Clusterin overexpression as a potential neuroprotective response to the pathological effects of high fat dieting on the brain reward system

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Abstract

High-fat diets (HFDs) can lead to pathological changes in the brain underlying several behavioral disturbances (e.g., reward deficiency). To further increase our knowledge of these associations, we studied the sucrose reward and the brain expression of clusterin, a protein that is overexpressed after several kind of brain damaging conditions. C57BL/6J male mice were differentially fed on an HFD or standard chow for 41 days and underwent 11 sucrose place conditioning sessions followed by 4 extinction sessions to monitor the effects of HFD on sucrose reward by means of free choice tests. We quantified clusterin expression by immunochemistry in the nucleus accumbens, dorsal striatum and cingulate cortex. HFD tended to provoke a transient potentiation in the acquisition of sucrose-conditioned place preference, but this effect was followed by a much more consistent reduction in sucrose preference, which spontaneously disappeared after 31 days of an HFD with no need for extinction learning. The HFD mice showed higher clusterin expression in the nucleus accumbens but not in the other brain areas studied. The results confirm that HFDs strongly influence the rewarding properties of palatable foods and suggest a direct connection with neurotoxic alterations in the brain reward system tagged by clusterin overexpression.

Key words: sucrose reward, high-fat diet, clusterin, nucleus accumbens

1. Introduction

Numerous studies have shown that low-quality diets can lead to brain dysfunction in humans, with subsequent potentiation of cognitive impairment, anxiety and affective disorders, among other neuropsychiatric abnormalities (see for instance the reviews by Beilharz et a., 2015 and Baker et al., 2017). The effects of different types of diets on human cognition have been broadly studied, and significant associations have been reported between cognitive dysfunction and a high intake of fat, refined sugar, Western-style food, saturated fatty acids and carbohydrates (particularly simple sugars) (Beilharz et al., 2015). These effects are not necessarily related to the diets' obesogenic properties. A single meal with a high glycemic load can impair memory performance in children and healthy young adults (Nabb and Benton, 2006; Micha et al., 2011), and only 5 days on a high-fat diet (HFD) is enough to cause detectably impaired attention, lower retrieval speeds and depressed moods among young adult men (Holloway et al., 2011).

Extensive research has been conducted using animal models to discover the neurochemical mechanisms underlying diet-induced brain dysfunction. Studies that have focused on HFDs have shown brain function impairment either by the direct influence of the diets' components on brain neurochemistry or indirectly by triggering obesity and type 2 diabetes. HFD-induced neuroinflammation was first reported in rodents by De Souza et al. (2005), who showed that rats on an HFD for 16 weeks exhibited higher concentrations of proinflammatory markers in the hypothalamus as well as insulin resistance. Chronic hypothalamic inflammation also contributes to leptin resistance associated with increased tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 levels (Howard and Flier, 2006). In addition to neuroinflammation and leptin/insulin insensitivity, a recent study has shown that the brains of C57BL6 mice fed an HFD for 12 weeks experience marked oxidative stress (increased oxidized glutathione/total glutathione ratio and decreased superoxide dismutase levels) and increased lipid peroxidation (higher malondialdehyde levels) and exhibited mitochondrial dysfunction (higher levels of reactive oxygen species and decreased

adenosine triphosphate content) (Ha et al., 2020). The biological accumulation of compounds abundant in HFDs, such as ceramides, diacylglycerols and saturated fatty acids, can cause direct damage to the brain as happens in the periphery, probably by triggering inflammation, oxidative stress, endoplasmic reticulum stress and disruption of essential metal homeostasis (Pewzner-Jung et al., 2010; Ginkel et al., 2012; Chakraborty and Jiang, 2013; Belegri et al., 2017; Paraiso et al., 2020; Mazzocco et al., 2020). The effects of saturated fatty acids appear to be mediated by the activation of toll-like receptors in microglia, which leads to the accumulation of proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6) and free radicals (e.g., nitric oxide) (Ransohoff and Brown, 2012; Maldonado-Ruiz et al., 2017). Palmitic acid in particular induces the release of cytokines from microglia via a toll-like receptor 4-initiated signaling pathway, which results in significant hypothalamic neurotoxicity as shown by decreased cell viability (Delint-Ramirez et al., 2015). Recently, the multifunctional protein clusterin has emerged as a putative biomarker of neurotoxicity associated to several types of noxious conditions such as ischemia (May et al., 1992), excitotoxicity (Park et al., 2007), cellular stress associated with amyotrophic lateral sclerosis (Zinkie et al., 2013), trauma (Huang et al., 2016), β-amyloid deposition and increased tau protein (Boggs et al., 1996; Shepherd et al., 2020). Up to our knowledge the effects of HFD on brain clusterin have not been explored, even though they could run in parallel with diet-induced pathological changes of brain function. The exact biological significance of changes in clusterin in the brain remains unclear; however, a number of authors have suggested that this protein protects neurons against cytotoxicity (Gregory et al., 2017). Beyond neurochemical alterations, HFD can also affect microglial and neuronal morphology, and thus influences the length of neurites and the number or type of dendritic spines (Bocarsly et al., 2015; Del Olmo and Ruiz Gayo, 2018).

The HFD-induced cognitive and behavioral disturbances depend on the brain area affected; thus, cognitive impairment is mostly related to functional alterations in the hippocampus and prefrontal cortex (Kanoski et al., 2007), whereas hypothalamic dysfunction underlies the disruption of

homeostatic eating (Delint-Ramirez et al., 2015). The brain structures functionally involved in driving behavior towards pleasurable stimuli delineate the brain reward system (Fakhoury, 2021), and numerous researchers have specifically studied the biological effects of HFDs on these neuronal circuits trying to establish a link with the hedonic disturbances involved in obesity and eating disorders. Many of these studies were focused on the nucleus accumbens of the ventral striatum, a key area of the mesolimbic system functionally involved in normal and pathological reward and aversion (Carlezon and Thomas, 2009) which is also the target of the present work. Barry et al. (2018) reported that only 2 weeks of an HFD are needed to induce significant reductions in rat striatal insulin receptor signaling, amphetamine responsiveness, dopamine D_2 receptor availability and mesolimbic/sensorimotor interactions. These findings confirmed and extended previous studies supporting the "reward deficiency hypothesis," which postulates that HFD (and other hypercaloric diets) provoke dopaminergic hypoactivity in the brain reward system, leading to reduced rewards and higher performance in active tasks to obtain palatable food and to compensate for reward hyposensitivity (Davis et al., 2004; Wang et al., 2002). A number of authors have found that reward deficiency is more dependent on the type of fat consumed than on body weight gain. Male rats fed on a saturated HFD (palm oil) for 8–9 weeks exhibited reduced sensitivity to the rewarding and locomotor-sensitizing effects of amphetamine and reduced dopamine D_1 receptor signaling in the nucleus accumbens, an effect that was not observed in the animals fed on an isocaloric monounsaturated HFD and was entirely independent of caloric intake, weight gain and plasma levels of leptin, insulin and glucose (Hryhorczuk et al., 2016). In another experiment, 4–6 weeks on diets high in stearic and palmitic acid increased rat lever pressing for sucrose without changing the levels of fasting glucose, insulin, cholesterol and triglycerides, the body fat percentage or the intravenous glucose tolerance test results (Figewilcz et al., 2018). Similarly, Décaire-Spain et al. (2018) reported that C57BL6 mice fed on a high-fat/high-sucrose diet enriched with saturated fat (palm but no monounsaturated olive oil-derived fat) exhibited compulsive sucrose seeking behavior and multiple

proinflammatory signs in the nucleus accumbens, including reactive gliosis and increased expression of cytokines, antigen-presenting markers and NFκB transcriptional activity. Maldonado-Avilés et al. (2018) suggested that HFDs can induce inflammation of nucleus accumbens cells by downregulating miRNA-155 and miRNA-146a, two modulators of inflammation. Reduced reward sensitivity (assessed by morphine place conditioning) has also been observed in animals exposed to interesterified fat supplements with a high content of saturated fatty acids from gestation to 31 days post-birth (Milanesi et al., 2017).

Despite the consistency of the previously mentioned reports, there are still a number of apparent controversies to resolve. The aforementioned studies tended to rule out the need for body weight gain to observe the HFD-induced reduction of reward; however, Takase et al. (2016) reported that 11 weeks of fat feeding reduced the preference for sucrose in those animals that became obese but not in paired nonobese HFD-fed mice, even when alcohol preference was blunted in both groups. More intriguingly, there is one report of decreased rather than increased operant responding for food in mice fed HFDs for 4 or 8 weeks (Íbias et al., 2016). The heterogeneity of the protocols employed in the previous studies makes it difficult to establish the influence of certain covariables on the effects of HFD on food reward (e.g., exposure time to the diet and age of the animals). In the field of obesity, it is especially important to examine the events that occur in early phases of a HFD, when body weight gain is still marginal, but changes in food rewards appear to drive the animals to seek palatable foods. In this work, we studied the effects of HFD on the acquisition, maintenance, and extinction of sucrose-conditioned place preference to provide a broad view of the effects of HFDs on food reward over 6 weeks, which coincided with the animals' life period equivalent to human adolescence (Campbell and Spear, 1972; Rudy and Morledge, 1994). Sucrose conditioned place preference paradigms enable the assessment of the acquisition and expression of context-reward associations, and thus are suitable to provide quantifications of the rewarding value of food (Patel et al., 2020). On the other hand, it is important to note that adolescents are highly vulnerable to develop

compulsive eating of palatable foods as a result of heightened reward drive and weaker inhibitory control (Kidd and Loxton, 2021), therefore our study could provide valuable data for such a critical period of neurodevelopment. Bearing in mind the previously mentioned neurochemical changes triggered by HFDs in the nucleus accumbens, we also checked the effects of an HFD in this brain area and two other brain regions employed as a reference by quantifying the expression of clusterin as a potential biomarker of neurotoxicity.

2. Material and Methods

2.1. Behavioral procedure

All animal experiments complied with the ARRIVE guidelines, were carried out in accordance European Union Directive 2010/63/EU and were approved by the Ethics Committee of the San Pablo CEU University. C57BL6 male mice aged 4 weeks had free access to water and standard chow (Global 18% Protein Rodent Diet, 18% calories from fat, energy density 3.1 kcal/g; Teklad Diets, Madison, WI, USA) and were maintained under a controlled environment (20–22°C, 12h/12h light/dark cycle). After one week of acclimation, animals were weighted and distributed into two groups with a similar body weight. Mice of the first group continued with free access to chow (CONTROL, n = 9), while those of the second group (n = 8) were shifted to an HFD (D12451 diet, 45% calories from fat, energy density 73 kcal/g; Research Diets, New Brunswick, NJ, USA). Most fat of this diet comes from lard and hence is rich in saturated fatty acids. Differential dieting was maintained throughout the rest of the study.

After two weeks of differential dieting, a behavioral study started involving a first phase of conditioned place preference (CPP) induction by sucrose, and a second phase of extinction of conditioned place preference (EXT). A plexigas box open on top and divided in two compartments of similar size (40 x 35 x 35 cm) separated by a guillotine door was used in both phases; one

compartment was black and dark, and the other was white, brightly illuminated and equipped with a small food tray in one corner to place sucrose pellets during conditioning.

CPP sessions started on day 21 and consisted on daily training with double 30-min place conditioning sessions separated at least 3 hours: in one of them mice were confined in the white compartment of the experimental box with 16 pellets of 64.37% sucrose (1811555-5TUT- Test Diet, Richmond, Indiana, USA) available in the food tray; in the other session, mice were placed in the dark compartment, which was food-unpaired. The order of these sessions was changed in consecutive days to avoid the influence of circadian rhythms. Eleven CPP sessions were conducted on days 21, 22, 23, 27, 28, 29, 30, 34, 35, 36 and 37. The number of pellets consumed during sucrose pairings was recorded. EXT sessions were identical to CPP sessions with the exception that food trays were empty; these sessions were conducted on days 41, 42 and 44.

Six free choice tests were performed along the experiment to monitor sucrose-conditioned place preference by placing the animals in the box with the guillotine door open and free access to the two compartments for 30 min. Animal behavior during these tests was monitored by video imaging and the time spent in the two compartments was recorded. The first free choice test was prior to the conditioning phase (PRE, day 20) and served to establish the spontaneous preference of the animals for the two compartments of the box. Three more tests were conducted during the CPP phase to assess the progression of sucrose-conditioned place preference for the white, sucrose-paired compartment (POST-1, day 24; POST-2, day 31 and POST-3, day 38), and the last two tests were conducted during the EXT phase to assess extinction learning (EXT-1, day 43 and EXT-2, day 45).

On day 48 animals were decapitated to obtain the brains, which were conserved in 70% ethanol until processing for immunohistochemistry. Figure 1 summarizes the experimental design used.

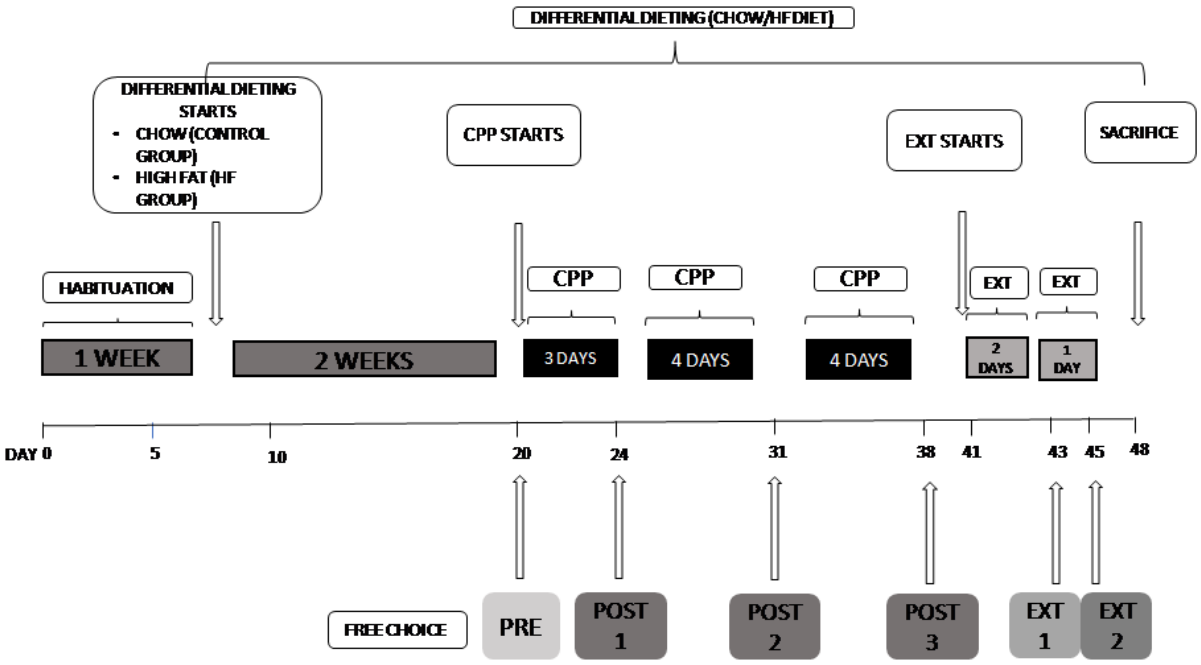

Figure 1. Experimental design of the study (see 2.1. for details). CPP (conditioned place preference): periods of place preference conditioning. EXT (extinction): periods of extinction learning. FREE CHOICE TESTS: PRE (pre-conditioning), free choice test before conditioning sessions; POST (post-conditioning) 1, POST 2, POST 3, free choice tests performed after the first, second and third periods of place preference conditioning; EXT 1, EXT 2: free choice tests performed after the first and second periods of extinction learning.

2.2. Immunohistochemistry

Brains were formalin-fixed, paraffin-embedded and then cut according to stereotaxic coordinates between bregma 7.44 mm and 6.24 mm (Paxinos and Franklin, 2012) containing the nucleus accumbens; two adjacent areas, the dorsal striatum and the cingulate cortex, were also studied as a reference (Fig. 2). Discontinuous sections (4 µm) from each selected block were cut and collected on clean gelatin-coated glass slides and left to air-dry overnight. Immunohistochemistry was performed

by using the EnVision TM FLEX (K8024) kit (Agilent Technologies, Glostrup, Denmark) according to the manufacturer's recommendations. Prior to staining, the tissues were deparaffinized to remove embedding media and rehydrated as follows: slides were placed in a xylene bath and incubated twice for 5 min, then in absolute ethanol for 3 min (twice) followed by 95 percent ethanol bath for 3 min (twice); finally, the slides were placed in distilled water for a minimum of 30 s and heat-induced epitope retrieval was performed by immersing them in a Tris/EDTA buffer (pH = 9) at 97°C for 30 min.

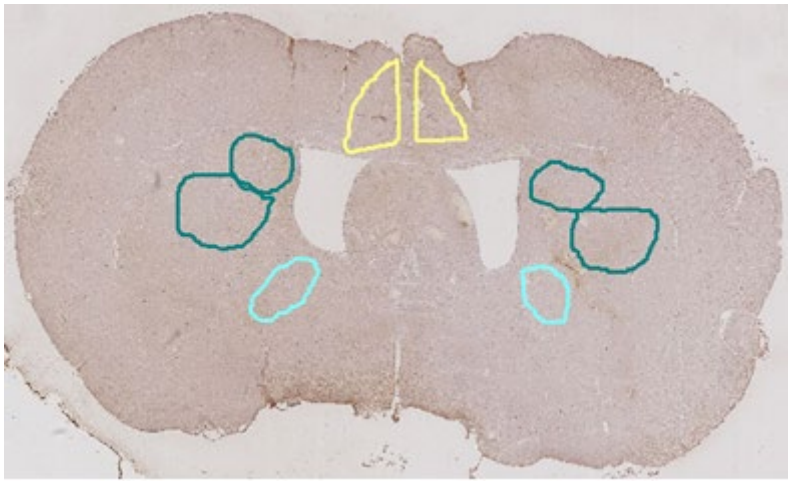

Figure 2. Location of brain areas studied in each section. Yellow: cingulate cortex. Light blue: nucleus accumbens. Dark blue: dorsal striatum.

Sections were then incubated with the primary antibody (clusterin 12289-1-AP 1:200, Proteintech, Rosemont, IL USA) for 1.5 h at 37°C then washed two times (5 min each) with the wash buffer provided by the kit. The secondary antibody included in the kit (Anti-Rabbit-HRP) was then added for 30 min at 37°C followed by two similar washes. Peroxidase-based immunohistochemical staining was performed with Liquid DAB+ Substrate Chromogen System (Dako, Agilent Technologies, Glostrup, Denmark) following manufacturer's instructions. Hematoxylin was used as a counterstain to facilitate the assessment of tissue morphology. Immunostained sections were then scanned in a

SCN 400 scanner (Leica Biosystems, Barcelona, Spain) and quantification of immunopositive cells was performed with the Aperio ImageScope Software (Leica Biosystems, Barcelona, Spain) by using two sections per animal and 8 selected areas per section (4 areas corresponded to dorsal striatum, 2 areas to nucleus accumbens and 2 areas to cingulate cortex). Figure 3 shows a representative image of clusterin staining. The correct detection of immunostaining was determined by using an algorithm previously designed for the antibody (Perez Ortiz et al., 2017) that enabled the calculation of the number of cells stained as well as the intensity of staining; we considered immunopositive cells those with positive (Np) and strong positive (Nsp) staining according to the thresholds determined by the mentioned algorithm and calculated the intensity of marks as the sum of the total intensity of positive cells (Ip) and the total intensity of strong positive cells (Isp).

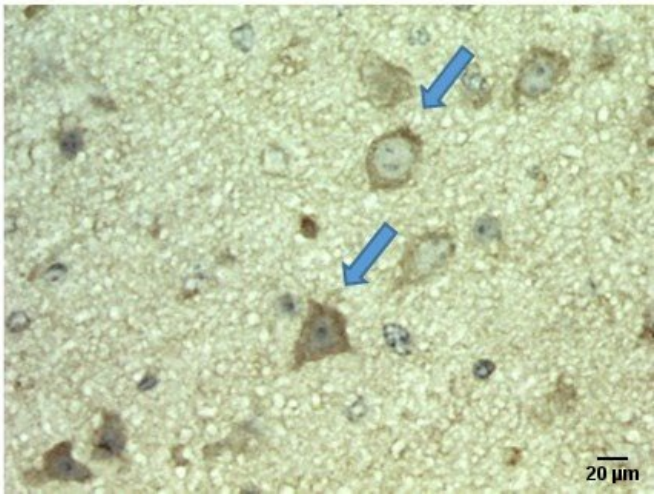

Figure 3. Clusterin immunostaining in nucleus accumbens neurons (blue arrows), 40x.

2.4. Statistics

The GraphPad PRISM software (version 8.4.2, GraphPad Software, San Diego, CA, USA) was used for statistics. Once confirmed the normality of data and homogeneity of variances with Kolmogorov-Smirnov and Levene's tests, respectively, we analyzed food intake, body weight, pellet consumption and sucrose preference by using two-way ANOVA (with Greenhouse-Geisserwith correction for

sphericity) followed by Fisher's LSD tests for multiple comparisons. Immunostaining in control and HF animals was compared separately in the three brain areas by using unpaired Student's t-tests. Statistical significance was considered at the 0.05 level.

3. Results

The mice with free access to an HFD showed a higher caloric intake than the controls from the start of the differential diet (Fig. 4A), as well as a significant increase in body weight from day 36 of the protocol onward (that is, from day 29 after beginning the differential diet: Fig. 4B).

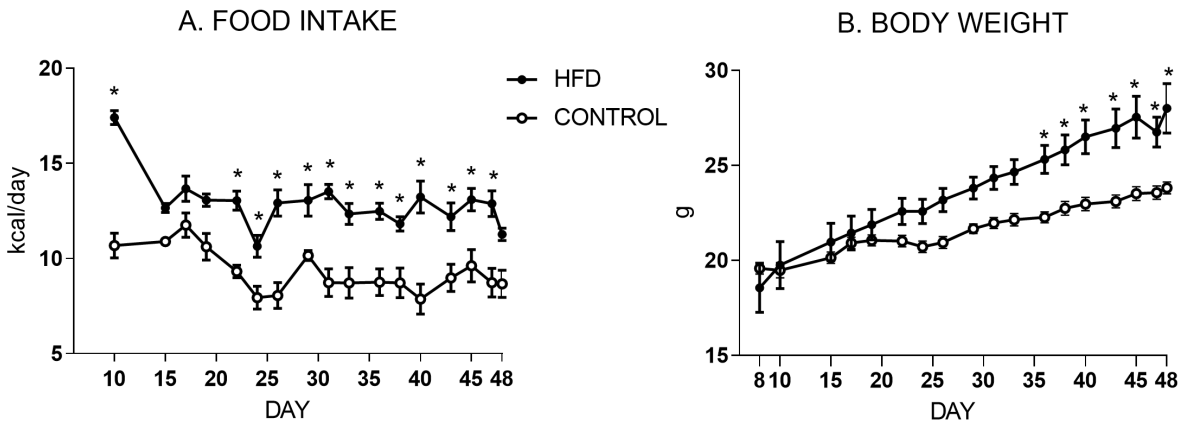

Figure 4. Evolution of caloric intake (panel A) and body weight (panel B) of mice fed on HFD or standard chow (CONTROL) during the experimental procedure (means ± SEM.* $p < 0.05$ versus CONTROL).

Figure 5 shows the number of sucrose pellets consumed during conditioning sessions as well as the acquisition, persistence, and extinction of sucrose-conditioned place preference. The HFD mice consumed far fewer sucrose pellets throughout the experiment (panel A). Concerning place preferences (panel B), HFD animals developed a significant preference for the sucrose-paired compartment very soon, just in the POST-1 free choice test, after only three conditioning sessions.

At this stage, control mice still did not exhibit significant changes with respect to preconditioning, however the time spent in the sucrose-paired compartment was not statistically different between HFD and control groups in the POST-1 test. More consistently, we found that the sucrose-conditioned place preference spontaneously disappeared in the HFD group in the POST-3 free choice test (even without the need for extinction learning), whereas the control mice required 3 extinction sessions to fully extinguish the sucrose-conditioned place preference in the EXT-2 free choice test.

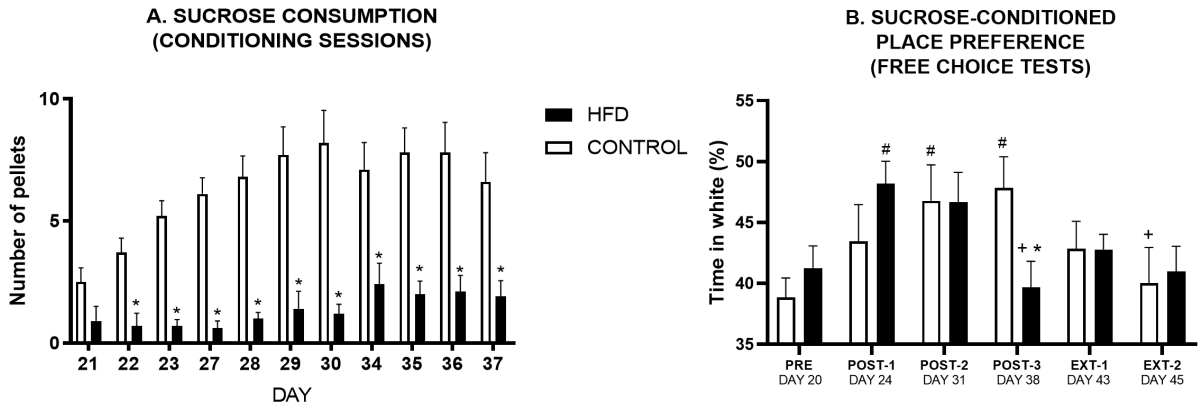

Figure 5. Panel A. Number of sucrose pellets consumed during the conditioning sessions by mice fed on HFD or standard chow (CONTROL). * $p < 0.05$ vs CONTROL. Panel B. Time spent in sucrose-paired compartment during free choice tests (mean ± SEM): PRE, preconditioning; POST: postconditioning; EXT-1 and 2: postextinction (see material and methods for details). * $p < 0.05$ vs CONTROL; # $p < 0.05$ vs PRE; + $p < 0.05$ vs last free choice test showing significant preference for the sucrose-paired compartment.

At the end of the procedure, the HFD mice exhibited higher clusterin expression than the controls in the nucleus accumbens but not in the cingulate cortex or the dorsal striatum (Fig. 6). The qualitative observation of the images by optical microscopy did not reveal any major change of the shape or size of cells, neither of the distribution of immunostaining after HFD. Figure 7 shows two representative images of clusterin immunostaining in the nucleus accumbens of the HFD and control mice.

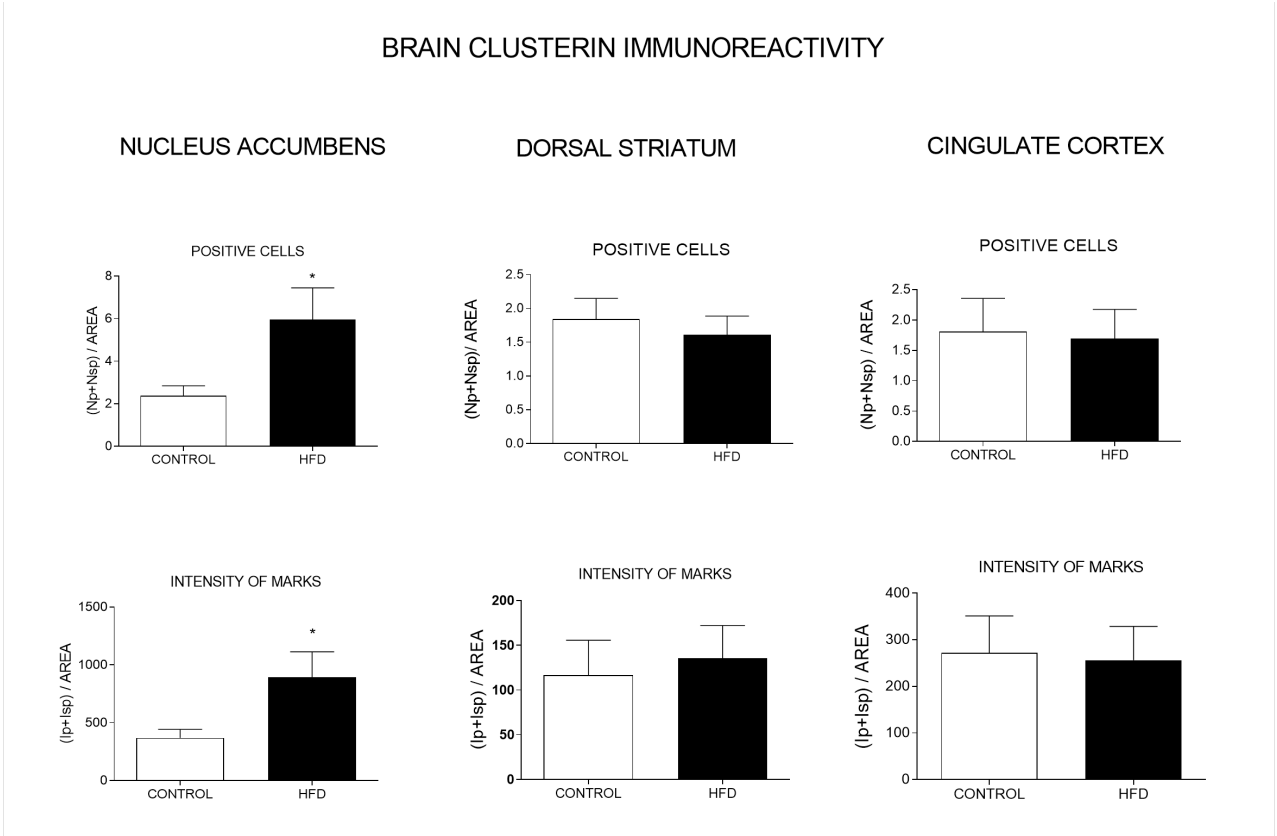

Figure 6. Clusterin expression in brain areas of mice fed on HFD or standard chow (CONTROL) (means ± SEM.* $p < 0.05$ versus CONTROL).

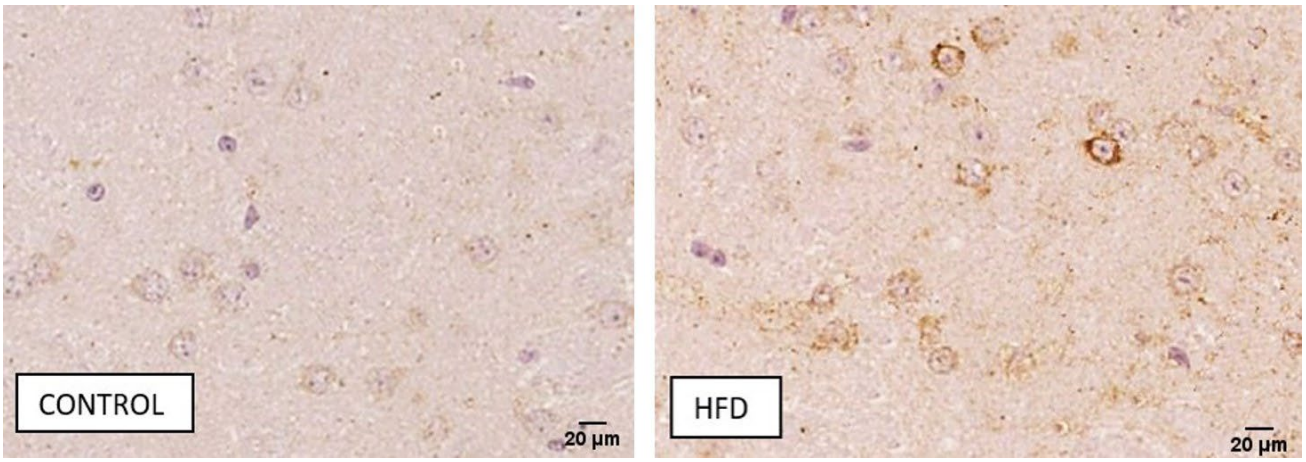

Figure 7. Clusterin immunostaining in nucleus accumbens neurons, 20x.

4. Discussion

The rapid onset of sucrose-conditioned place preference in the mice fed an HFD after just 3 conditioning sessions could be interpreted as a transient increase in food reward sensitivity compared with the controls, given that the latter required more sessions to develop a significant place

preference. It is important to note, however, that the time spent by the HFD mice and controls in the sucrose-paired chamber did not significantly differ, either in the first or second free election. Robust conclusions cannot therefore be reached on this subject. More focused experiments are needed to confirm this difference, given the lack of such findings in the literature.

Once established, the sucrose-conditioned place preference was maintained in the controls but exhibited a decrease in the HFD mice to completely disappear in the third free choice test of the conditioning phase, with no need for extinction learning. This finding reveals a marked reduction in food reward after only 31 days of the HFD, which is consistent with the fast decrease reported in previous studies (Barry et al., 2018). A similar decrease has also been reported with cafeteria diets, which are able to increase the brain stimulation reward threshold in rats from day 28 of unrestricted access to the diet (Johnson and Kenny, 2010). On the other hand, our results do not permit to clarify the controversy around the actual involvement of body weight changes and obesity in the appearance of food reward alterations; the observed reduction of food reward in HFD mice was indeed parallel to a significant increase of body weight with respect to controls, however the difference between experimental groups was not striking and HFD mice could hardly be considered obese.

We also observed a pronounced decrease in sucrose pellet consumption during all conditioning sessions in the HFD mice, a result that is probably unrelated to the food reward but rather to increased satiety. According to this concept, La Fleur et al. (2007) studied sucrose seeking in rats fed high-fat/high-sugar diets and found that the sucrose solution consumed in the housing cages did not correlate with the amount of active lever presses to obtain a sucrose pellet. Furthermore, when the authors tested the rats under fixed ratio schedules, they observed a reduced number of total presses in the rats fed a high-fat/high-sugar diet, a finding that was attributed to the influence of satiety signals in the response to food.

Based on the previous behavioral results, we expected significant neurochemical alterations in the nucleus accumbens after an HFD. We found a marked increase in clusterin expression in the HFD mice by the end of the experiment. As has been previously noted, changes in the brain expression of clusterin have been associated with the onset of diverse noxious conditions and are possibly related to a neuroprotective homeostatic reaction (Gregory et al., 2017), however other explanations cannot be discarded if we consider the multiple biological functions of this protein (see review by Rodríguez-Rivera et al., 2021). One of the putative mechanisms involved in clusterin neuroprotection is the ability to sequester activated Bax and inhibit the formation of the Bax–Bak complex, thereby creating an antiapoptotic effect (Zhang et al., 2005). Accordingly, the local clusterin overexpression in the nucleus accumbens could reflect a neurotoxic effect of the HFD. This hypothesis needs to be further addressed by carefully studying possible correlations between clusterin overexpression and cell viability or integrity of dopaminergic transmission, but it fits well with the previously reported increase in plasma clusterin levels in patients with morbid obesity who specifically exhibit poor control over eating (Rodríguez-Rivera et al., 2019). Another important finding is the region-specific clusterin overexpression, which appears to indicate that the nucleus accumbens could be particularly sensitive to the effects of HFD. Comparative studies between brain regions on the damaging effects of HFD are scarce; however, a dissimilar distribution of reactive oxygen species (assessed by NAD(P)H quinone oxidoreductase activity) has been reported, showing a higher sensitivity of the striatum compared with the cerebellum, frontal cortex, hypothalamus and hippocampus (Valdez et al., 2018). The higher sensitivity of the nucleus accumbens in our report with respect to the cingulate cortex is therefore not surprising. Moreover, previous comparative studies between the dorsal and ventral striatum also support a more marked effect of HFD in the latter region. Thus, rats fed an HFD showed reduced levels of dopamine D_2 receptors, cAMP response element-binding protein (CREB) and phosphorylated CREB (Ser133) in the ventral (but not dorsal) striatum (Adams et al., 2015). One limitation of this study is the use of male but not female

mice; this was decided to prevent excessive variability upon designing a completely new protocol, but obviously the introduction of female groups is highly recommended for further experiments.

5. Conclusions

The results of these experiments reveal a strong effect of high-fat diets on palatable food rewards and clusterin expression in the nucleus accumbens. The observed decrease of sucrose place preference agreed with the reward deficiency hypothesis and suggests that HFD markedly reduces food reward. On the other hand, clusterin overexpression could be indicative of a parallel, pathological change in the function of nucleus accumbens neurons, if we bear in mind that clusterin upregulation has been repeatedly observed in brain damaging conditions. Further experiments are needed to confirm possible associations between clusterin overexpression, dysfunction of the brain reward system and vulnerability to related diseases (e.g., food and drug addiction).

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