Lipid excess affects chaperone-mediated autophagy in hypothalamus

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Abstract

Obesity is a major health problem worldwide. Overweight and obesity directly affect health-related quality of life and also have an important economic impact on healthcare

systems. In experimental models, obesity leads to hypothalamic inflammation and loss of metabolic homeostasis. It is known that macroautophagy is decreased in the hypothalamus of obese mice but the role of chaperone-mediated autophagy is still unknown. In this study, we aimed to investigate the role of hypothalamic chaperone-mediated autophagy in response to high-fat diet and also the direct effect of palmitate on hypothalamic neurons. Mice received chow or high-fat diet for 3 days or 1 week. At the end of the experimental protocol, chaperone-mediated autophagy in hypothalamus was investigated, as well as cytokines expression. In other set of experiments, neuronal cell lines were treated with palmitic acid, a saturated fatty acid. We show that chaperone-mediated autophagy is differently regulated in response to high-fat diet intake for 3 days or 1 week. Also, when hypothalamic neurons are directly exposed to palmitate there is activation of chaperone-mediated autophagy. High-fat diet causes hypothalamic inflammation concomitantly to changes in the content of chaperone-mediated autophagy machinery. It remains to be studied the direct role of inflammation and lipids itself on the activation of chaperone-mediated autophagy in the hypothalamus *in vivo* and also the neuronal implications of chaperone-mediated autophagy inhibition in response to obesity.

Keywords

Autophagy; Hypothalamus; Obesity; Palmitic acid; Chaperone-mediated autophagy.

Abbreviations

CMA, chaperone-mediated autophagy; FBS, fetal bovine serum; HCS, High-content screening; HFD, High-fat diet.

1. Introduction

Obesity became a health problem worldwide. In experimental models of this disease, it is well established that hypothalamus plays a pivotal role in the regulation of energy homeostasis and is directly affected by lipid excess and other obesogenic stimuli (1, 2, 3, 4). Despite major advances in the understanding of the pathophysiology of obesity, it is still unknown the role of chaperone-mediated autophagy (CMA) in the hypothalamus in experimental models of diet-induced obesity. In liver, for example, it was shown that dietary lipid excess is able to impair this type of autophagy (5). CMA blockage also leads to dysregulation of hepatic metabolism and hepatosteatosis in mice (6).

Autophagy is an important process that regulates protein quality control among other aspects of cellular homeostasis (7). CMA specifically targets cytosolic proteins that can be recognized by the chaperone HSC70. Once the complex of target protein and chaperone is formed, it reaches the lysosomal membrane and binds to LAMP2a, a lysosome-associated protein. Then, the substrate protein translocates into lysosomal lumen where is degraded. Different stimuli activate CMA, such as starvation and oxidative stress (8, 9).

In the central nervous system, CMA has been extensively studied and is responsible to protect neurons against injuries and chronic neurodegeneration (10, 11). For example, CMA is activated during hypoxic and ischemic stress to promote neuronal survival (12). Likewise, dysfunction of CMA leads to the accumulation of abnormal proteins and is involved in the pathogenesis of neurodegenerative diseases (13).

The aim of this study was to evaluate CMA in HFD-fed mice and in hypothalamic neurons treated with saturated fatty acids. We have found that HFD leads to early modifications in CMA machinery in the hypothalamus. Interestingly, palmitate is able to directly activate CMA in hypothalamic neurons.

2. Material and Methods

2.1 Experimental animals, diets and metabolic parameters

Swiss mice were provided by the State University of Campinas Animal Breeding Center (CEMIB, Brazil). All animal procedures followed the *Guide for the Care and Use of Laboratory Animals* published by National Institute of Health and the guidelines of the Brazilian College for Animal Experimentation. All the experiments were approved by the State University of Campinas Ethics Committee (protocol 4245-1). Animals were maintained on a 12-hour light-dark cycle and each mouse was individually housed in cages with *ad libitum* food and water. All murine experiments were performed with 7 weeks old of age animals. Mice received a chow diet that was composed of 77% kcal carbohydrate, 12% kcal protein and 11% kcal lipids or a high-fat diet that was composed of 27% kcal carbohydrate, 12% kcal protein and 61% kcal lipids for 3 days or 1 week. Body weight was monitored daily. At the end of experimental period animals were fasted overnight and blood glucose was measured with a glucometer (Accucheck, Roche).

2.2 Western-blotting and Co-Immunoprecipitation

Co-immunoprecipitaion was performed with 100 µg of protein and 1,5 µl of LAMP2 antibody (Santa Cruz Biotechnology, USA) with overnight rotation. Next, 20 µl of Protein A Sepharose beads (Sigma-Aldrich) were added and the mix was incubated for 3 hours.

After centrifugation, the pellet was washed for 3 times and the extract was used for western blotting with HSC-70 antibody. Immunoblotting was performed to analyze the content of HSC70 (sc7298 from Santa Cruz Biotechnology) and LAMP2a (ab18528 from Abcam) proteins. Polyacrylamide gels (SDS-PAGE) were used to separate the proteins in samples. A Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) was used to transfer the samples from the gel to a nitrocellulose membrane (Bio-Rad) by semi-dry blotting the gel in a transfer buffer with methanol and SDS. Membranes were then blocked in a solution containing 5% skim milk in Tris-buffered saline (TBS)-Tween 20 (TTBS; 10 mmol Tris/L, 150 mmol NaCl/L, 0.5% Tween 20) for 2 hours at room temperature. The membranes were then incubated overnight with primary antibodies at 4°C, which was followed by 1-hour incubation of horseradish peroxidase-conjugated secondary antibody at room temperature. Bands were detected by chemiluminescence and quantified by densitometry with (UN-Scan-it Gel 6.1, Silk Scientific Inc, Orem, Utah USA).

2.3 Gene expression

Hypothalamic tissue was homogenized in Trizol (Invitrogen 15596018, Sao Paulo, Brazil) and RNA was precipitated and quantified (Nanodrop, Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized with 3 µg of total RNA sample with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368813). qRT-PCR analysis was performed in an ABI Prism 7700 sequence detection system (Applied Biosystems). GAPDH primer was used as the housekeeping gene. Each PCR contained 3.0 ng of reverse-transcribed RNA, 200 nM of each primer, TaqMan™

(Applied Biosystems) and RNase free water. Data were analyzed with 7500 System SDS Software (Applied Biosystems – Life Technologies).

2.4 Cell culture

The GT1-7 and mHypoA-2/29 mouse hypothalamic cell lines were maintained in DMEM High Glucose (4.5 mg/L of glucose), containing 10% fetal bovine serum (FBS), 100 u/mL penicillin and 100 mg/mL streptomycin. In all experiments, cells were plated at a final concentration of 70% cells/plate. Cells were treated with bovine serum albumin (BSA) in the control group or palmitate at different concentrations for 24 hours. For the experiments, only 1% of FBS was added to DMEM. As positive controls, cells were treated with DMEM without FBS for 24 hours.

2.5 Palmitate preparation

A 25 mM solution of palmitate was prepared with 10% of sodium hydroxide 5 M. Solubilization was achieved by heating the solution at 80°C for 10 minutes. While the solution was still warm, palmitate was diluted to the final concentration in DMEN with 1% FBS and BSA (palmitate to BSA molar ratio at 3:1). The solution was incubated for 40 minutes at 37°C to allow palmitate-BSA conjugation prior to cell treatment.

2.6 High-content screening

For High-content screening (HCS) analysis, cells were fixed in cold methanol for 2 minutes. To prevent non-specific binding of the antibodies, a blocking solution containing 3% BSA in PBS was incubated for 2 hours at room temperature. Then, the cells were incubated overnight with primary antibodies at 4°C, which was followed by 1-hour incubation with Alexa Fluor 488 secondary antibody at room temperature. Images

were acquired with Molecular Device (ImageXpress Micro Confocal High-Content Imaging®) and analyzed with MetaXpress software.

2.7 Ceramide quantification

Ceramide levels were measured by the diacylglycerol kinase method. Lipid extracts were incubated in the presence of DAG kinase and [γ- 32P]-ATP as previously described (14). The reaction was stopped, and [γ- 32P]-ceramide phosphate was resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, by vol.) and quantified using a FLA700 PhosphorImager (GE Healthcare). Total phospholipid levels were also quantified.

2.8 Propidium Iodide

GT1- 7 and mHypoA 2/29 neurons were seeded in 6 well-plates and viability was assessed by staining cells with propidium iodide (PI) diluted in PBS. Briefly, neurons were treated with palmitate 24 hours. Medium with treatment was then removed and reserved to resuspend the cells. The neurons were washed with PBS, trypsinized and resuspended in the reserved medium. The cells were centrifuged and the pellet was resuspended with PI solution (1 ug/ mL). Cells were kept on ice and then subjected to flow cytometry analysis (BD Accuri C6) using FL2 (585/40 nm) and FL3 (610/20 nm channel). Cells with fragmented DNA were bound with PI and detected.

2.9 Statistical Analysis

Results are expressed as mean values ± standard error mean (SEM). Levene's test for the homogeneity of variance determined if the data could be used for parametric analysis of variance. Student's unpaired t-tests were used to compare the differences between two groups. When there were more than two groups, One-Way ANOVA was

160 performed. Tukey post hoc tests were used to evaluate significant main effects. In all
161 cases, $p < 0.05$ was considered statistically significant. Data were analyzed with
162 GraphPad Prism 6 software (GraphPad Software Inc, USA).
163
164 **3. Results**
165 ***3.1 High-fat diet leads to early obesity onset in Swiss mice***
166 High-fat diet (HFD) was offered to mice at different time points to investigate the
167 outcomes of lipid excess on metabolic parameters. After 3 days on HFD mice gained
168 more weight than those in chow group and this difference was maintained with 1 week
169 on HFD (Figure 1 A). Fasting blood glucose was also higher in HFD fed mice compared
170 to chow group since 3 days of HFD, but no difference was found in epididymal fat in this
171 experimental time (Figure 1 B and C). Interestingly, mice fed HFD for 1 week showed
172 increase epididymal fat gain, but no difference was found in fasting glucose (Figure 1 B
173 and C). Food intake was similar between the groups (Figure 1 D).
174

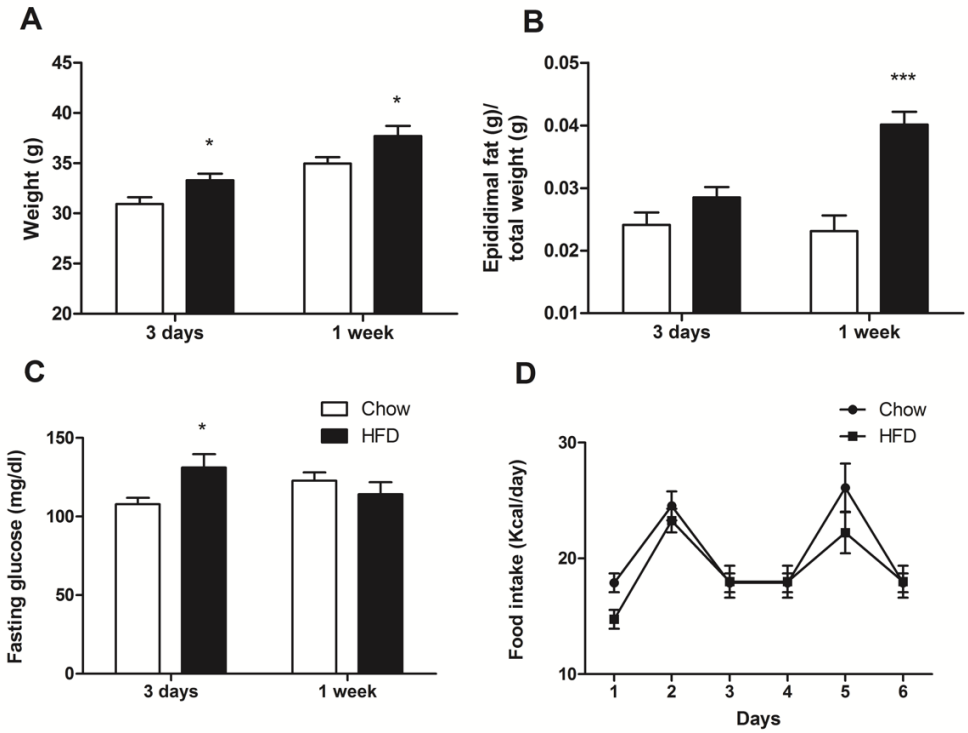

Figure 1. Metabolic parameters of mice on HFD. **A)** Weight (g) of chow and HFD mice after 3 days and 1 week on experimental diets. **B)** Epididymal fat (g) normalized by body weight (g) of mice after experimental diets. **C)** Fasting glucose of animals after experimental diets. **D)** Daily food intake (n=10). Values are shown as mean ± SEM, n=15 animals per group in 3 days experimental group and n= 10 animals per group in 1-week experimental group * p<0,05 and *** p<0,0001.

3.2 Short-term exposure to high fat diet leads to differential regulation of CMA in hypothalamus

Next, we aimed to investigate the effects of HFD on hypothalamic chaperone-mediated autophagy. By co-immunoprecipitation, there was a significant higher association in LAMP2 with HSC70 after 3 days on HFD in the hypothalamus (Figure 2 A). The protein content of LAMP2a and HSC70 was decreased at this time point (Figure 2 B and C). On the other hand, there was a decrease in the association of these proteins in the

hypothalamus of mice fed HFD for 1 week (Figure 2 D), and the protein content of HSC70 was decreased with no difference in the content of LAMP2a after 1 week of experimental diet (Figure 2 E and F).

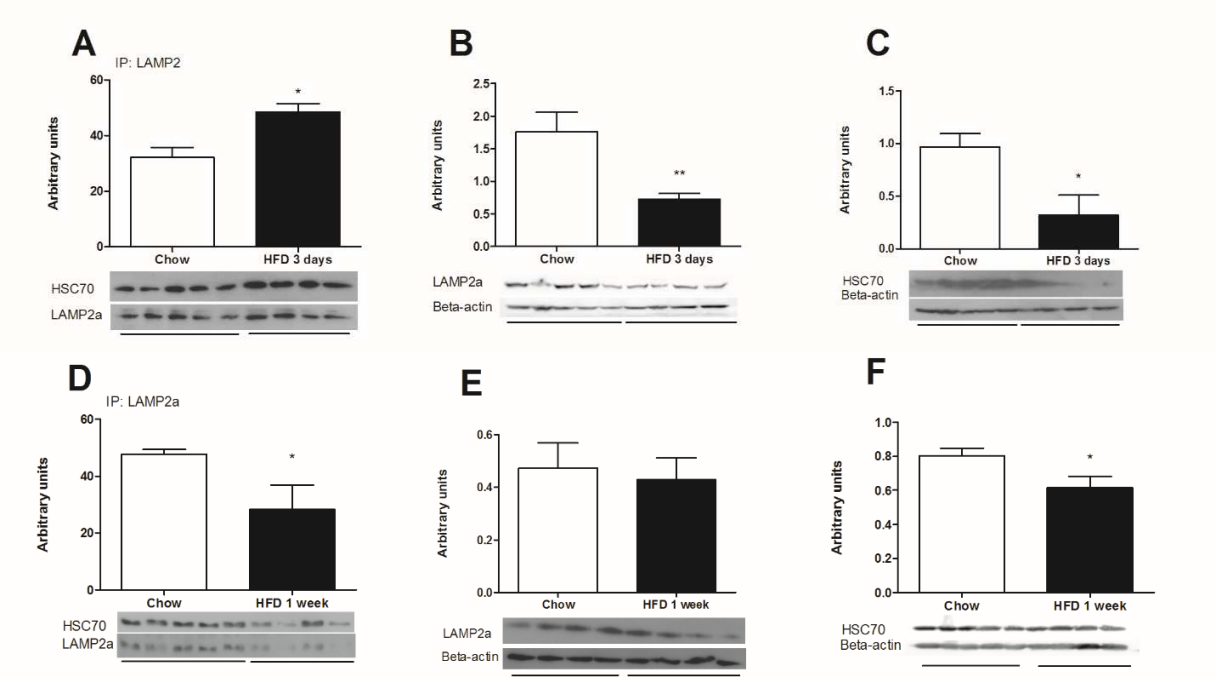

Figure 2. Hypothalamic CMA on mice fed HFD. **A)** LAMP2 immunoprecipitation in mice after 3 days on HFD. **B)** LAMP2a content by western blotting in mice after 3 days on HFD. **C)** HSC70 content by western blotting in mice after 3 days on HFD. **D)** LAMP2 immunoprecipitation in mice after 1 week on HFD. **E)** LAMP2a content by western blotting in mice after 1 week on HFD. **F)** HSC70 content by western blotting in mice after 1 week on HFD. Values are shown as mean ± SEM, n= 5 (Chow) n= 4 (HFD) * p<0,05.

It is well established that obesity leads to hypothalamic inflammation in animal models (1, 4). In our experimental model, there was a transient increase in mRNA of *Il1 beta*, *Il6* and also *Il10* in hypothalamus of mice after 3 days on HFD compared to chow diet group. No change in the hypothalamic expression levels of *Tfeb* gene in hypothalamus,

an important regulator of lysosome biogenesis, was found between the groups. Lamp2 relative expression was also not different from the controls in HFD mice in neither time points.

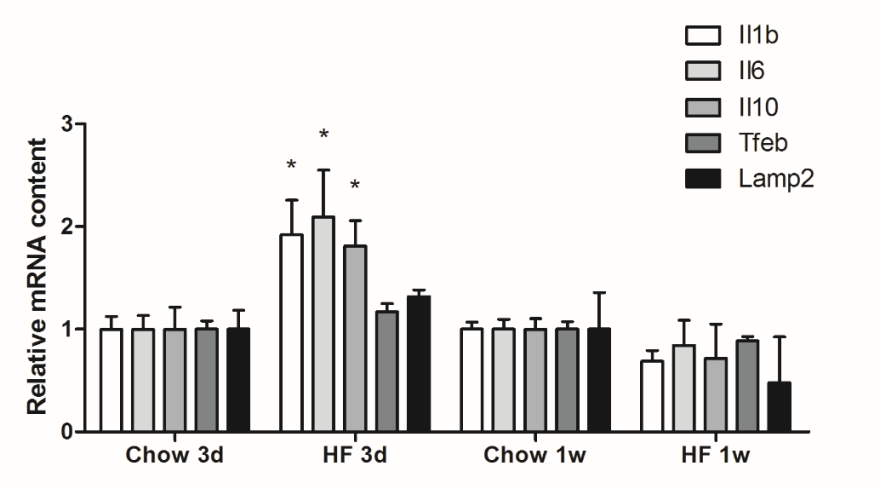

Figure 3. Cytokines and autophagy-related gene expression on mice fed HFD for 3 days and 1 week. qRT-PCR was used to analyze the gene expression of Il1b, Il6, Il10, Tfeb and Lamp2 in the hypothalamus of mice with 3 days of treatment. Values are shown as mean ± SEM, n= 4-5 animals per group in HFD 3 days and n=3 animals per group in HFD 1 week group, *p < 0.05.

3.4 Palmitate induces chaperone-mediated autophagy in hypothalamic neurons

To investigate the effect of saturated fatty acids on neurons directly, we treated mHypoA-2/29 cells with different concentrations of palmitate for 24 hours. We first assessed cell viability using PI staining. We observed a decrease in viable cells when we used P 250 µM after 36 hours (Supplementary Figure 1). Next, we used a treatment with medium without serum (-FBS) as positive control to our palmitate treatment. Similar to previously reported, serum deprivation led to redistribution and accumulation of LAMP2a in the perinuclear region of the neuron (15), reflecting activation of CMA. When cells were incubated with 50 µM of palmitate, there was also an increase in LAMP2a

fluorescence near the nucleus. In contrast, the treatment with 100 µM of palmitate decreased LAMP2a staining in mHypoA-2/29 cells (Figure 4 A and B).

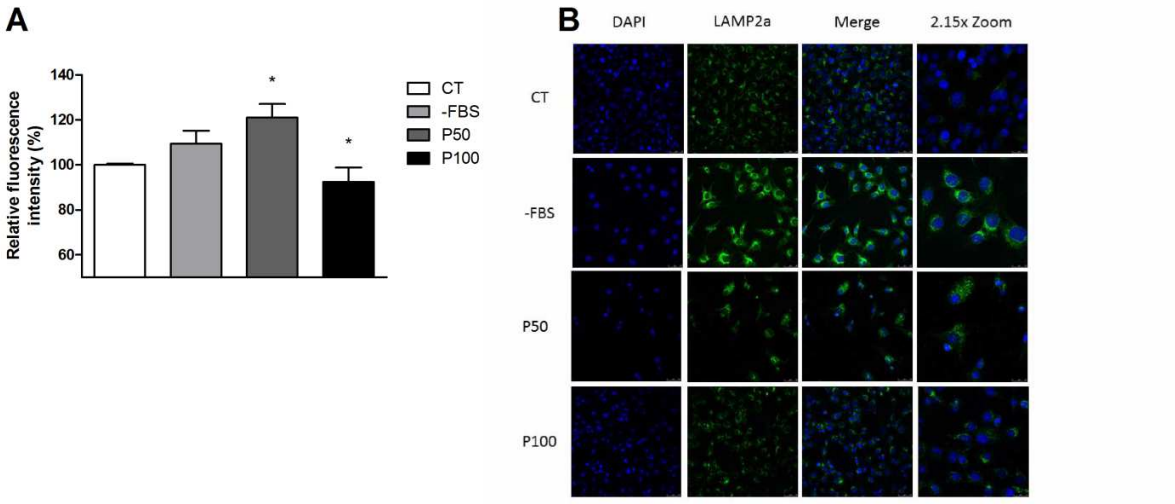

Figure 4. LAMP2a immunofluorescence in hypothalamic neurons. **A)** Relative LAMP2a fluorescence after serum deprivation (-FBS) or treatment with 50 µM (P50) and 100 µM (P100) of palmitate for 24 hours. **B)** Representative images of cell nuclei labeled with Dapi (blue) and LAMP2a with AlexaFluor488 (green) with serum deprivation and palmitate treatment in mHypoA-2/29 cells. Three independent experiments were performed. Values are shown as mean ± SEM, * $p < 0.05$.

Another neuronal cell line, the GT1-7 cells, were also treated with different concentrations of palmitate. To analyze chaperone-mediated autophagy in this model, High Content Screening was performed. This method consists of a cellomics approach that involves automated microscopy and image analysis of several compounds in parallel, the output includes the measurement of fluorescent intensity of LAMP2a within each treatment. First, we confirmed that 24 hours of fasting was sufficient for maximum activation of chaperone-mediated autophagy in GT1-7 cells (Figure 5 A and C). Also,

palmitate at 50, 250 and 500 μM for 24 hours led to increasing in LAMP2a staining, suggesting an increase on CMA in hypothalamic neurons after supra-physiological exposure to saturated fat (Figure 5 A and C). Recent studies suggest that palmitate regulate ceramide levels, a potent regulator of macroautophagy (16). Concomitantly, there was an increase in ceramide content in a dose-dependent manner in GT1-7 cells treated with palmitate (Figure 5 B). Interestingly, PA treatment did not affect GT1-7 cell viability in the time/concentration studied (Supplementary Figure 01).

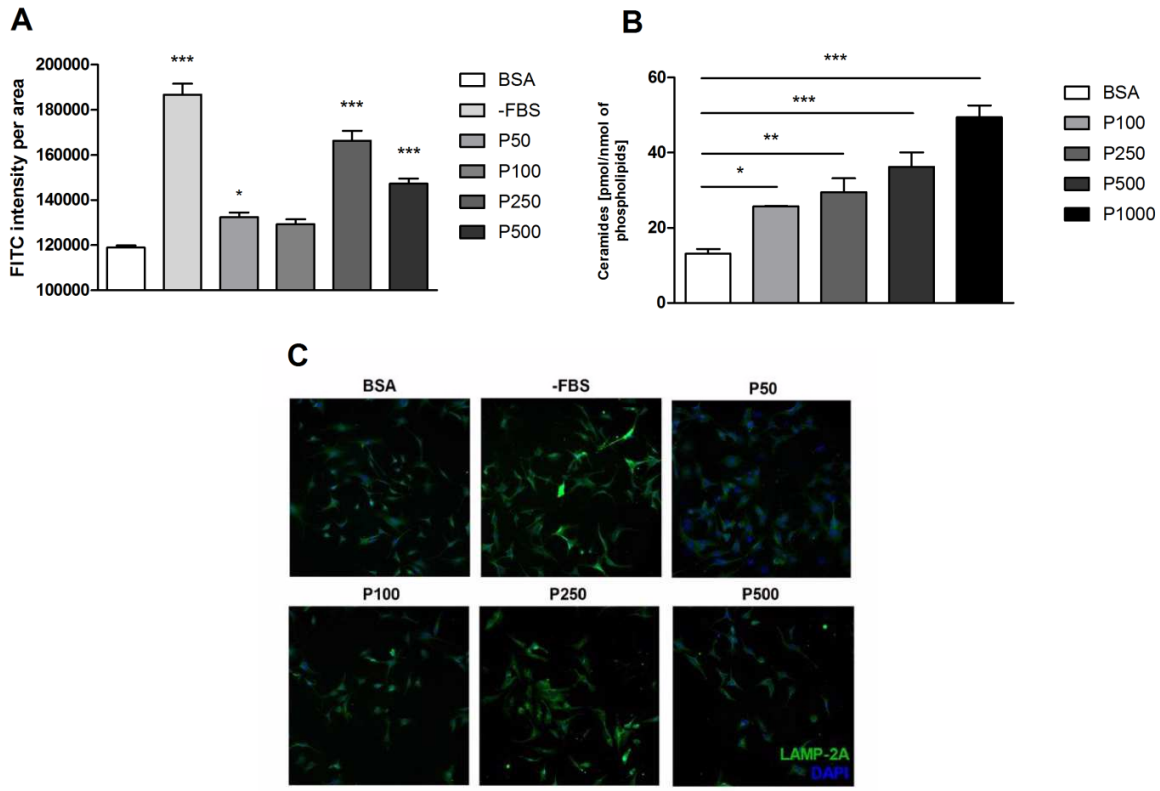

Figure 5. HCS for CMA quantification in hypothalamic neurons. A) LAMP2a quantification by HCS after prolonged starvation or different concentrations of palmitate exposure for 24 hours. B) Ceramide quantification after palmitate treatment for 24 hours. C) Representative images of HCS analysis. Three independent experiments were performed. Values are shown as mean ± SEM, * $p < 0.05$, ** $p < 0,01$, *** $p < 0,001$.

4. Discussion

Inflammation is a hallmark of obesity (17) and evidence shows that hypothalamus is rapidly affected by lipid excess (4, 18). In this work, we show that inflammatory markers are induced after short-term HFD feeding in agreement with previous reports (4).

The rapidly onset of inflammation in hypothalamus after HFD is sustained by the activation of several mechanisms including the induction of endoplasmic reticulum stress (2), activation of TLR4 (3) and activation of IKKβ (2, 19, 20). More recently, it was reported that macroautophagy (commonly named autophagy), is also affected by HFD and plays an important role in the inflammatory response (21, 22). Here we show that chaperone-mediated autophagy is also affected by HFD. After three days of HFD, there was an increase in the association between LAMP2a and HSC70, the most important proteins of CMA machinery, although the content of both proteins was decreased.

In contrast, there was a decreased association between LAMP2a and HSC70 after 1 week of HFD. Decreased CMA activity is often related to poor protein quality control and neurotoxicity as reviewed elsewhere (23), suggesting that inhibition of CMA by long-term HFD could contribute to its deleterious effect in hypothalamus. These data suggest that short-time HFD decreased CMA in hypothalamus. This is in agreement with the recent evidence that CMA activity is decreased after nutritional challenges like lipid overload (5). It remains to be studied if sustained activation of hypothalamic CMA in diet-induced obese mice has a potential therapeutic target in obesity and its correlated diseases.

It is important to point out that these changes in CMA components reflect a steady-state read out of hypothalamic CMA. Although the association between LAMP2a and HSC70

is a critical step in CMA, a functional analysis, such as protein degradation assessment, would complement these results. Next, we assessed if palmitate, a saturated fatty acid, could also modulate CMA directly in hypothalamic neurons by using two different neuronal cell lines, mHypoA-2/29 and GT1-7. Previous work has showed that saturated fatty acids exposure in cultured hypothalamic neurons leads to endoplasmic reticulum stress and lipotoxic effects, although they are resistant to inflammation and insulin resistance (24). We found that palmitate is able to activate CMA in neurons after 24 hours of exposure.

Palmitate treatment also increased the content of ceramide in neurons. Similar results were found in previous work (25). This is an important finding since increase ceramide production in hypothalamic neurons is linked to insulin resistance and glucose intolerance (25). Additionally, exogenous ceramide causes lipotoxicity and endoplasmic reticulum stress in the hypothalamus, which leads to weight-gain in experimental animals (26). Interestingly, a positive correlation between decreased glucocerebrosidase activity and decreased LAMP2 content was found in the anterior cingulate cortex of early stage Parkinson's disease patients, although the mechanistic explanation needs to be further addressed (27). It would be interesting to investigate if increased ceramide levels in response to palmitate regulates hypothalamic CMA. However, the role of ceramide levels could be ambiguous since remodeling of ceramide acyl chain length has been shown to down-regulate Hsc70 levels in astrocytes (28). Therefore, palmitate induced ceramide accumulation in hypothalamus could primarily activate CMA which be latter on down-regulated by alteration of HSC70 levels.

Importantly, cell viability was not affected in GT1-7 cells treated with palmitate for 24 hours, although longer periods of treatment (36 hours) led to viability impairment in these cells (data not show). However, mHypoA 2/29 presented a decrease in the number of viable cells after 24 hours, suggesting palmitate is affecting neuronal function. Those differences could be related to the differential profile of immortalization process, since mHypoA 2/29 was immortalized using the retrovirus SV40 and GT1-7 cells are derived from tumoral cells (29).

5. Conclusions

Altogether, our results show that high-fat diet induces CMA in the hypothalamus in short-term feeding, but prolonged exposure to HFD leads to CMA impairment. Failure to degrade misfolded and oxidized proteins can lead to dysfunction of hypothalamus but also neurodegeneration, although this needs further investigation in diet-induced obesity models.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Contributions

MM designed the work. MP performed most of the experiments, with assistance from AR and JEM. MPH assisted with cell culture experiments. LAS and MP performed the immunofluorescent experiments. MC the performed ceramide experiments. MP, MM, LMIS and HLS interpreted the data. AST, CM and MAT supervised the project and helped with technical advice. MP and MM drafted the manuscript. RFL and HLS reviewed the manuscript. All authors have approved the submitted version.

Ethics approval

All mice experiments were approved by the State University of Campinas Ethics Committee (protocol 4245-1).

Competing interests

The authors declare that they have no competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Figure

Supplementary Figure 1. Cell viability detected by flow cytometry. GT1-7 cells were treated with 50 or 250 µM PA or control (H_20) following the described period. After, cell viability (%) was assessed using PI solution (1 µg/ mL). (A-C) % of viable cells after PA treatment. (D) Morphological parameter (Size, termed FSC-A) after PA treatment in both embryonic and adult cells, respectively. (E-H) Representative morphological parameters (FSC-A X SSC-A). Data are expressed as mean ± SEM; *P < 0.05. The experiment was performed one time using 3 wells/group. Statistical analysis was performed by one-way ANOVA with Dunnet post hoc test comparing the mean of each column with the means of the control column.

Supplementary Figure 2. Cell viability detected by flow cytometry. mHypoA 2/29 cells were treated with 50 or 250 µM PA or control (BSA) following the described period. After, cell viability (%) was assessed using PI solution (1 µg/ mL). (A) Representative morphological parameters (FSC-A X SSC-A). (B) % of viable cells after PA treatment. Data are expressed as mean ± SEM; *P < 0.05. The experiment was performed three times using 3 wells/group. Statistical analysis was performed by one-way ANOVA with

453 Dunnet post hoc test comparing the mean of each column with the means of the control
454 column.

Highlights

- Short-term high-fat diet enhances hypothalamic cytokines expression.
- High-fat diet induces changes in chaperone-mediated autophagy machinery.
- Acute palmitate treatment enhances neuronal chaperone-mediated autophagy.

Conflicts of Interest Statement

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.