucose were measured by using enzymatic colorimetric methods (CHOD-PAP; GPO-PAP; GOD POD) following the manufacturer's protocol (Biogramma srl; Biotecnica Instruments, Rome, Italy). This part and the evaluation of body weight, liver, and abdominal fat were used to correlate the values of such parameters to the effects of DZ on metabolic disorders of obese hamsters.

Neurodegenerative Analysis

The fact that behavioral alterations of HFD hamsters ± DZ may be related to neurodegenerative events was verified by applying the amino cupric silver stain (ACS) method. Such a selective technique, largely used for the detection of both necrotic and apoptotic processes, provided early and semi-acute neurodegeneration morphological indications consisting of advanced damaged cell bodies, dendrites, axons, and terminals together with the recruitment of new structures in progressive pathologies (Mele et al. 2015). It is based on the formation of silver precipitated granules (argyrophilic reaction) in damaged neuronal fields (Zizza et al. 2017). For this part, coronal sections (30 µm) of various brain areas namely of HTH, HIP, and AMY for HFD hamsters ($n = 2$), 30 days HFD + DZ hamsters ($n = 3$), and CTRLs ($n = 3$) were selected at an interval of 240 µm (3 slides/subgroup) for ACS procedures (Alò et al. 2017). Afterward, stained sections were analyzed at a bright-field Dialux EB 20 microscope (Leitz, Stuttgart, Germany) according to a previous study (Zizza et al. 2018).

Statistical Analysis

All behavioral and molecular biochemical results of HFD hamsters ± DZ were compared with CTRLs (*) and to HFD (letters) using ANOVA followed by post hoc Newman-Keuls multiple range test when $p < 0.05$. $^{*,a}p < 0.05$; $^{**,b}p < 0.01$; $^{***,c}p < 0.001$.

Results

DZ Effects on Feeding/Home-Cage Performances

HFD hamsters treated with the phytoestrogen DZ exhibited notable feeding and motor performances. Regardless of the elevated quantity of food consumed, this isoflavone accounted for a moderate reduction [$F_{(7,50)} = 2.17$; $p < 0.05$] of body weight (-37%) at the end of treatment with respect to only HFD animals (Fig. 1a). Interestingly enough, the significantly evident HFD-dependent reduced exploration

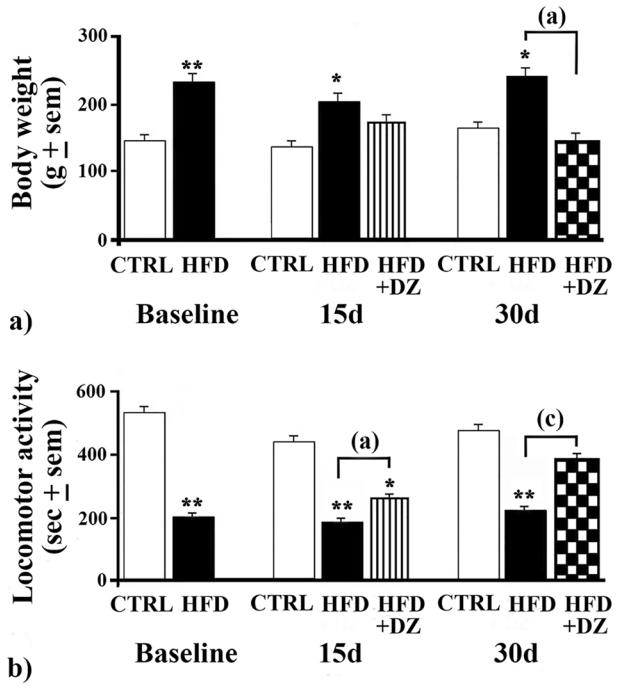

Fig. 1 DZ effects on **a)** body weight and **b)** locomotor activity in HFD hamsters. Changes in baseline body weight of HFD ($n=6$) and CTRL ($n=5$) were compared with that of other HFD hamsters 15 ($n=6$) plus 30 days ($n=6$) along with HFD + DZ 15 days ($n=10$) and 30 days ($n=10$) with respect to CTRLs [CTRL ± DZ 15 days ($n=8$); CTRL ± DZ 30 days ($n=8$)]. Time (s) engaged for locomotor activities in the cage were evaluated in HFD hamsters ± DZ with respect to both CTRL (*) and to HFD alone (letters). The values (mean ± sem) of all differences were determined by ANOVA plus a post hoc Newman-Keuls test when $p<0.05$. *·$^{a}p<0.05$; **$p<0.01$; $^{c}p<0.001$

activities appeared to be inverted following the addition of DZ to animals during permanence in their home-cage. In this case, motor behaviors, such as rearing, and spontaneous movements, were extremely numerous (+ 90%; $p<0.001$) after 30 days of treatment with respect to animals treated with HFD while the shorter treatment period (15 days) was only responsible for a moderate increase (+ 30%) of movements (Fig. 1b).

Anxiolytic-like Behaviors Induced by DZ

Assessment of anxiety during animal performances in EPM allowed us to evaluate beneficial effects of DZ [$F_{(2,21)}=3.45$ $p<0.05$] as indicated by HFD + DZ animals exhibiting a moderate reduction of time (− 35%) spent in closed arms following 15 days of treatment with respect to HFD animals (Fig. 2a). A moderate enhancement of the number of entries (+ 47%) in EPM arms seemed to underlie the ability of this isoflavone to increase explorative activities (Fig. 2b), especially as shown by the more pronounced DZ effects [$F_{(2,21)}=3.41$; $p<0.05$] after 30 days of treatment with respect to HFD animals. In this case, HFD + DZ hamsters exhibited a notably evident ($p<0.01$) increase and decrease of permanence in open (+ 70%) and closed (− 65%) arms, respectively, when compared with HFD animals (Fig. 2c). Such a trend proved to be of an extremely ($p<0.001$) greater entity when DZ-dependent greater anxiolytic-like responses in HFD hamsters during the entries in the arms of the maze (+ 140%) were compared, this time to HFD group (Fig. 2d).

DZ Effects on Cognitive Alterations Induced by HFD

A protective role of this isoflavone was also detected as early as 15 days of treatment on mnemonic performances using NOR test [$F_{(5,42)}=3.47$; $p<0.01$] as indicated by a longer exploration time together with a better ability to recognize the new objects (Fig. 3a). In particular HFD + DZ hamsters displayed an extremely evident increase in DI (+ 95%; $p<0.001$) with respect to HFD group. In the case of the 30-day treatment session, the protective role of DZ still continued to be of an extremely evident nature, despite the numerically greater value (+ 255%) when DI value HFD + DZ was compared with that of HFD hamsters (Fig. 3b).

DZ Effects on Blood Parameters Plus Body and Tissue Weight

From the evaluations of blood parameters, it appeared that DZ was able to reduce the notably altered lipid profile in hamsters fed with HFD for 12 weeks with respect to CTRL. In particular, the protective effects [$F_{(5,36)}=2.49$; $p<0.05$] of this phytoestrogen seemed to be responsible for moderate reductions of triglycerides (− 47%; Fig. 4a) and cholesterol (− 35%; Fig. 4b) as early as 15 days that became more consistent (− 61%; − 43%, respectively) at 30 days when compared with HFD hamsters (Fig. 4a, b). Even for glucose levels, DZ lowered this trend for both periods (− 36%; − 49%, respectively; Fig. 4c). The extremely elevated abdominal fat (+ 280%) in HFD hamsters with respect to CTRL was moderately diminished at 15 days (− 42%) following treatment with DZ while it was extremely reduced (− 94%) after the 30-day treatment session when compared with HFD animals (Fig. 4d). Conversely, the notably elevated liver weight (+ 60%; $p<0.01$) of HFD with respect to CTRL remained more or less constant even after DZ treatment (Fig. 4d, e).

Neurodegeneration Analyses

The altered behaviors evoked by HFD seemed to be strongly correlated to the differentiated degenerative responses detected in the various HTH, HIP, and AMY neuronal fields. Application of ACS approaches supplied an elevated

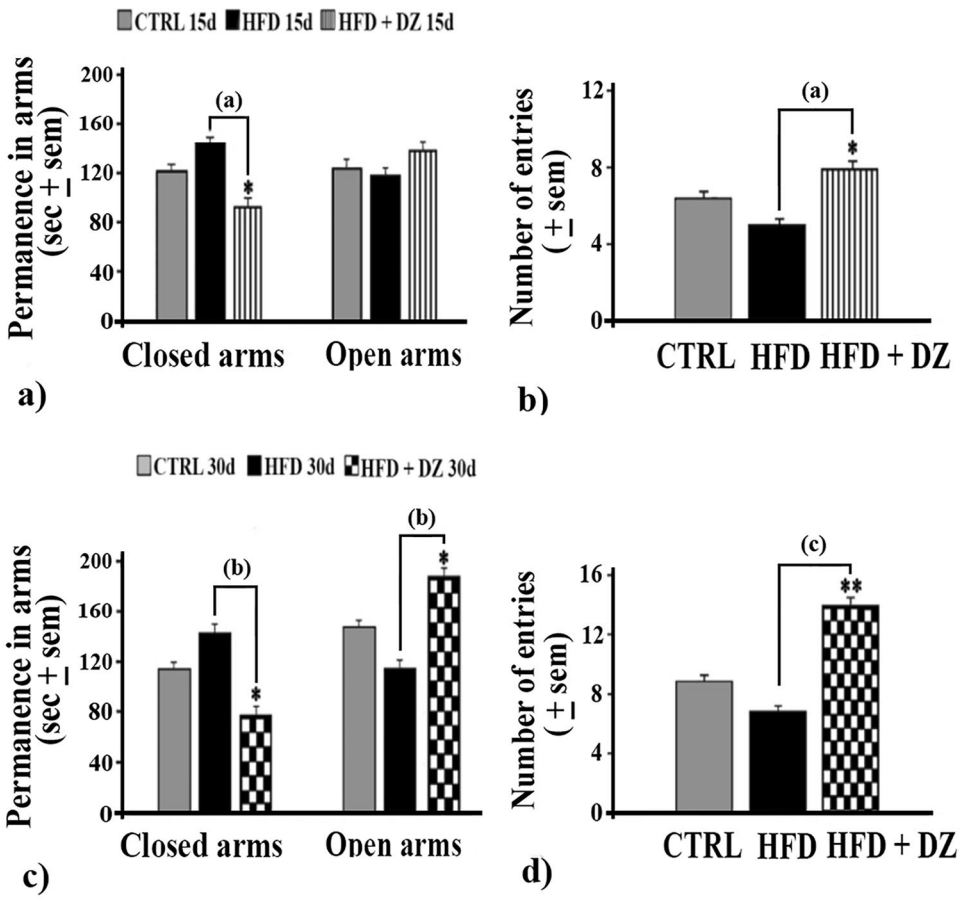

Fig. 2 Anxiolytic-like responses of DZ. Behavioral actions of DZ on (**a**, **c**) permanence (sec ± sem) in open and closed arms together with (**b**, **d**) number of entries (± sem) in EPM after 15 (**a**, **b**) and 30 days (**c**, **d**) of treatment. Mean time spent in two arms of EPM with respect to total duration of the test (300 s) plus average value of total entries of the same above hamsters—HFD 15 days ($n=6$), HFD + DZ 15 days ($n=10$), HFD 30 days ($n=6$), HFD + DZ 30 days ($n=10$), with respect to CTRL [*, CTRL 15 days ($n=8$), CTRL 30 days ($n=8$)] and to group exposed to HFD ($n=6$, baseline) alone (letters) were determined using ANOVA plus post hoc Newman-Keuls test when $p<0.05$. $^{*,a}p<0.05$; $^{**,b}p<0.01$; $^{***,c}p<0.001$. For this analysis, values of CTRL group for both HFD and HFD + DZ were very similar and so to avoid confusion, only CTRL + DZ (indicated as CTRL) was used for comparison purposes

argyrophilic reaction in the various neuronal fields of all limbic areas in HFD animals. Indeed, the numerous damaged fields in HTH (Fig. 5a (ii)), and to a less extend in HIP (Fig. 5b (ii)) plus AMY (Fig. 5c (ii)) were typical of obese hamsters with respect to their CTRLs (Fig. 5a (i), b (i), c (i)). Conversely, HFD hamsters that received DZ for 30 days displayed few scattered, if any, ACS granules in HTH (Fig. 5a (iii)) comparable with those of CTRLs (Fig. 5a (i)). This trend was also observed for the other two brain areas as indicated by an elevated number of degenerated neuronal fields in HIP and AMY that exhibited, in a similar manner as the HTH neuronal fields, few if any dense dark granules (Fig. 5b (iii) and 5c (iii), respectively).

DZ-Induced NTR1 Expression Changes

A protective role of the isoflavone on mnemonic abilities appeared to be further related to reduced expression levels of NTR1 [$F_{(3,22)}=4.85$; $p<0.01$] in AMY, HIP, and HTH at both 15 days and 30 days of DZ treatment. The moderate and notable reductions of this receptor was detected in HTH at both 15 days (− 44%; $p<0.05$) and 30 days (− 72%; $p<0.01$), respectively, of HFD + DZ hamsters as compared with CTRLs (Fig. 6a). However, such a relationship became somewhat more evident when the levels of this neuropeptide in HFD + DZ hamsters were compared with that of HFD group as shown by a moderate decrease at 15 days (− 53%), which became extremely greater at 30 days (− 93%; $p<0.001$). Similarly for HIP, in which a moderate reduction of NTR1 levels at 15 days (− 49%) resulted to be extremely reduced (− 79%) at 30 days of treatment with respect to CTRLs. Even for this brain area, such a trend resulted to be still greater when its NTR1 levels were compared with HFD as indicated by notably lower levels at 15 days (− 74%; $p<0.01$) while extremely diminished levels (− 110%) were featured at 30 days of treatment (Fig. 6b). As for AMY, DZ did not improve the diminishing trend as pointed out by a moderate reduction (− 45%) at 15 days while an extremely evident reduction was observed at 30 days (− 81%) even when compared with HFD hamsters (Fig. 6c).

DZ-Modified Expression of Il-10

Interestingly, the neurodegeneration events typical of obesity and the notable inflammatory actions characterizing this metabolic disorder coincided with elevated levels [$F_{(3,22)}=3.07$; $p<0.05$] of the anti-inflammatory factor IL-10 in all limbic areas. Indeed, low expression levels of this factor

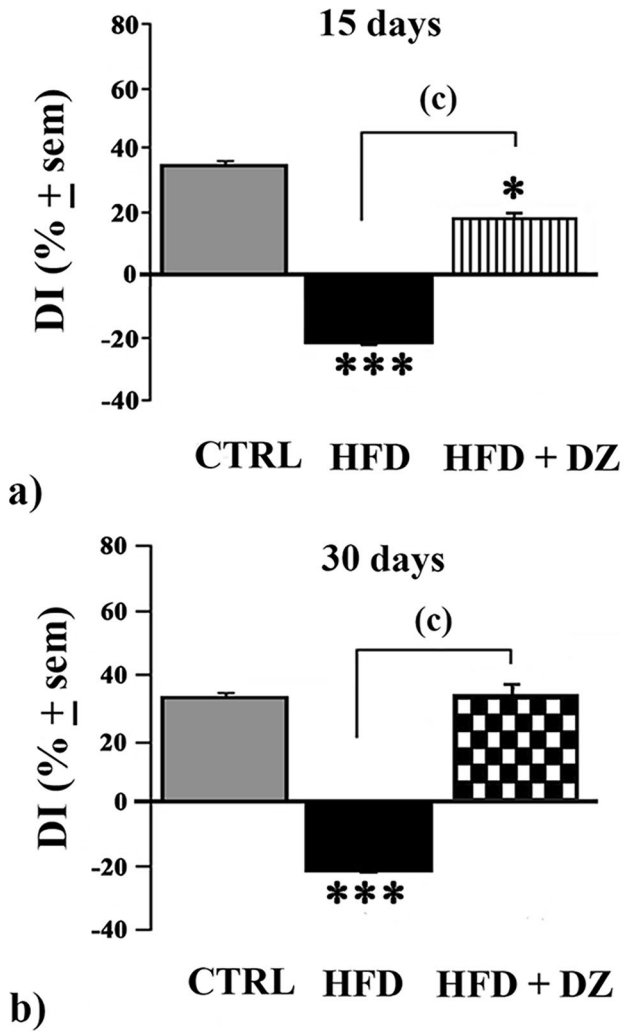

Fig. 3 DZ modified NOR test responses in HFD hamsters. Discrimination index (DI; %) was calculated as follows: time spent exploring novel object − time spent exploring familiar object / total exploration time of both objects in above HFD ± DZ hamsters with respect to CTRL (*) and to hamsters exposed to HFD alone (letters). Data were expressed as mean ± sem, and changes were estimated by ANOVA plus a post hoc Newman-Keuls test when $p < 0.05$; $^{*}p < 0.05$; $^{***,c}p < 0.001$

were detected in HTH of HFD hamsters with respect to CTRLs (Fig. 7a) after NOR test. Conversely, when these hamsters were treated with DZ, a notable percentage increase of IL-10 was reported for both treatment periods as indicated by moderate expression levels at 15 days (+51%) and extremely evident expression levels 30 days (+125%) when compared with CTRL. The expression levels for this area turned out to be somewhat greater at 15 days (+69%) but extremely greater at 30 days (+209%) when their levels were compared with HFD group. A similar situation was also reported for HIP in which the moderately reduced (− 37%) IL-10 levels in HFD group, after NOR test with respect to CTRL (Fig. 7b), were completely reversed in HFD + DZ-treated hamsters (Fig. 7b) as shown by moderate increases of IL-10 levels at 15 days (+40%) and extremely evident greater levels at 30 days (+91%) with respect to CTRLs. In a comparable fashion, the increased expression levels in HIP resulted to be extremely greater when compared with HFD-treated hamsters for both 15 days (+87%) and 30 days (+225%) of treatment. As for AMY, significant differences were only reported at 30 days as indicated by a moderate (+55%) increase of IL-10 expression level with respect to its CTRL while an extremely evident (+121%) increase was detected when compared with HFD animals (Fig. 7c).

Discussion

These first indications corroborate a consistent protective effect of DZ on our rodent model (*Mesocricetus auratus*) fed with HFD suggesting it to be a safe natural agent for the treatment of severe metabolic dysfunctions and at the same time improving anxiety plus mnemonic-cognitive alterations in obese individuals. It was worthy to note that while HFD hamsters showed an evident increase of body weight, this physiological disorder was restored, especially after 30 days following treatment with DZ as shown by a reduced body weight similarly to that obtained in mice treated with DZ (Luo et al. 2018). Such an effect appears to derive from the interaction of this isoflavone (or its metabolites) with estrogen receptors (ERs) in critical brain feeding areas like HTH since the specific deletion of the steroid site induces, aside from promoting a feeding stimulus, a greater energy demand and consequently an increase of body weight that is typical of anabolic states (Musatov et al. 2007; Xu et al. 2015; Zeng et al. 2010). The protective action of this phytoestrogen on body weight may also be related to the attenuated blood lipid and glycemic levels especially after 30 d of treatment, which is in accordance with diminished cholesterol levels in rats exposed to the same isoflavone (Bhattarai et al. 2017). Likewise, with this effect, the agonistic influences of isoflavones on ER receptors could also be responsible for the profound reduction of the altered lipoprotein profile as previously observed in HFD mice (Guo et al. 2009). Contextually, the hypoglycemic action exerted by DZ appears to be tightly linked to the suppression of anti-inflammatory factors that are responsible for the lowering of glucose-dependent oxidative states (Park et al. 2016).

As for the behavioral role of this phytoestrogen, it seemed to coincide with the successful recovery of the poor locomotor performances of HFD hamsters during the entire treatment period. This improvement was especially evident at 30 days of treatment, as pointed out by increased spontaneous locomotor activities in a comparable manner with the elevated open field and plus-maze performances

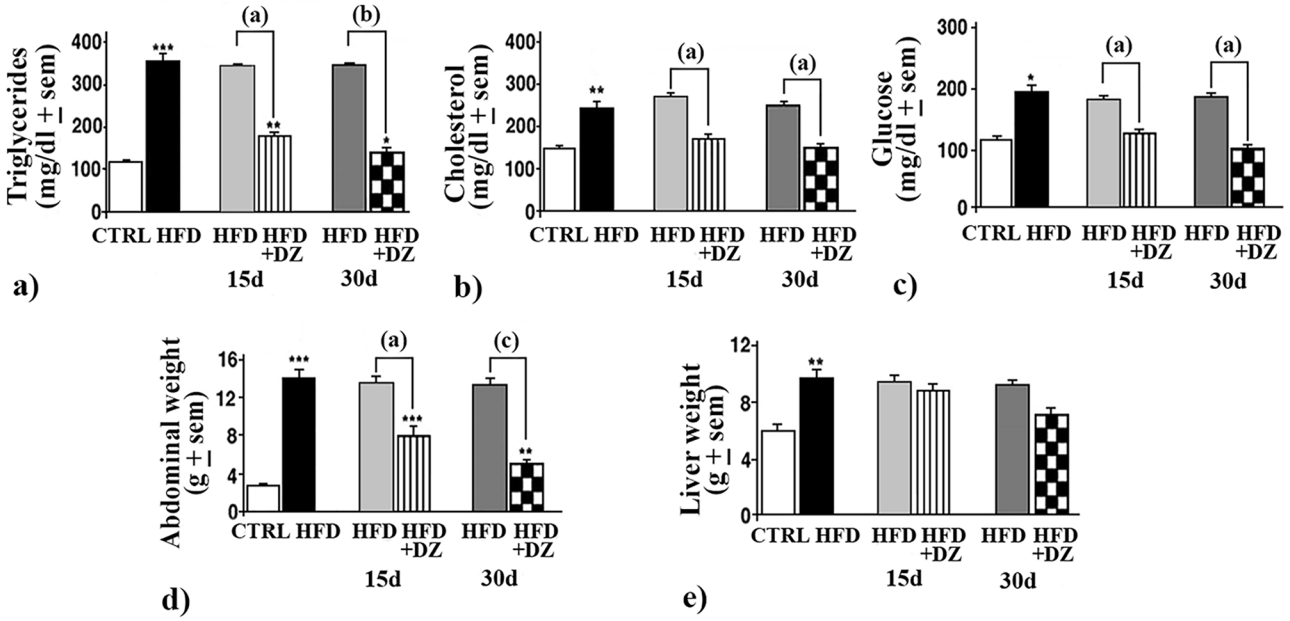

Fig. 4 Effects of DZ on lipid and glucose levels as well as on abdominal fat and liver weight. **a)** Plasma triglycerides, **b)** cholesterol, **c)** glucose levels (mg/dl ± sem) along with **d)** abdominal fat and **e)** liver weight (g) were determined in HFD 15 days ($n=6$), HFD 30 days ($n=6$), HFD + DZ 15 days ($n=8$), and HFD + DZ 30 days ($n=7$) with respect to both CTRL (*, $n=7$) and to HFD hamsters alone (baseline, letters; $n=6$) using the same statistical indications reported in Fig. 2

exhibited by mice following treatment with DZ (Zeng et al. 2010). The recovery ability of the isoflavone seems to be in line with the effects of other phytoestrogens (genistein and Puerarin), which via their synergic interactions with neuronal signaling mechanisms of key encephalic-related motor centers like HIP were able to invert the abnormal Morris water maze, EPM, and locomotor activities into normally smoother plus elevated and longer mobility performances (Tao et al. 2017; Pierzynowska et al. 2019). In the case of our experimental model, DZ tended to re-establish HFD hamster's reactivity to EPM as early as 15 days of treatment thus causing animals to spend less time in closed arms plus showing a consistent increase in number of total entries in all arms. HFD animals treated with this phytoestrogen for a longer period evoked anxiolytic-like effects as pointed out by a longer time period in open arms along with a higher number of exploration intervals. This relationship fits well with that of the early exposure to genistein promoting anxiolytic-like effects while reducing depressive states as suggested by stressed hamsters executing climbing, swimming, and overall exploratory behaviors (Alò et al. 2019; Le Moëne et al. 2019). Additionally, the predominant DZ-dependent anxiolytic effect tends to be in line with the interaction of this phytoestrogen with ERβ site (anxiolytic) rather than ERα, as indicated by a prevalence of anxiolytic and antidepressant states in mice featuring elevated encephalic ERβ levels (Sharma and Thakur 2015) thus favoring considerable exploratory performances in open field tests (Gleason et al. 2015; Mosquera et al. 2014). As for mnemonic performances, HFD hamsters treated with DZ exhibited a greater and rapid capacity to recognize the novel object during NOR test thus supporting this phytoestrogen's ability to invert cognitive deficits (Matias et al. 2016; Neese et al. 2014).

The altered physical-behavioral functions appeared to be tightly linked to neuronal damages induced by HFD in the limbic areas as indicated by the notable accumulations of dark dense granules obtained in neurodegenerative argentophilic ACS reactions. These obesogenic-dependent effects were readily reversed after 30 days of treatment with DZ in a similar fashion to its restoration of pro-neurogenic factors such as BEX2 and tyrosine hydroxylase, which actively prevent neuronal cell death (Li et al. 2017). It may very well be that DZ by dampening the activation of microglial elements and release of pro-inflammatory factors ROS, p38 MPAK phosphorylation and NF-KB activation tends to promote anti-apoptotic events (Chinta et al. 2013; He et al. 2018). Indeed, elevated expression levels of IL-10 with respect to the low levels in HFD hamsters strengthens the beneficial cognitive activities of DZ (Carlson et al. 2019; Zhang et al. 2018), due most likely to this cytokine promoting anti-obesity and anti-inflammatory effects (Toita et al. 2016). It may very well be that the inhibition of Jun N-terminal kinase phosphorylation, which by reducing the levels of soluble pro-inflammatory factors (Sakamoto et al. 2016) is accounting for altered mnemonic and motor performances (Zhang et al. 2018). A greater protective role of DZ was

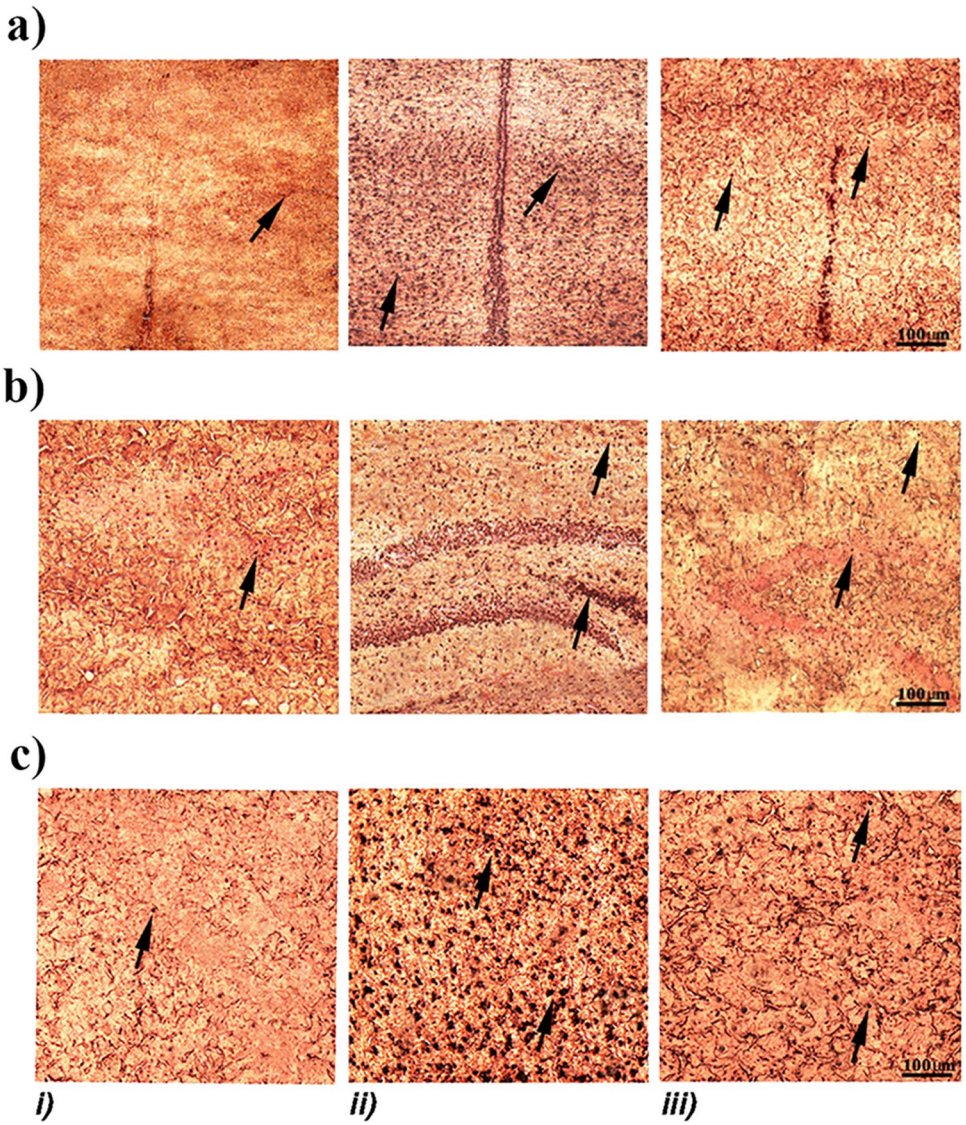

Fig. 5 Actions of DZ on HFD-linked neurodegeneration in areas such as the **a)** hypothalamus (HTH), **b)** hippocampus (HIP) and **c)** amygdala (AMY). The argentophilic reaction of amino cupric silver staining (ACS) provided a heterogeneous distribution of granules, in which strongly marked fields () were detected in the various above brain areas of animals treated with (ii) HFD ($n=2$) for 12 weeks with respect to (i) CTRL ($n=3$) while few damaged neuronal fields () were observed in the different brain sections of (iii) 30 days HFD + DZ ($n=3$) animals

also largely supported by the integration of soy isoflavones (containing mainly DZ and its metabolite daidzin) being able to modify anti-inflammatory gene expression profiles in adipose tissues of postmenopausal women (Van der Velpen et al. 2014). In this context, the main role played by the elevated levels of DZ-dependent IL-10 changes appear to be tightly linked to reduced apoptosis through diminished oxidative stressful conditions, which largely favor pro-neurogenic events (Meng et al. 2017).

Elevated HFD consumption appeared to further coincide with an increased encephalic NT signaling event like that obtained in another study (Fazzari et al. 2018) plus in Wister obese rats (Saiyasit et al. 2020), which was inverted by DZ as indicated by decreased NTR1 expression levels in the same above limbic areas. Surprisingly enough, an upregulation of this receptor was reported in HIP of HFD animals after the NOR test, which appear to go in the same direction of studies highlighting an inhibitory role of HFD on episodic and spatial memory tasks due to upregulated NTR1 levels (Vadnie et al. 2014; White et al. 2012). It is tempting to speculate that reduced levels of this anorectic receptor by DZ, which are linked to the activation of ERβ site, may be promoting increased open field exploring activities (Mosquera et al. 2014), induction of anxiolytic state (Sharma et al. 2015), and restoration of memory deficits (Bastos et al. 2015). As a result, DZ-linked upregulation of ERβ sites (Musatov et al. 2007; Xu et al. 2015) constitute a likely element restoring memory abilities via their binding with DZ, which in turn by reducing the expression of NTR1 tend to favor enhanced neurogenic processes of key cognitive brain areas like HIP (Bastos et al. 2015; Yamada et al. 2016). This aspect is supported by structural affinities of isoflavones and estrogens favoring the participation of its preferential site (ERβ) regulating the expression of protein

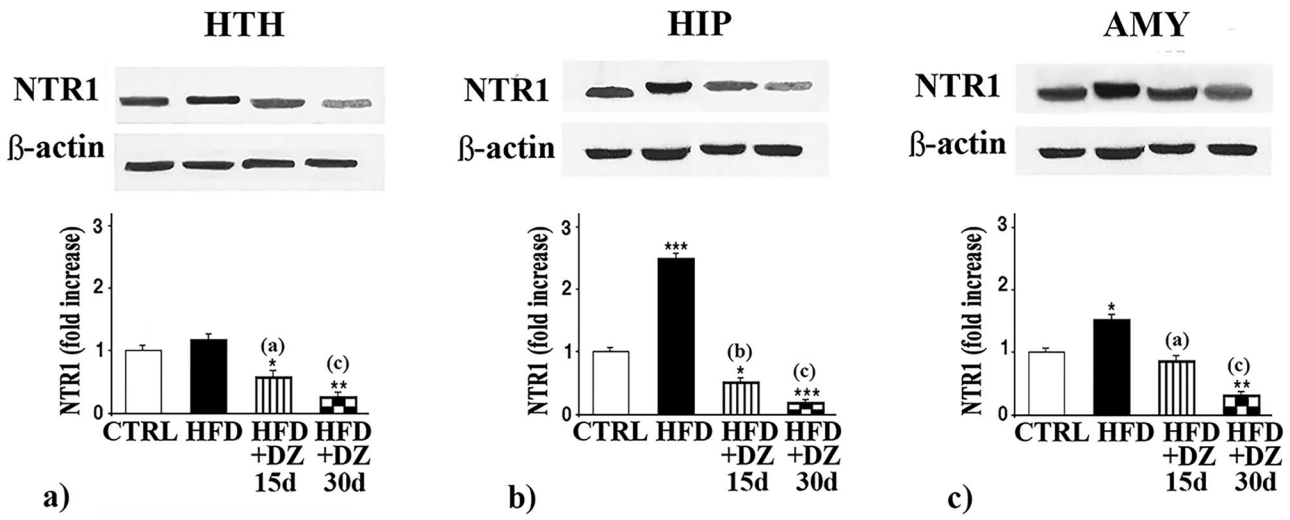

Fig. 6 Changes of NTR1 (Fold increase) levels. The role of DZ on NTR1 expression levels was evaluated in **a)** HTH, **b)** HIP, and **c)** AMY neuronal fields of HFD animals after NOR test. Data were expressed as mean optical density (O.D.) and differences in HFD + DZ 15 ($n = 8$) and 30 days ($n = 7$) were compared with CTRL [* ($n = 7$)] and to HFD alone [letters, baseline ($n = 4$)], using the same statistical indications reported in Fig. 2

kinase–dependent NTR1 levels (Cheong et al. 2014) thereby promoting cross-talking neuronal events (Martini et al. 2019). Hence, it should not amaze us if administration of diets based on DZ or genistein is becoming promising therapeutic agents that improve cognitive and motor activities above all for dementia syndromes (Kobilo et al. 2014) through diminished NTR1 levels (Xiao et al. 2014).

Overall, these first data support the recovery role of DZ on HFD-induced neurobehavioral changes and above all on its neuroprotective effects toward inflammation plus neurodegeneration features of obesity conditions. As for behavioral effects, this phytoestrogen restored, aside from the different morphological features of HFD animals (body, abdominal and liver weight), the altered locomotor activities associated with anxiety conditions. Furthermore, mnemonic deficits observed in the NOR test were also strongly recovered following diets combined with DZ, which by strongly reducing anxiety-like episode improved explorative behaviors toward new environments and objects (Khodamoradi et al. 2017). From the differentiated transcriptional activities of IL-10 and

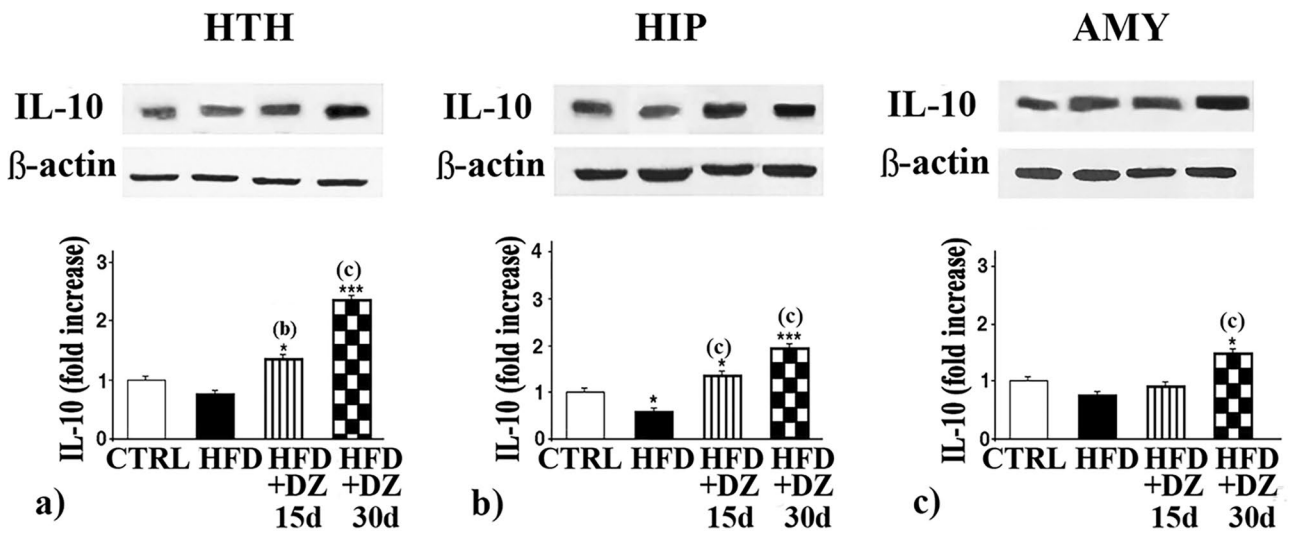

Fig. 7 Changes of IL-10 (Fold increase) levels. The role of DZ on IL-10 levels was evaluated in **a)** HTH, **b)** HIP, and **c)** AMY neuronal fields of the same HFD animals after NOR test. The data was handled in the same manner as in Fig. 6

NTR1 being associated with the recovery of DZ-dependent behavior, it is possible that more than one pathway, in an estrogen-dependent manner, modulates both the effects of HFD and the recovery role of DZ in discrete brain areas controlling not only anxiogenic activities but also mnemonic performances (Kim et al. 2020; Yang et al. 2020). These results, highlighting novel molecular mechanisms that coincide with DZ-related neuroprotective responses, tend to point to the synergic interaction of IL-10 and NTR1 as alternative therapeutic targets for the treatment of obesity states.

Conflict of Interest

The authors declare that that there are no conflicts of interest.

Disclaimer

Study sponsors had no involvement in collection, analysis and interpretation of data or writing of the manuscript.

Authors' Contributions All the authors discussed the results and commented and approved the final manuscript. G. Fazzari and M. Canonaco arranged and designed the experiments, developed and performed the behavioral tests, and wrote and edited the manuscript. M. Zizza and E. Avolio handled the behavioral tests as well as ACS. A. Di Vito, G. Cuda, and T. Barni handled the western blotting evaluations, while R. Bruno estimated the serum molecular parameters. R. Alò and R.M. Facciolo performed the statistical analysis and contributed with the editing of the manuscript.

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