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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

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

42 **Keywords**

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

44

45 **Abbreviations**

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

48

49 **1. Introduction**

50 Obesity became a health problem worldwide. In experimental models of this disease, it
51 is well established that hypothalamus plays a pivotal role in the regulation of energy
52 homeostasis and is directly affected by lipid excess and other obesogenic stimuli (1, 2,
53 3, 4). Despite major advances in the understanding of the pathophysiology of obesity, it
54 is still unknown the role of chaperone-mediated autophagy (CMA) in the hypothalamus
55 in experimental models of diet-induced obesity. In liver, for example, it was shown that
56 dietary lipid excess is able to impair this type of autophagy (5). CMA blockage also
57 leads to dysregulation of hepatic metabolism and hepatosteatosis in mice (6).

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

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

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

74 **2. Material and Methods**

75 **2.1 Experimental animals, diets and metabolic parameters**

76 Swiss mice were provided by the State University of Campinas Animal Breeding Center
77 (CEMIB, Brazil). All animal procedures followed the *Guide for the Care and Use of*
78 *Laboratory Animals* published by National Institute of Health and the guidelines of the
79 Brazilian College for Animal Experimentation. All the experiments were approved by the
80 State University of Campinas Ethics Committee (protocol 4245-1). Animals were
81 maintained on a 12-hour light-dark cycle and each mouse was individually housed in
82 cages with *ad libitum* food and water. All murine experiments were performed with 7
83 weeks old of age animals. Mice received a chow diet that was composed of 77% kcal
84 carbohydrate, 12% kcal protein and 11% kcal lipids or a high-fat diet that was
85 composed of 27% kcal carbohydrate, 12% kcal protein and 61% kcal lipids for 3 days or
86 1 week. Body weight was monitored daily. At the end of experimental period animals
87 were fasted overnight and blood glucose was measured with a glucometer (Accucheck,
88 Roche).

89 **2.2 Western-blotting and Co-Immunoprecipitation**

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

93 After centrifugation, the pellet was washed for 3 times and the extract was used for
94 western blotting with HSC-70 antibody. Immunoblotting was performed to analyze the
95 content of HSC70 (sc7298 from Santa Cruz Biotechnology) and LAMP2a (ab18528
96 from Abcam) proteins. Polyacrylamide gels (SDS-PAGE) were used to separate the
97 proteins in samples. A Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) was used to
98 transfer the samples from the gel to a nitrocellulose membrane (Bio-Rad) by semi-dry
99 blotting the gel in a transfer buffer with methanol and SDS. Membranes were then
100 blocked in a solution containing 5% skim milk in Tris-buffered saline (TBS)-Tween
101 20 (TTBS; 10 mmol Tris/L, 150 mmol NaCl/L, 0.5% Tween 20) for 2 hours at room
102 temperature. The membranes were then incubated overnight with primary antibodies at
103 4°C, which was followed by 1-hour incubation of horseradish peroxidase-conjugated
104 secondary antibody at room temperature. Bands were detected by chemiluminescence
105 and quantified by densitometry with (UN-Scan-it Gel 6.1, Silk Scientific Inc, Orem, Utah
106 USA).

107 **2.3 Gene expression**

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

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

117 **2.4 Cell culture**

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

125 **2.5 Palmitate preparation**

126 A 25 mM solution of palmitate was prepared with 10% of sodium hydroxide 5 M.
127 Solubilization was achieved by heating the solution at 80°C for 10 minutes. While the
128 solution was still warm, palmitate was diluted to the final concentration in DMEM with
129 1% FBS and BSA (palmitate to BSA molar ratio at 3:1). The solution was incubated for
130 40 minutes at 37°C to allow palmitate-BSA conjugation prior to cell treatment.

131 **2.6 High-content screening**

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

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

139 **2.7 Ceramide quantification**

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

146 **2.8 Propidium Iodide**

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

155 **2.9 Statistical Analysis**

156 Results are expressed as mean values \pm standard error mean (SEM). Levene's test for
157 the homogeneity of variance determined if the data could be used for parametric
158 analysis of variance. Student's unpaired t-tests were used to compare the differences
159 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).

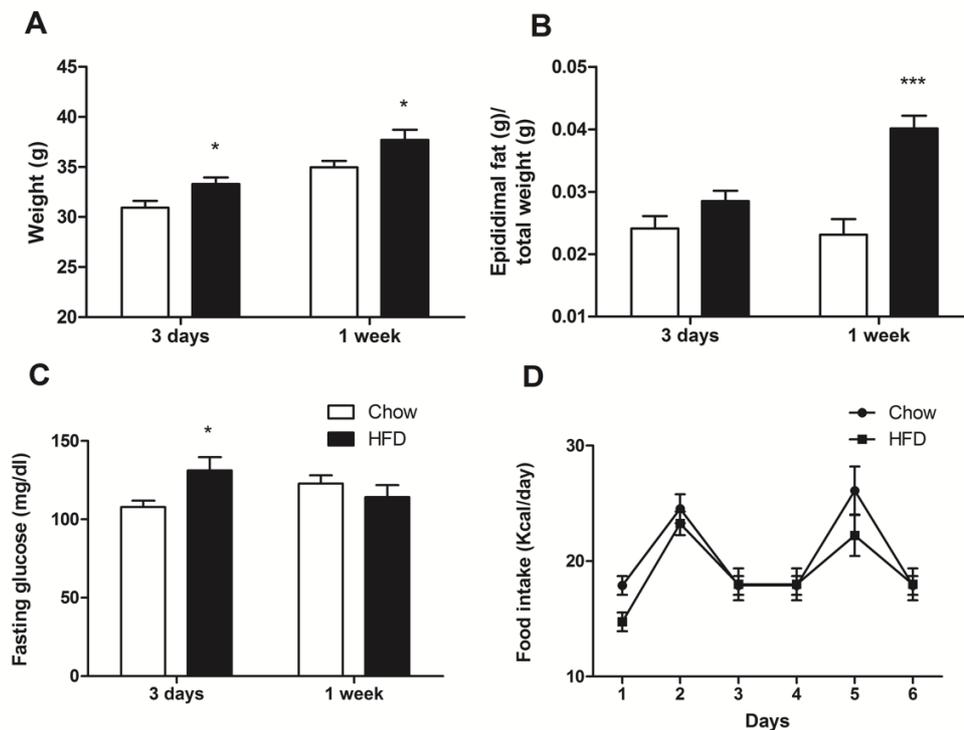
163

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



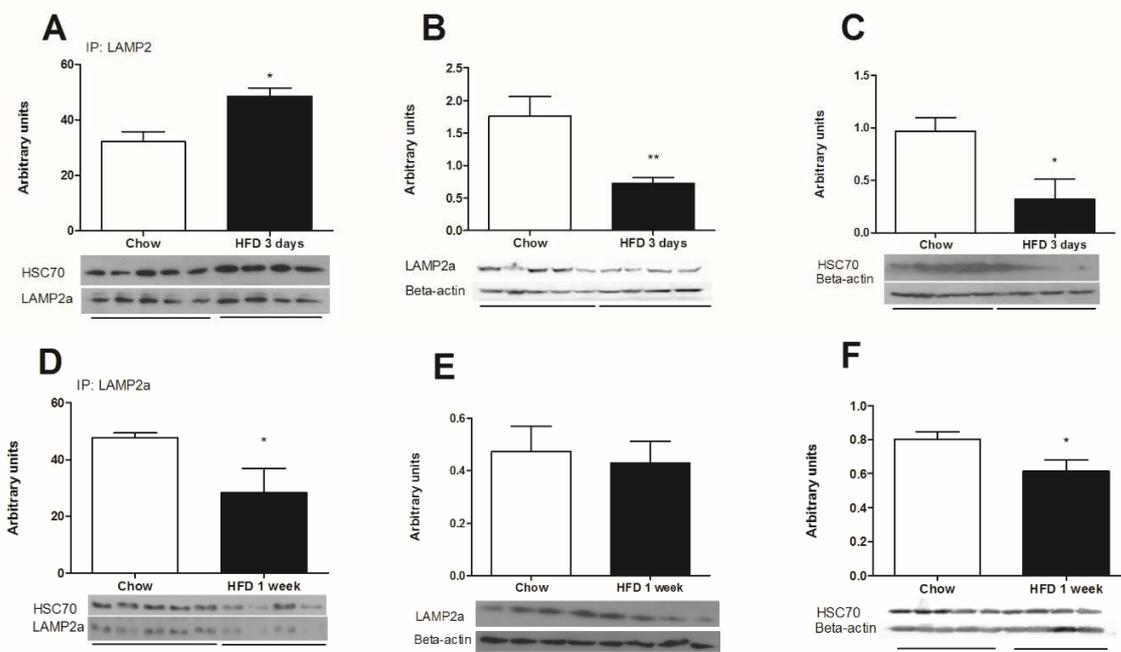
175

176 **Figure 1.** Metabolic parameters of mice on HFD. **A)** Weight (g) of chow and HFD mice after 3
 177 days and 1 week on experimental diets. **B)** Epididymal fat (g) normalized by body weight (g) of
 178 mice after experimental diets. **C)** Fasting glucose of animals after experimental diets. **D)** Daily
 179 food intake (n=10). Values are shown as mean \pm SEM, n=15 animals per group in 3 days
 180 experimental group and n= 10 animals per group in 1-week experimental group * $p < 0,05$ and ***
 181 $p < 0,0001$.

182 **3.2 Short-term exposure to high fat diet leads to differential regulation of CMA in** 183 **hypothalamus**

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

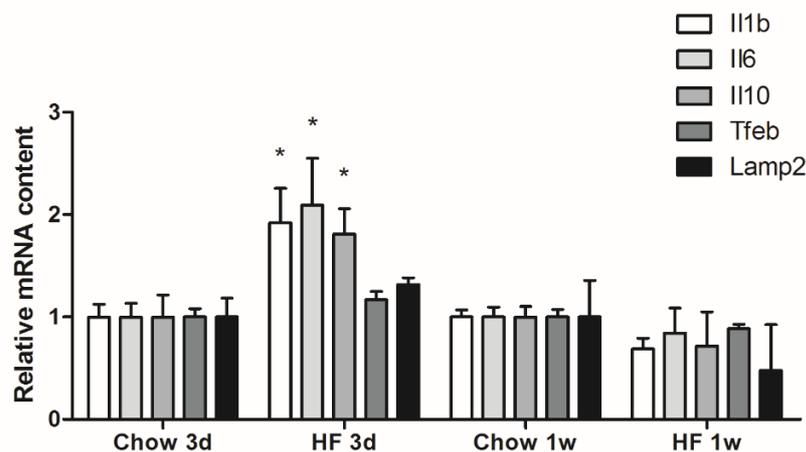
189 hypothalamus of mice fed HFD for 1 week (Figure 2 D), and the protein content of
 190 HSC70 was decreased with no difference in the content of LAMP2a after 1 week of
 191 experimental diet (Figure 2 E and F).



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

199
 200 It is well established that obesity leads to hypothalamic inflammation in animal models
 201 (1, 4). In our experimental model, there was a transient increase in mRNA of *I11 beta*, *I16*
 202 and also *I10* in hypothalamus of mice after 3 days on HFD compared to chow diet
 203 group. No change in the hypothalamic expression levels of *Tfeb* gene in hypothalamus,

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



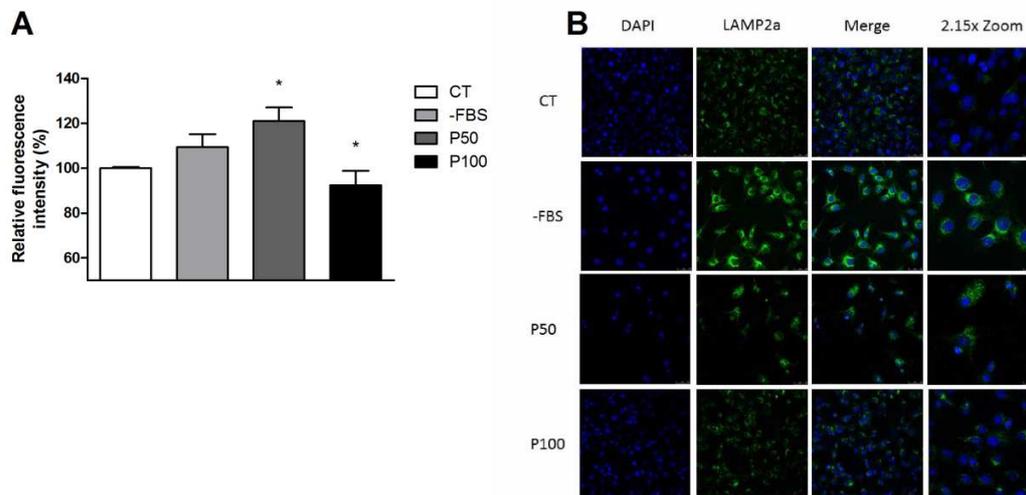
207
 208 **Figure 3.** Cytokines and autophagy-related gene expression on mice fed HFD for 3 days and 1
 209 week. qRT-PCR was used to analyze the gene expression of Il1b, Il6, Il10, Tfeb and Lamp2 in
 210 the hypothalamus of mice with 3 days of treatment. Values are shown as mean \pm SEM, n= 4-5
 211 animals per group in HFD 3 days and n=3 animals per group in HFD 1 week group, *p < 0.05.

212

213 **3.4 Palmitate induces chaperone-mediated autophagy in hypothalamic neurons**

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

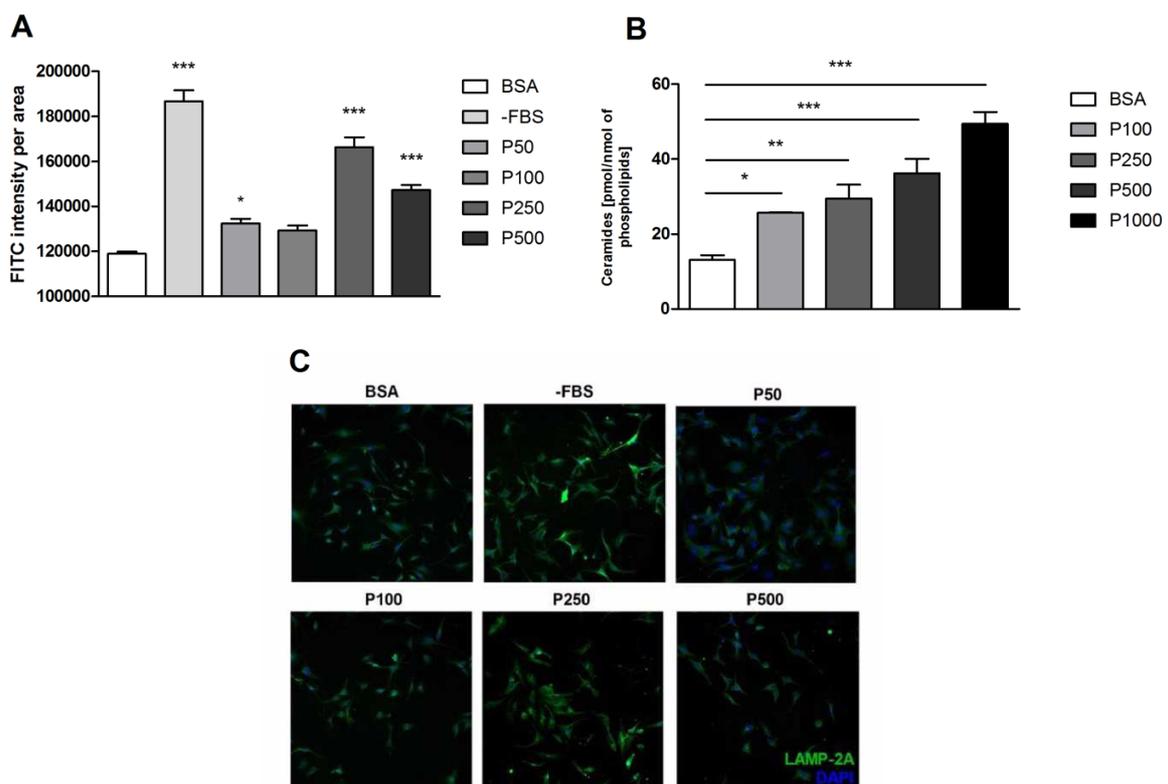
222 fluorescence near the nucleus. In contrast, the treatment with 100 μ M of palmitate
 223 decreased LAMP2a staining in mHypoA-2/29 cells (Figure 4 A and B).



224
 225 **Figure 4.** LAMP2a immunofluorescence in hypothalamic neurons. **A)** Relative LAMP2a
 226 fluorescence after serum deprivation (-FBS) or treatment with 50 μ M (P50) and 100 μ M (P100)
 227 of palmitate for 24 hours. **B)** Representative images of cell nuclei labeled with Dapi (blue) and
 228 LAMP2a with AlexaFluor488 (green) with serum deprivation and palmitate treatment in
 229 mHypoA-2/29 cells. Three independent experiments were performed. Values are shown as
 230 mean \pm SEM, * $p < 0.05$.

231
 232 Another neuronal cell line, the GT1-7 cells, were also treated with different
 233 concentrations of palmitate. To analyze chaperone-mediated autophagy in this model,
 234 High Content Screening was performed. This method consists of a cellomics approach
 235 that involves automated microscopy and image analysis of several compounds in
 236 parallel, the output includes the measurement of fluorescent intensity of LAMP2a within
 237 each treatment. First, we confirmed that 24 hours of fasting was sufficient for maximum
 238 activation of chaperone-mediated autophagy in GT1-7 cells (Figure 5 A and C). Also,

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



246

247 **Figure 5.** HCS for CMA quantification in hypothalamic neurons. A) LAMP2a quantification by
 248 HCS after prolonged starvation or different concentrations of palmitate exposure for 24 hours.
 249 B) Ceramide quantification after palmitate treatment for 24 hours. C) Representative images of
 250 HCS analysis. Three independent experiments were performed. Values are shown as mean \pm
 251 SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

252 **4. Discussion**

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

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

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

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

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

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

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

304 **5. Conclusions**

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

310

311 **Availability of data and materials**

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

314

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318

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323 Pessoal de Nível Superior – CAPES [#001].

324

325 **Contributions**

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

332

333 **Ethics approval**

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

336

337 **Competing interests**

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

340

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436

437 **Supplementary Figure**

438 **Supplementary Figure 1.** Cell viability detected by flow cytometry. GT1-7 cells were
439 treated with 50 or 250 μ M PA or control (H_2O) following the described period. After, cell
440 viability (%) was assessed using PI solution (1 μ g/ mL). (A-C) % of viable cells after PA
441 treatment. (D) Morphological parameter (Size, termed FSC-A) after PA treatment in
442 both embryonic and adult cells, respectively. (E-H) Representative morphological
443 parameters (FSC-A X SSC-A). Data are expressed as mean \pm SEM; *P < 0.05. The
444 experiment was performed one time using 3 wells/group. Statistical analysis was
445 performed by one-way ANOVA with Dunnet post hoc test comparing the mean of each
446 column with the means of the control column.

447 **Supplementary Figure 2.** Cell viability detected by flow cytometry. mHypoA 2/29 cells
448 were treated with 50 or 250 μ M PA or control (BSA) following the described period.
449 After, cell viability (%) was assessed using PI solution (1 μ g/ mL). (A) Representative
450 morphological parameters (FSC-A X SSC-A). (B) % of viable cells after PA treatment.
451 Data are expressed as mean \pm SEM; *P < 0.05. The experiment was performed three
452 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.

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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.

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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.

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