Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 34 (2016) 30–41

Journal of Nutritional Biochemistry

Autophagy proteins are modulated in the liver and hypothalamus of the offspring of mice with diet-induced obesity☆,☆☆

Andressa Reginato[a], Thaís de Fante[a], Mariana Portovedo[a], Natália Ferreira da Costa[a], Tanyara Baliani Payolla[a], Josiane Érica Miyamotto[a], Laís Angélica Simino[a], Letícia M. Ignácio-Souza[b], Márcio A. Torsoni[a], Adriana S. Torsoni[a], Marciane Milanski[a,*]

[a]Faculdade de Ciências Aplicadas, Universidade Estadual de Campinas, UNICAMP, Limeira, SP, Brazil
[b]Faculdade de Nutrição, Universidade Federal do Mato Grosso, UFMT, Cuiabá, MT, Brazil

Received 8 August 2015; received in revised form 6 April 2016; accepted 7 April 2016

Abstract

Nutritional excess during pregnancy and lactation has a negative impact on offspring phenotype. In adulthood, obesity and lipid overload represent factors that compromise autophagy, a process of lysosomal degradation. Despite knowledge of the impact of obesity on autophagy, changes in offspring of obese dams have yet to be investigated. In this study, we tested the hypothesis that maternal obesity induced by a high fat diet (HFD) modulates autophagy proteins in the hypothalamus and liver of the offspring of mice. At birth (d0), offspring of obese dams (HFD-O) showed an increase in p62 protein and a decrease in LC3-II, but only in the liver. After weaning (d18), the offspring of HFD-O animals showed impairment of autophagy markers in both tissues compared to control offspring (SC-O). Between day 18 and day 42, both groups received a control diet and we observed that the protein content of p62 remained increased in the livers of the HFD-O offspring. However, after 82 days, we did not find any modulation in offspring autophagy proteins. On the other hand, when the offspring of obese dams that received an HFD from day 42 until day 82 (OH-H) were compared with the offspring from the controls that only received an HFD in adulthood (OC-H), we saw impairment in autophagy proteins in both tissues. In conclusion, this study describes that HFD-O offspring showed early impairment of autophagy proteins. Although the molecular mechanisms have not been explored, it is possible that changes in autophagy markers could be associated with metabolic disturbances of offspring.

Keywords: High-fat diet; Obesity; Offspring; Autophagy; Hypothalamus; Liver

1. Introduction

Despite the fact that it is well known that obesity (body mass index ≥ 30 kg/m^2) is a global health problem, its worldwide prevalence almost doubled between years 1980 and 2008 [1]. Several studies in recent years describe how prenatal obesity or malnutrition is a strong risk factor for the development of chronic diseases in offspring, creating the concept of "metabolic programming" [2,3], when an abnormal metabolic environment occurs at a critical or sensitive period of development. During these early periods of "plasticity", fetal metabolic changes that promote survival in an inappropriate environment may remain into adulthood. Thus, in adulthood, the nutritional abundance promoted by the consumption of a western diet (which is very common nowadays) may lead to metabolic disturbances that promote the development of diseases, such as hypertension, obesity and diabetes [3–5].

Autophagy, an important cellular pathway, has implications in the development of chronic disease. It is one of the most powerful cell cleaning systems during which cellular organelles, proteins and invading microorganisms are degraded by lysosomes, maintaining a balance between the synthesis, degradation and subsequent recycling of cellular components, providing homeostasis and cell survival [6,7]. It has been shown that autophagy is divided into three types: macroautophagy, microautophagy and chaperone-mediated autophagy, depending on the physiological functions and the molecular components involved in each of these steps [7–10]. Macroautophagy (hereafter referred to as autophagy) is responsible for more than 90% of cellular autophagy [11]; thus, this paper focuses on this mechanism.

☆ The present study was supported by São Paulo Research Foundation (FAPESP) process 2011/51.205-6.

☆☆ A.R. contributed to design research, conducted research, reviewed the literature, conducted the statistical analysis and wrote the manuscript. T.F., M.P., N.F.C., T.B.P., J.E.M. and L.A.S. contributed to experimental planning and discussion of results. L.M.I.-S., M.A.T., A.S.T. and M.M. provided essential reagents, materials and others' financial support besides providing advice regarding interpretation of the results. L.M.I.-S. edited the manuscript and J.E.M. edited the reviewer's answer. M.M. was responsible for design research, conduct of research and data collection and provided advice regarding and edited the manuscript.

* Corresponding author. Laboratório de Distúrbios do Metabolismo, Faculdade de Ciências Aplicadas, Universidade Estadual de Campinas, Pedro Zaccarias, 13484-350, Limeira, SP, Brazil. Tel.: +55-19-37016705.
E-mail address: marciane.milanski@fca.unicamp.br (M. Milanski).

http://dx.doi.org/10.1016/j.jnutbio.2016.04.002
0955-2863/

Recent studies have highlighted the importance of the association between autophagy and lipid content. A high fat diet (HFD) is responsible for the reduction of autophagosome, with lysosome fusion suggesting that the lipid composition of membranes is important in the homeostasis of autophagy [12]. Other animal experiments show that chronic exposure to an HFD is associated with inflammation and impairment in autophagy activity in the region of the basal medium hypothalamus, resulting in changes in the control of energy balance, which are responsible for accelerating the development of obesity and other associated metabolic disorders [13]. Additionally, our group recently reported that hypothalamic autophagy dysfunction occurs after chronic exposure to an HFD *via* the direct effects of saturated fatty acids [14].

In peripheral tissues, such as the liver, autophagy is also crucial for normal function in addition to preventing the development of liver diseases, such as steatosis and nonalcoholic fatty liver disease [15]. Autophagy inhibition in both hepatocytes and the livers of mice leads to increased triglyceride storage in lipid droplets, demonstrating that autophagy plays a critical role in lipid metabolism with important implications for human diseases characterized by lipid overaccumulation, such as those that comprise the metabolic syndromes [16]. Consistent with this idea, other researchers have found a decrease in liver autophagy accompanied by an increase in Beclin-1, Atg7 and Atg5 protein degradation, which may be an important mechanism explaining the impairment of autophagy during the development of obesity after exposure to an HFD [17].

Therefore, we hypothesized that obesogenic intrauterine and lactation environments can have a negative impact on autophagy modulation. We described, for the first time, if exposure to an HFD *in utero*, during lactation and in adulthood can alter autophagy proteins in the offspring of mice. Our results show that, at birth (d0), the offspring from obese dams (HFD-O) show increases in p62 protein content and decreases in LC3-II in the liver when compared to control offspring (SC-O). After weaning (d18), we found modulations of autophagy markers in both the hypothalamus and the liver of HFD-O animals. At 42 days, the animals from both groups received a control diet and the results show that only the livers of the HFD-O animals showed an accumulation of p62 protein. However, at 82 days, control animals (OC-C *vs.* OH-C) showed no impairment of autophagy proteins in either of the tissues analyzed. We also fed the animals with an HFD from 42 days until 82 days and observed that offspring from obese dams (OH-H) had impairments in autophagy proteins when compared with offspring that received the HFD only in adulthood (OC-H). Thus, the changes observed in offspring autophagy modulation, as well as the experimental models in adulthood as previously reported, also appear to be linked to exposure to an HFD and dam obesity.

2. Materials and methods

2.1. Animal procedures

All experiments were approved by the Ethical Committee for Animal Use (ID protocol: 3383-1) of the Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil, following all the recommendations in the Brazilian College of Animal Experimentation guidelines. Virgin female and male (5-week-old) Swiss mice were obtained from the breeding colony at UNICAMP and maintained in individual polypropylene cages at 22°C±1°C with lights on from 6:00 a.m. to 6:00 p.m. Before mating, the females were randomly divided into two groups: females fed with standard chow (SC) or females fed an HFD. Both groups had free access to food and water for 3 weeks to allow them to adapt. The HFD was prepared according to the AIN-93G, modified for a high fat (45%) content based on a protocol previously published by our group [18,19]. Mating was performed by housing females with adult males (fed with SC) for 1 week. Pregnancy was confirmed by monitoring the dams' weight. Pregnant females were separated again into individual cages and received the same diet (SC or HFD) *ad libitum* during pregnancy and lactation. On the first day after birth (d0), some animals were killed for experimental analyzes (d0 group). Then, the litter size of both groups was adjusted to eight animals per litter using males and females. However, after this point, only the males were used to evaluate metabolic parameters. Male offspring were weaned and killed at 18 days, representing the second experimental group (d18). At both time points, the hypothalamus and liver were extracted; however, at 18 days, adipose tissue was also extracted from both groups (SC-O and HFD-O). After weaning, male offspring were fed with SC until 42 days old. At this age, we also examined metabolic parameters and performed tissue extraction from both groups (SC-O and HFD-O). We divided the remaining animals into four groups: offspring of control dams fed with SC (OC-C), offspring of control dams fed an HFD (OC-H), offspring of HFD dams fed with SC (OH-C) and offspring of HFD dams fed an HFD (OH-H). The animals received each type of diet until 82 days of life. At the end of the experimental period, all the male mice were killed and the hypothalamus, liver and adipose tissue were extracted, giving the third experimental group (d82). The weight of the adipose tissue in all the experimental groups was expressed as a percentage, adjusted by animal weight. The full design of the study is shown in Fig. 1.

2.2. Metabolic assessments

Maternal weight was measured throughout the experimental period. After weaning, we also measured the adiposity of the dams. Offspring fasting glucose was measured at 18 days (with 4 h of fasting) and at 42 and 82 days (with 12 h of fasting) using an Accu-Chek Performa glucometer. To obtain the Lee Index [from the ratio of $BW(g)^{1/3}$/nasoanal length (cm)×1000], we measured the body weight (BW) and the length of the offspring at birth (d0) and at weaning (d18) [20]. The consumption of breast milk was obtained based on the weight of fasted and fed pups at 16 days, following the procedure previously described [21]. In adulthood (d42 and d82), food intake was evaluated. In the d42 group, we assessed the consumption of the control diet, and in the d82 group, we assess the consumption of the SC and HFD, after 2 weeks of adaptation, by calculating the difference between the initial and final weight of the food over 5 days. Values of food intake were expressed as a percentage of calories corrected by animal weight. Commercial kits were used, in line with the manufacturer's instructions, for the quantification of serum cholesterol (CHOD-PAP/Roche Diagnostics), serum triglycerides (GPO-PAP, Roche Diagnostics) and serum or plasma TNF-α (Mouse TNF-α, DY410-05, R&D Systems). The number of animals used in each metabolic assessment is described in detail in each figure legend. In summary, we used 10–30 animals to weight assessments and 5–15 animals to adiposity and glucose measures.

2.3. Immunoblotting

After extraction, tissue samples from the hypothalamus and liver (d0, d18, d42 and d82) were homogenized in freshly prepared ice-cold buffer [1% (v/v) Triton X-100, 0.1 mol/L Tris, pH 7.4, 0.1 mol/L sodium pyrophosphate, 0.1 mol/L sodium fluoride, 0.01 mol/L EDTA, 0.01 mol/L sodium vanadate, 0.002 mol/L PMSF and 0.01 mg/ml aprotinin]. Insoluble material was removed by centrifugation (10,000*g*) for 25 min at 4°C. The protein concentration of the supernatant was determined using the Bradford dye-binding method (hypothalamus) or Biuret (liver). The supernatant was suspended in Laemmli sample buffer and boiled for 5 min before separation by SDS-PAGE using a miniature gel apparatus (BioRad, Richmond, CA, USA). Following electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were treated with a blocking buffer (5% nonfat dried milk, 10 mM Tris, 150 nM NaCl and 0.02% Tween 20) and were subsequently incubated overnight at 4°C with specific antibodies (anti-SQSTM1/ab91526 and anti-LC3B/ab48394). The membranes were then incubated with HRP-conjugated secondary antibodies (KPL, Gaithersburg, MD, USA). The proteins recognized by the secondary antibodies were detected by chemiluminescence (Amersham ECL, kit RPN 2232, Buckinghamshire, UK) and visualized by exposing the blot to Kodak XAR film. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software, ScionCorp, Walkersville, MD, USA) and the intensities of the bands were normalized to the loading of control β-actin (ab8227). All analyses were done using three to six animals per group.

2.4. Real-time quantitative PCR

A real-time quantitative PCR was performed to measure the mRNA levels of the TNF-α. Hypothalamic and liver total RNA was extracted using a Trizol reagent, in line with the manufacturer's instructions (Invitrogen Corporation, California, USA). Total RNA was quantified on a Nanodrop ND-2000 (Thermo Electron, Madison, WI, USA). Reverse transcription was performed using total RNA from the hypothalamic and liver samples. A real-time PCR analysis of gene expression was performed in an ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems). The primers used were obtained from Applied Biosystems, TNF-α (Mm00443258_m1), IL-1β (Mm00434228_m1), IL-10 (Mm00439614_m1) and GAPDH as the endogenous control (4352339E mouse GAPDH, Life Technologies Corporation). Nos2 was obtained from Integrated DNA Technologies (MmPT5843744771). Each PCR contained 20 ng of reverse-transcribed RNA and was run in line with the manufacturer's recommendations using the TaqMan PCR Master Mix (Applied Biosystems). Target mRNA expression was normalized to GAPDH expression and expressed as a relative value using the comparative threshold cycle (Ct) method ($2-\Delta\Delta Ct$) in line with the manufacturer's instructions. All analyses were carried out using four to six animals per group.

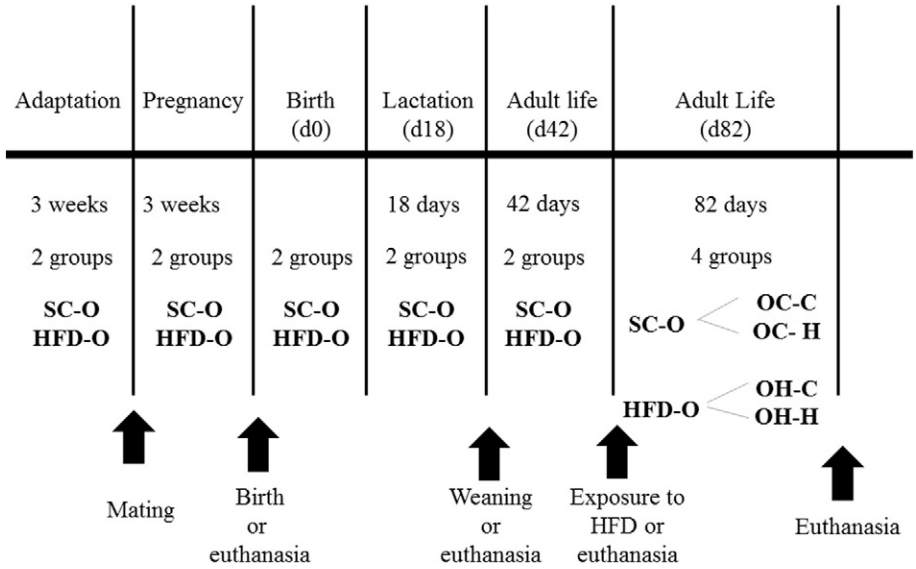

Fig. 1. Design of the study followed to obtain offspring control (SC-O) or HFD (HFD-O)wherein dams were fed with SC or HFD 45%. After 42 days of life, offspring were divided into four groups and received SC or HFD 45% until 82 days.

2.5. Statistical analysis

The results are expressed as the mean ± SE of the indicated number of experiments. Blot results are presented as direct band comparisons in autoradiographs and quantified by densitometry using Scion Image software. The statistical analyses were carried out using Student's *t* tests for unpaired samples or an ANOVA and Tukey *post hoc* tests for multiple comparisons, and the level of significance was set at $P<.05$.

3. Results

3.1. HFD alters weight and adiposity of dams

To examine the effects of maternal obesity induced by diet (DIO) on the hypothalamus and liver autophagy of offspring, female mice were fed with an HFD (45%). As expected, the dams fed an HFD showed an increase in body mass after 2 weeks, maintaining the difference throughout pregnancy (56.93 g ± 3.93 *vs.* 30.63 g ± 2.91, respectively) and lactation (50.33 g ± 1.18 *vs.* 40.27 g ± 0.55, respectively), compared to control dams (Fig. 2A). The increase in body mass was accompanied by increased gonadal adipose tissue (1.55 g ± 0.20 in SC dams *vs.* 3.54 g ± 0.39 in HFD dams) and rWAT (0.74 g ± 0.13 in SC dams *vs.* 0.25 g ± 0.04 in HFD dams) (Fig. 2B).

3.2. Metabolic parameters and autophagy evaluation of offspring on the day of birth

The weight of offspring from obese dams was significantly lower (11%) than those from lean dams (Fig. 3A). This result was accompanied by a decrease in the Lee Index (Fig. 3B), although no differences were observed in TNF-α plasma levels, cholesterol or triglycerides serum levels (Fig. 3C–E). After assessing metabolic parameters, we investigated the modulation of inflammatory and autophagy markers. The gene expression of TNF-α, IL-1β and IL-10 as well as p-JNK did not differ in any of the tissues analyzed (Fig. 4). However, HFD-O animals exhibited a decrease in liver Nos2 relative gene expression (Fig. 4I). No differences in autophagy markers in the hypothalamus were observed between the groups (Fig. 5A and B). With this result, we decided to investigate whether the liver showed

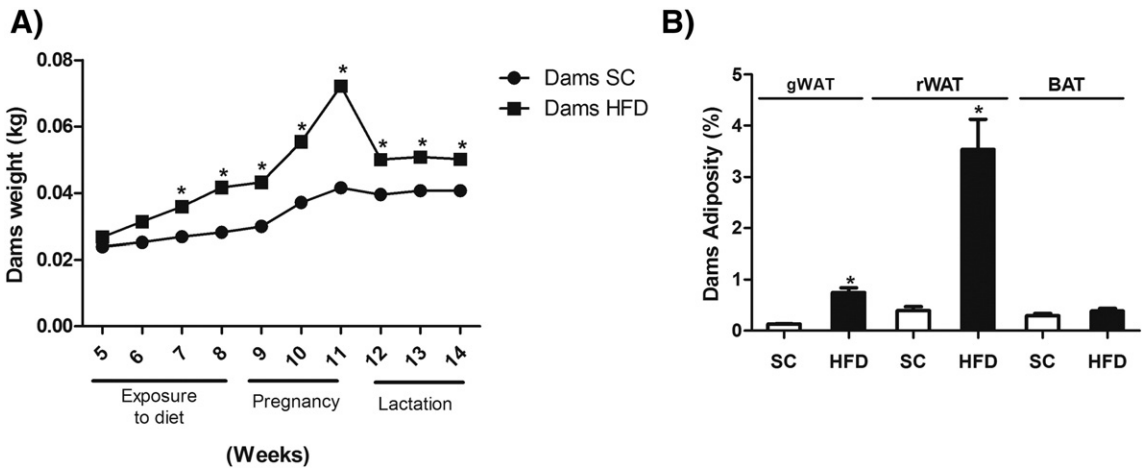

Fig. 2. Body weight and adiposity of dams (SC or HFD) during pregnancy and after weaning. **(A)** Weight of dams since exposure to SC or HFD until the weaning. **(B)** Adiposity (%) of dams after weaning (gWAT, gonadal white adipose tissue; rWAT, retroperitoneal white adipose tissue; BAT, brown adipose tissue). Values are mean ± SEM, $n = 5–7$ dams per group. *$P<.05$; *SC *vs.* HFD.

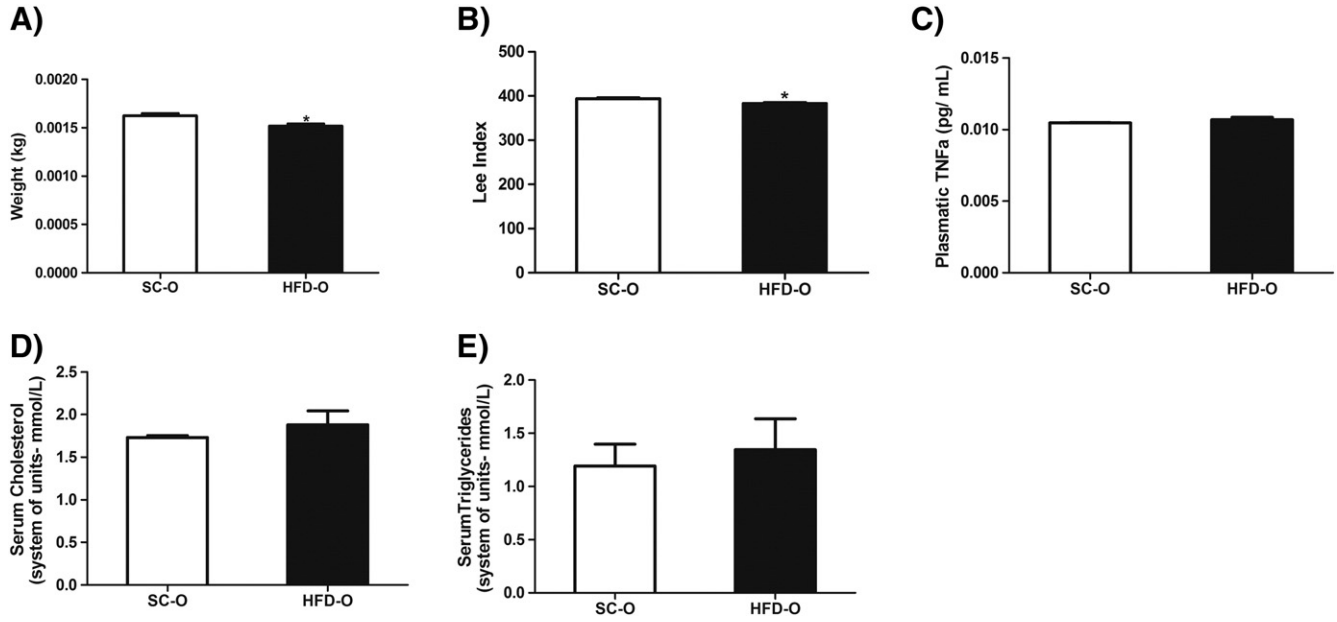

Fig. 3. Metabolic characterization of male offspring (SC-O or HFD-O) at birth (d0). **(A and B)** Body weight and Lee Index, respectively ($n=30$ pups per group). **(C)** Plasma TNF-α ($n=6$ per group). **(D)** Serum cholesterol ($n=6$ per group). **(E)** Serum triglycerides ($n=6$ per group). Values are mean ± SEM, *$P<.05$; *SC-O *vs.* HFD-O.

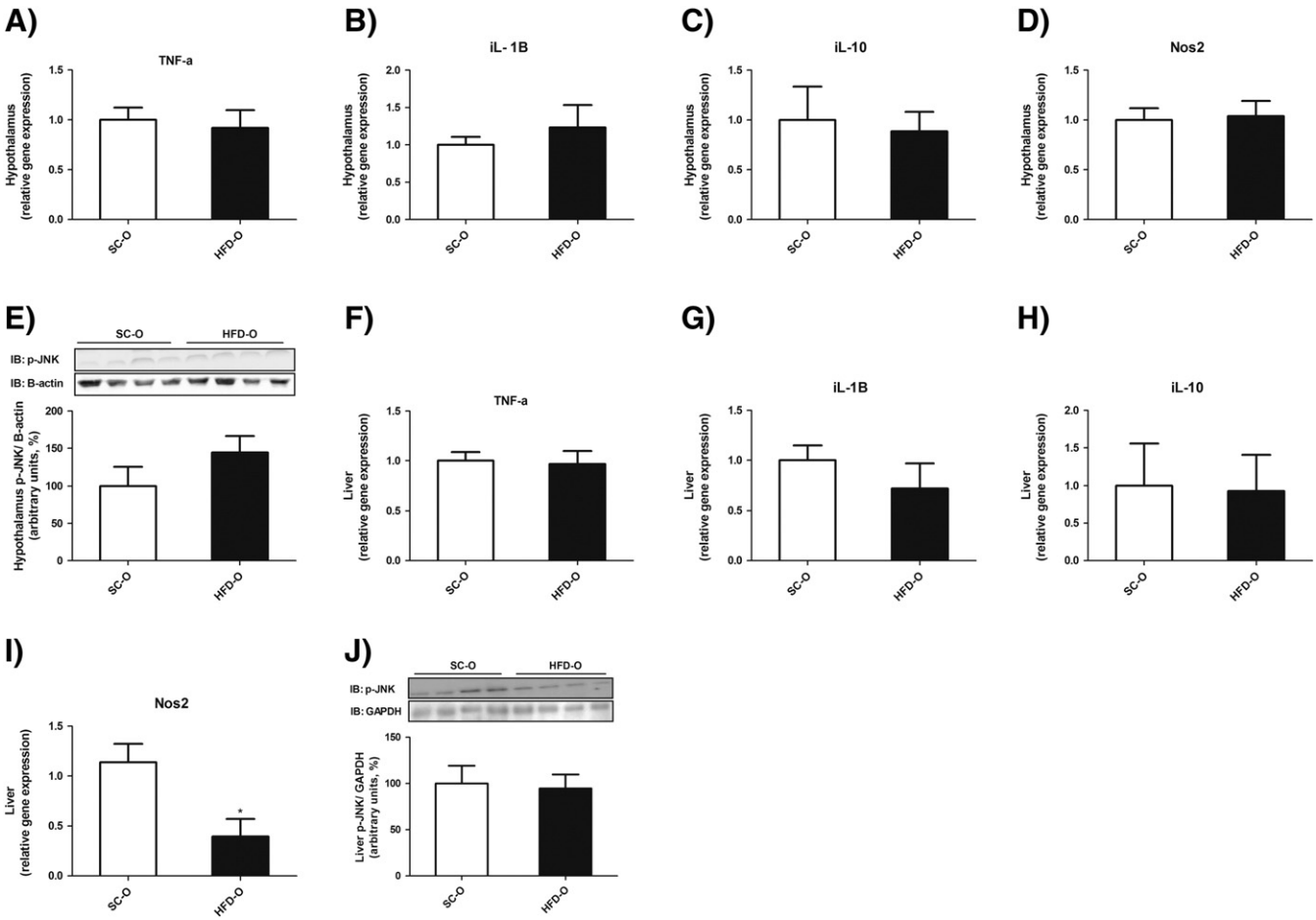

Fig. 4. Inflammatory markers in hypothalamus and liver of male offspring (SC-O or HFD-O) at birth (d0). **(A)**, **(B)**, **(C)** and **(D)** represent hypothalamus relative gene expression and **(F)**, **(G)**, **(H)** and **(I)** represent liver relative gene expression of TNF-α, IL-1β, IL-10 and Nos2, respectively. Representative western blotting of hypothalamus **(D)** and liver **(H)** of p-JNK corrected by β-actin or GAPDH. Values are mean ± SEM, $n=3$–5 pups per group. *$P<.05$; *SC-O *vs.* HFD-O.

any changes in autophagy. Interestingly, we observed an increase in protein content of p62 (Fig. 5C) and a decrease in the conversion of LC3-I to LC3-II (Fig. 5D), suggesting a decreased autophagy process in the liver of offspring from obese dams at birth. Additionally, at birth, HFD-O animals presented an increase in relative gene expression of markers of triglycerides synthesis such as GPAM and AGPAT (unpublished data).

3.3. Metabolic parameters of male offspring at lactation

Next, we evaluated the metabolic characteristics of the offspring during lactation. The offspring of obese dams showed differences in body weight from day 14 until day 18 compared to control mice (1.4-fold in both ages) (Fig. 6A). This was accompanied by an increase in the Lee Index (Fig. 6B) and an increase in eWAT (0.18 g ± 0.08 in SC-O mice *vs.* 1.42 g ± 0.26 in HFD-O mice), rWAT (0.10 g ± 0.12 in SC-O mice *vs.* 0.41 g ± 0.10 in HFD-O mice) and BAT (0.49 g ± 0.07 in SC-O mice *vs.* 0.69 g ± 0.13 in HFD-O mice) tissues (Fig. 6C). We also observed an increase in fasting glucose, serum cholesterol and triglycerides (Fig. 6D–F). No difference in serum TNF-α was found (Fig. 6G). In addition, the consumption of breast milk in the offspring of obese mothers was higher compared with the control animals (Fig. 6H). Animals at 18 days did not show any differences in energy expenditure, measured by indirect calorimetry (data not showed).

3.4. Autophagy evaluation of male offspring at weaning

At weaning, we observed an increase in the gene expression of TNF-α in the hypothalamus and decrease of IL-10 and Nos2 gene expression in the livers of animals (Fig. 7A, H and I, respectively). The livers of HFD-O animals receiving diet control to 10 days after weaning presented increase in hepatic lipid content when compared to SC-O group (0,41,431 ± 0,06,334 *vs.* 0,15,037 ± 0,01,718 arbitrary units, respectively). Regarding autophagy markers such as observed at birth, liver autophagy remained decreased in the HFD-O group compared to the SC-O group (Fig. 8C and D). In addition, lactation resulted in negative regulation of the hypothalamic autophagy, which was demonstrated by an increase in p62 protein (Fig. 8A) and a decrease in LC3-II (Fig. 8B) in the HFD-O offspring compared to the SC-O offspring.

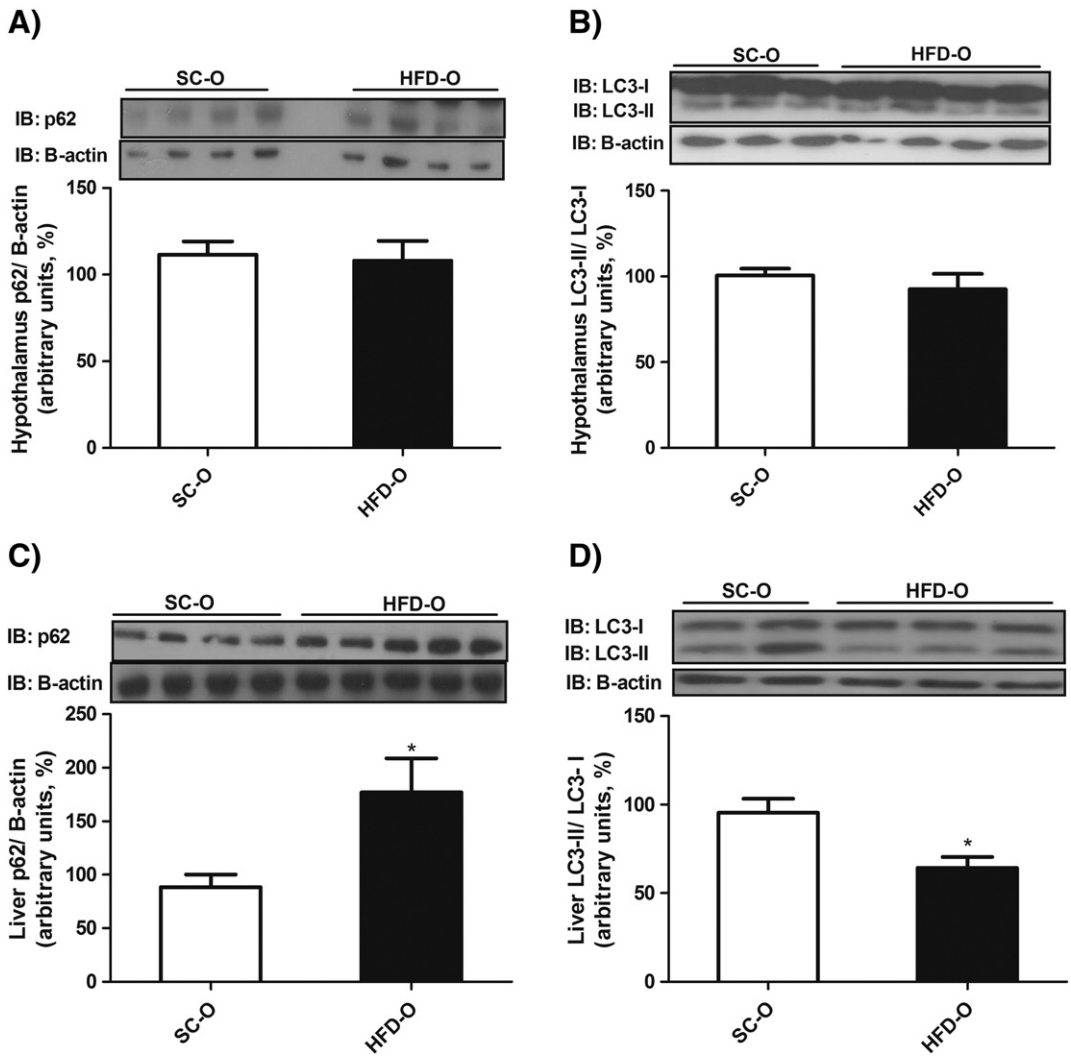

Fig. 5. Protein content of autophagy markers in hypothalamus and liver of male offspring (SC-O or HFD-O) at birth (d0). Representative western blotting of hypothalamus **(A)** p62/β-actin. **(B)** LC3-II/LC3-I. Representative western blotting of liver **(C)** p62/β-actin. **(D)** LC3-II/LC3-I. Values are mean ± SEM, $n = 3–6$ pups per group. *$P<.05$; *SC-O *vs.* HFD-O.

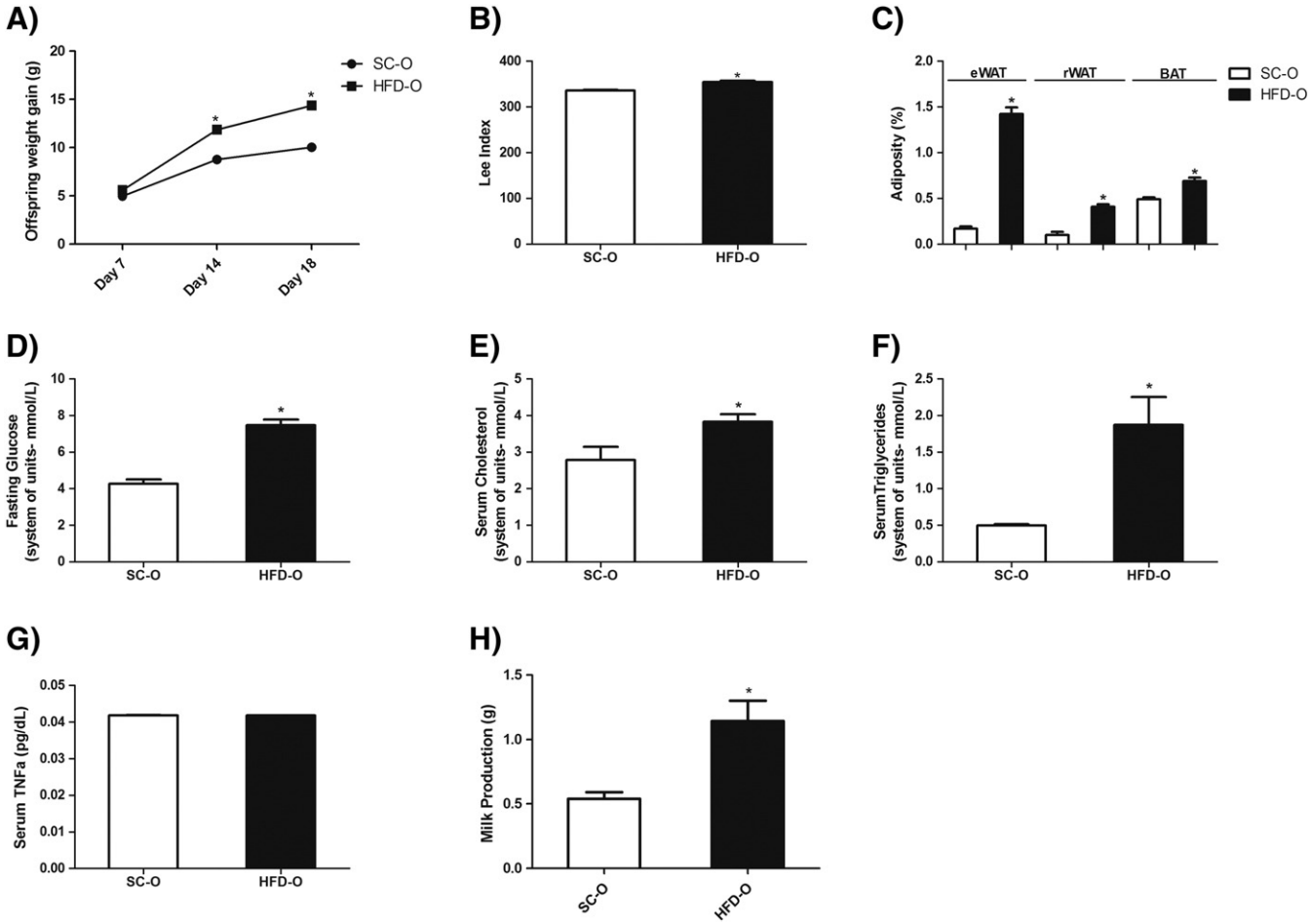

Fig. 6. Metabolic parameters of male offspring (SC-O or HFD-O) during lactation until weaning (d18). **(A)** Weight during lactation ($n=30$ per group). **(B)** Lee Index during lactation ($n=30$ per group). **(C)** Adiposity corrected by weight at day 18 ($n=14$ per group) (eWAT, epididymal white adipose tissue; rWAT, retroperitoneal white adipose tissue; BAT, brown adipose tissue). **(D)** Fasting glucose at day 18 ($n=16$ per group). **(E)** Serum cholesterol at day 18, **(F)** serum triglycerides at day 18 and **(G)** serum TNF-α at day 18 ($n=4$–6 per group). **(H)** Milk production at day 16 ($n=20$ per group). Values are mean ± SEM, *$P<.05$; *SC-O *vs.* HFD-O.

3.5. Metabolic parameters and autophagy evaluation of offspring in adulthood

After weaning, we fed animals from both groups (SC-O and HFD-O) with SC until day 42. At this age, offspring from the obese dams (HFD-O) still had a higher body mass (12%), which had developed during lactation (Fig. 9A). The HFD-O animals also had higher fasting glucose and adiposity (5%) (Fig. 9C and D, respectively). The relative caloric intake was not different between the groups (Fig. 9B). Interestingly, although we found changes in the protein content of p62 and LC3-II in the hypothalamus at weaning, no differences were found at 42 days. Thus, only the liver had an accumulation of p62 protein (Fig. 9F–H).

To evaluate the effects of HFD reexposure in the modulation of autophagy in adulthood in the offspring from SC and HFD-fed dams, the animals were fed with SC or HFD diet from day 42 until day 82, resulting in four experimental groups (as described in Fig. 1). We found that the reexposure to HFD was effective in increasing the body mass of offspring in adulthood (by using the ANOVA variance analysis). After this analysis, we performed comparisons between groups that remained on the same diet in adulthood (d82). HFD offspring (OH-C) fed with SC maintained their increased body mass (11.3%) (Fig. 10A), fasting glucose (1.4-fold) (Fig. 10C) and adiposity (eWAT, 1.3-fold; rWAT, 1.4-fold; BAT, 1.3-fold) (Fig. 11D) compared to OC-C despite no differences in the relative caloric intake (Fig. 11B). The OH-C offspring did not show any modulation in autophagy markers in the hypothalamus (Fig. 11A and B). Unexpectedly, the liver showed no alterations in the protein content of autophagy markers (Fig. 11C and D). When the SC-O and HFD-O groups were exposed to an HFD (OC-H and OH-H groups), the comparison between the OH-H *vs.* OC-H showed an increase in body mass (11%) (Fig. 10E), accompanied by an increase in caloric intake (1.2-fold) (Fig. 10F), fasting glucose (1.5-fold) (Fig. 10G) and adiposity (eWAT, 1.2-fold; rWAT, 1.3-fold) (Fig. 10H). Regarding autophagy markers, we saw an increase in the protein content of p62 in hypothalamus (Fig. 11E) and liver (Fig. 11G) tissues in the OH-H group compared to the OC-H group. However, the protein content of the LC3-II only decreased in the hypothalamus (Fig. 11F and H).

4. Discussion

It is well documented that autophagy has several physiological and pathological properties, including the adaptive response to starvation, quality control of intracellular proteins and organelles, antiaging, suppression of tumor formation, elimination of intracellular pathogens and antigen presentation [8,22]. In physiological conditions, autophagy plays important roles during development by driving fast cellular changes. Thereafter, the autophagy process provides a favorable environment for differentiation and development [23]. When autophagy is defective during these periods, some abnormalities in various organisms, such as fungi [24], protozoa [25] and insects

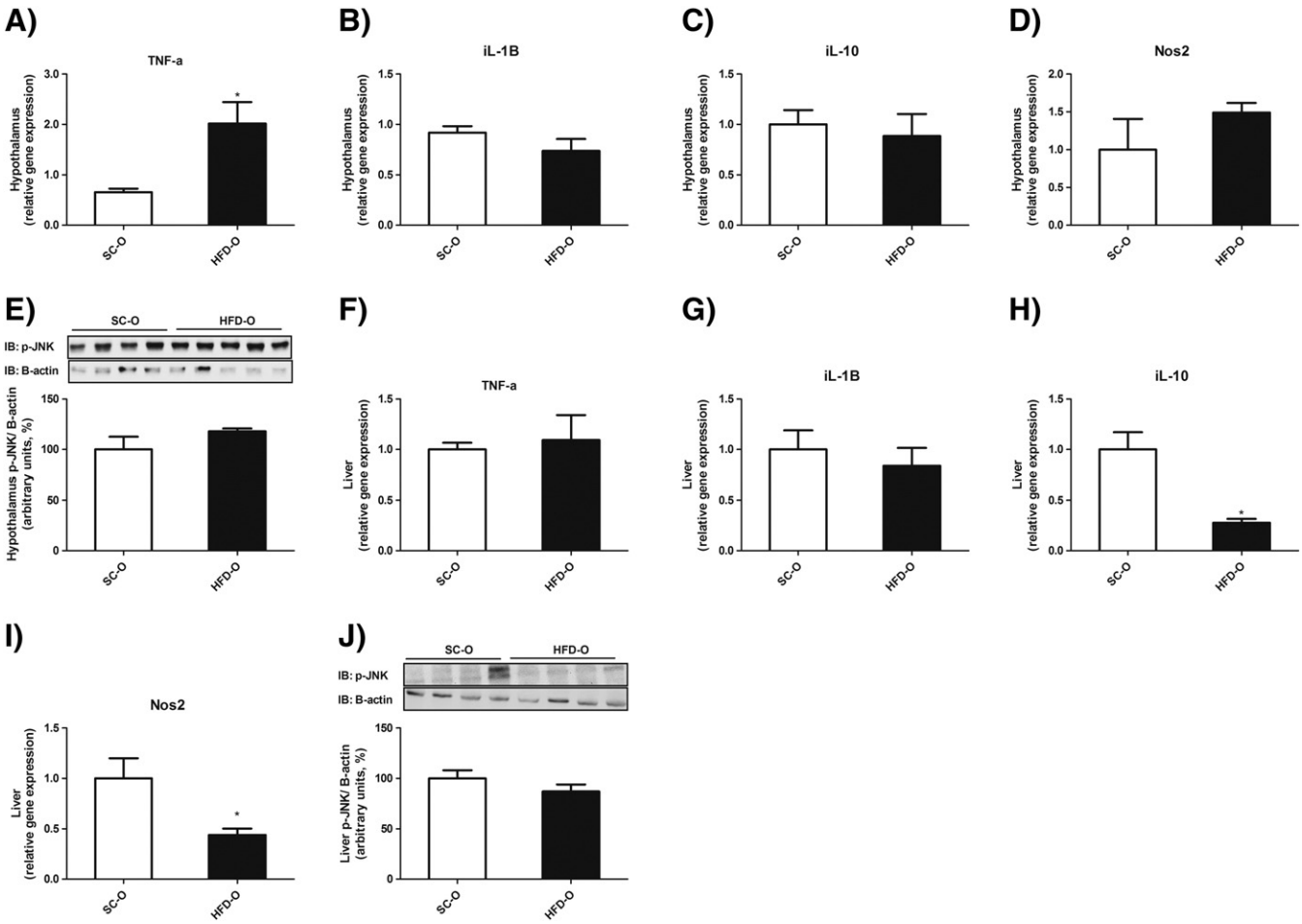

Fig. 7. Inflammatory markers in hypothalamus and liver of male offspring (SC-O or HFD-O) at weaning (d18). **(A)**, **(B)**, **(C)** and **(D)** represent hypothalamus relative gene expression and **(F)**, **(G)**, **(H)** and **(I)** represent liver relative gene expression of TNF-α, IL-1β, IL-10 and Nos2, respectively. Representative western blotting of hypothalamus **(D)** and liver **(H)** of p-JNK corrected by β-actin. Values are mean ± SEM, $n = 3–5$ pups per group. *$P<.05$; *SC-O *vs.* HFD-O.

[26], have been reported. In the context of obesity, some studies have shown that autophagy is often compromised in the central nervous system [13] and peripheral tissues [15,17], probably contributing to the progression of this disease. This study investigated the hypothesis that maternal obesity, induced by an HFD, modulates autophagy proteins in the hypothalamus and liver of the offspring of mice.

Our first result demonstrates that the offspring of obese dams have reduced body mass, as previously reported [19,27], and show a decrease in the Lee Index despite no changes in any of the other metabolic parameters evaluated. Although the reason for the reduced body size is unclear, we hypothesize that the placental function or the inefficient reutilization of nutrients during embryogenesis may be important in maternal obesity. Interestingly, animals with ablation of some Atgs genes, essential to the autophagy pathway, showed a similar reduction in body mass at birth [28,29].

Furthermore, we also observed down-regulation of autophagy in the livers of offspring from obese dams but no differences in hypothalamus tissue. Importantly, exposure to HFD only during pregnancy was already enough to induce early ectopic lipid accumulation (Simino *et al.*, unpublished data) and negative modulation of autophagy markers in our experimental model. Also, these inflammatory markers were not increased between groups leading us to suspect that autophagy markers may be linked to earlier hepatic lipid accumulation. Moreover, some date of neonates deficient in Atg5 or Atg7 demonstrated that, after exposure to starvation, deficient animals had shorter survival rate when compared to wild-type mice, showing that autophagy homeostasis is important from the first moments of a neonate's life [28,29].

Additionally, it has been reported that autophagy is involved in placenta development during human pregnancy [30]. Maternal obesity during pregnancy contributes to increased placental permeability by up-regulation of placental amino acid transporters, resulting in the activation of mTOR signaling [31]. This activation could down-regulate autophagy in the liver tissue of neonates as mTOR signaling is an important negative regulator of this system [32]. Interestingly, a study in nonhuman primates [33] found that an HFD led to an increase in TLR4 activation in the placenta when compared to animals fed SC. In addition, the authors found changes in blood flow, leading us to believe that the activation of TLR4 *via* an HFD could also be other important factor that influences the birth weight and autophagy activity of offspring.

On the other hand, rodents complete the development of their neural circuits during lactation [34], which may influence the protein content of the hypothalamic autophagy markers at birth. In accordance with this idea, metabolic parameters were compromised after weaning and hepatic autophagy remained decreased in the hypothalamus tissue of the HFD-O group. Indeed, lactation seems to be highly sensitive in rodents, providing important changes in response to maternal HFD [35]. The use of an HFD during lactation also has a negative impact on the development of neuronal projections in offspring, which results in metabolic alterations, such as changes in food intake and glucose homeostasis [36]. Importantly, the deletion of

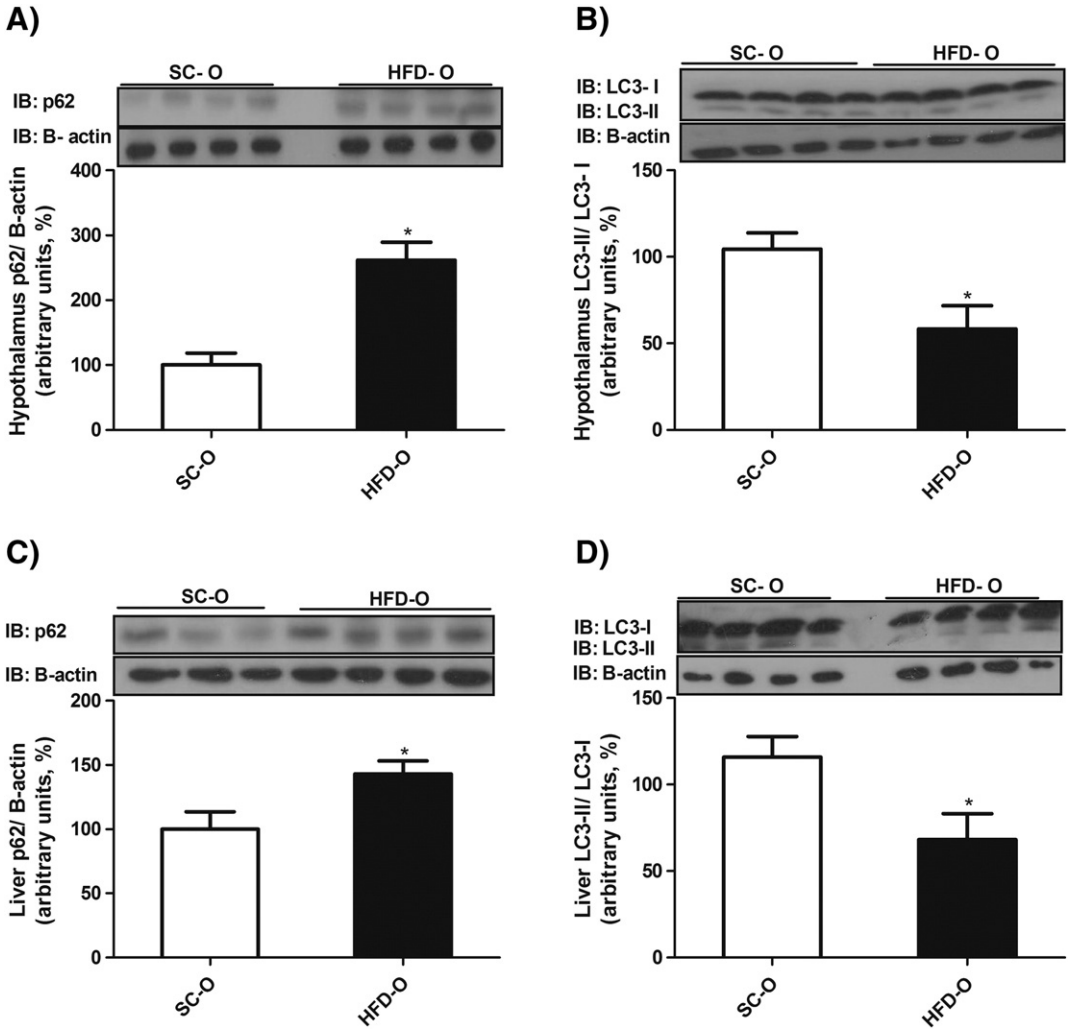

Fig. 8. Protein content of autophagy markers in hypothalamus and liver of male offspring (SC-O or HFD-O) at birth (d0). Representative western blotting of hypothalamus **(A)** p62/β-actin. **(B)** LC3-II/LC3-I. Representative western blotting of liver **(C)** p62/β-actin. **(D)** LC3-II/LC3-I. Values are mean ± SEM, $n = 3–6$ pups per group. *$P < .05$; *SC-O *vs.* HFD-O.

Atg7 in POMC neurons causes higher postweaning body mass, increased adiposity and a marked reduction in POMC fiber density. Thus, it has been suggested that autophagy is an important mechanism for the normal development of neuron projections [37].

As there were no differences in energy expenditure between the groups at d18 (data not shown), we suggest that the higher body mass of the HFD-O animals can be explained by breast milk intake, but this should be confirmed by direct measurement of milk intake. Then, it would be interesting to evaluate the composition of breast milk in our model, as the maternal dietary source of fat can affect the amount and composition of fatty acid in the breast milk of rats [38] since several reports described an important crosstalk between lipids and autophagy. In addition to other interactions, the lipid profile can directly affect the physicochemical properties of lipid bilayers, interfering with its fluidity and in the remodeling of the cell membrane [12,39]. Thus, even though it is poorly understood, it is known that lipids can influence various steps of autophagy, including initiation, autophagosome biogenesis and maturation [39,40]. Accordingly, we suspect that altered milk from obese dams can alter lipid membrane composition, leading to impairments in the autophagosome–lysosome fusion. In the future, it may be interesting to study the lipid composition of membranes in our experimental model.

Regarding metabolic changes, our group has recently demonstrated that weaned offspring from obese dams show some alterations accompanied by activation of inflammatory pathways, represented by an increase in p-JNK and p-IKK in the hypothalamus and the liver [18,19]. Also, insulin resistance and modulation in endoplasmic reticulum stress in the hypothalamus has been reported [19]. These animals showed hepatic steatosis, indicated by the presence of vacuoles that contained lipids within hepatocytes as shown by hematoxylin–eosin-stained liver sections. Furthermore, maternal consumption of an HFD was responsible for early lipid metabolism in offspring by modulating the expression of hepatic β-oxidation-related genes and microRNA [18].

In accordance with these changes, we also observed an important difference in hepatic lipid content in HFD-O compared to control group after weaning (unpublished data). Taking into account that autophagy has an important link with hepatic lipid metabolism; we believe that the origins of this alteration are associated with the earlier down-regulation of liver autophagy, as found here in our study. This hypothesis is consistent with studies that show that mice with a hepatocyte-specific knockout of Atg7 fed an HFD developed markedly increased liver triglycerides and cholesterol [17], indicating that defects in autophagy can promote hepatic steatosis [16,17] and that autophagy induction by rapamycin treatment protects the liver from HFD-induced lipotoxicity and may be an important therapeutic target for liver disease associated with obesity [41].

In addition, some studies reported that the suppression of autophagy increases inflammation in metabolic tissues, with the overexpression of certain types of cytokines; however, the exact

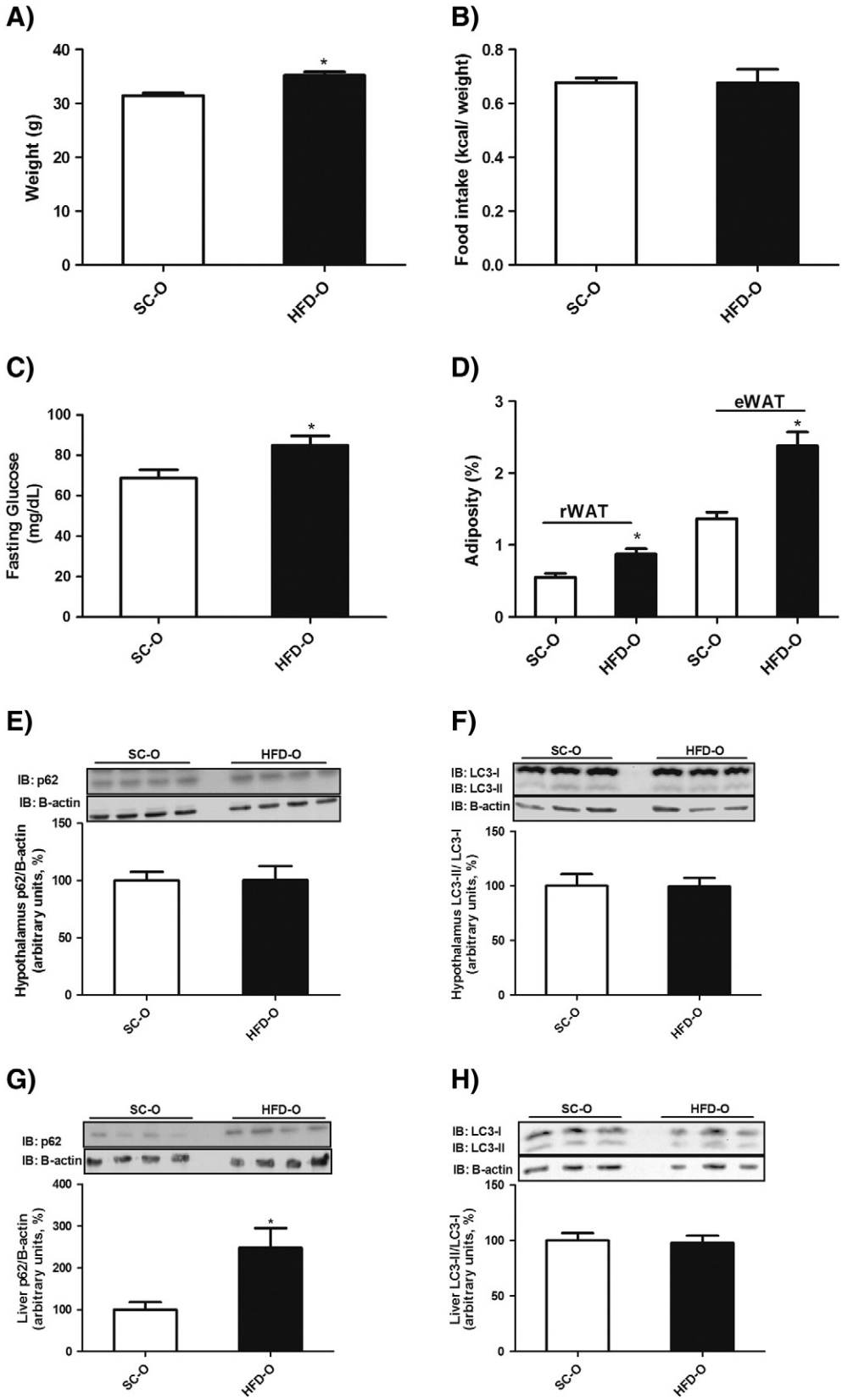

Fig. 9. Metabolic characterization and autophagy markers of male offspring at adulthood (d42). **(A)** Weight of SC-O and HFD-O mice. **(B)** Food intake corrected by weight of SC-O and HFD-O mice. **(C)** Fasting glucose of SC-O and HFD-O mice. **(D)** Adiposity correct by weight (eWAT, epididymal white adipose tissue; rWAT, retroperitoneal white adipose tissue; BAT, brown adipose tissue) of SC-O and HFD-O mice. Representative western blotting of hypothalamus **(E)** p62/β-actin. **(F)** LC3-II/LC3-I. Representative western blotting of liver **(G)** p62/β-actin. **(H)** LC3-II/LC3-I. Values are mean ± SEM, $n = 4$ pups per group to western blotting and $n = 10$ pups per group to metabolic parameters. *P<.05; *SC-O *vs*. HFD-O.

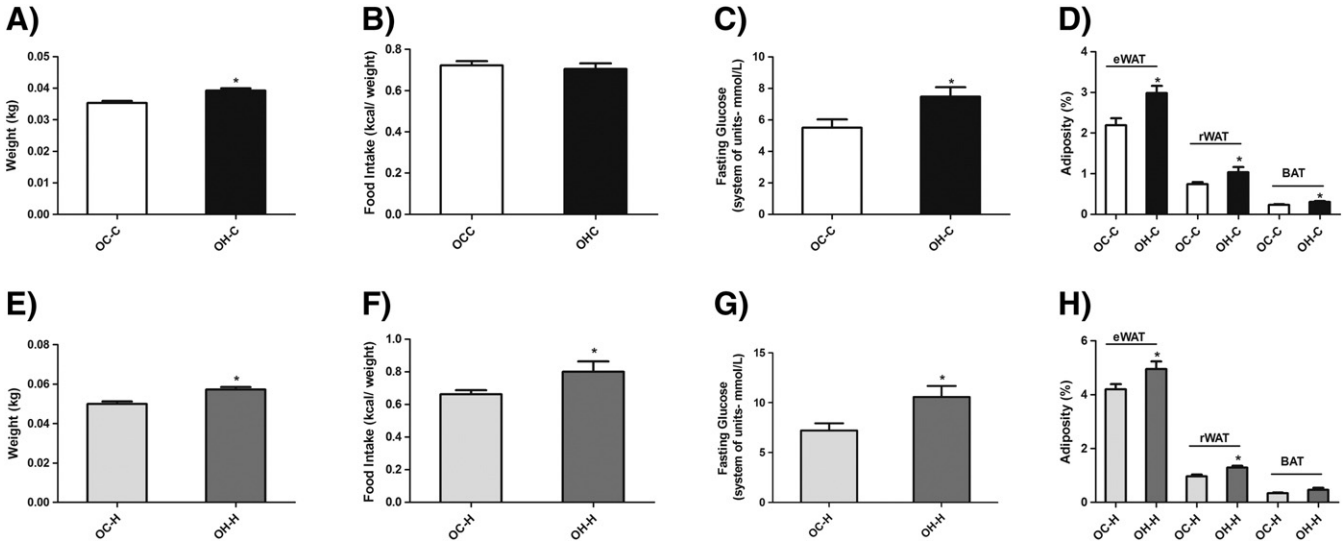

Fig. 10. Metabolic characterization of male offspring at adulthood (d82). **(A)** Weight of OC-C and OH-C mice. **(B)** Food intake corrected by weight of OC-C and OH-C mice. **(C)** Fasting glucose of OC-C and OH-C mice. **(D)** Adiposity correct by weight (eWAT, epididymal white adipose tissue; rWAT, retroperitoneal white adipose tissue; BAT, brown adipose tissue) of OC-C and OH-C mice. **(E)** Weight of OC-H and OH-H mice. **(F)** Food intake corrected by weight of OC-H and OH-H mice. **(G)** Fasting glucose of OC-H and OH-H mice. **(H)** Adiposity correct by weight of OC-H and OH-H mice. Values are mean ± SEM, $n = 5–15$ pups per group to metabolic parameters. *$P<.05$; *OC-C *vs.* OH-C or *OC-H *vs.* OH-H.

mechanisms by which cytokines mediate autophagy regulation and immune cell function remain unknown [42]. TNF-α content was not different in our analyses, in line with data published elsewhere by our group [18,19]. Perhaps the levels were too low to be detected by the methods used in this study. This may, at least in part, explain our results for TNF-α between the groups. Although, Rother *et al.* [43] reported that inflammatory markers may be restricted to the hypothalamus, since they did not find changes in other peripheral tissues analyzed in their study. In our work, we were not able to establish a clear relationship between markers of inflammation and autophagy markers.

During adulthood (d42 and d82), both groups (SC or HFD with control diet) exhibited significant alterations in the metabolic parameters measured, replicating findings from previous studies [44–46]. Ashino *et al.* reported higher liver triglyceride content as well as large lipid vacuoles within hepatocytes, accompanied by increased p-IKK and p-JNK, in the livers of offspring from obese dams at d82 [44]. Even after 42 days, the livers still exhibited p62 accumulation, while maternal DIO, at d82, during pregnancy and lactation appeared not to be sufficient to negatively modulate the autophagy proteins in the SC group compared to the OCC and OHC groups. Thus, we suspect that an HFD during adult life is responsible for enhancing possible epigenetic

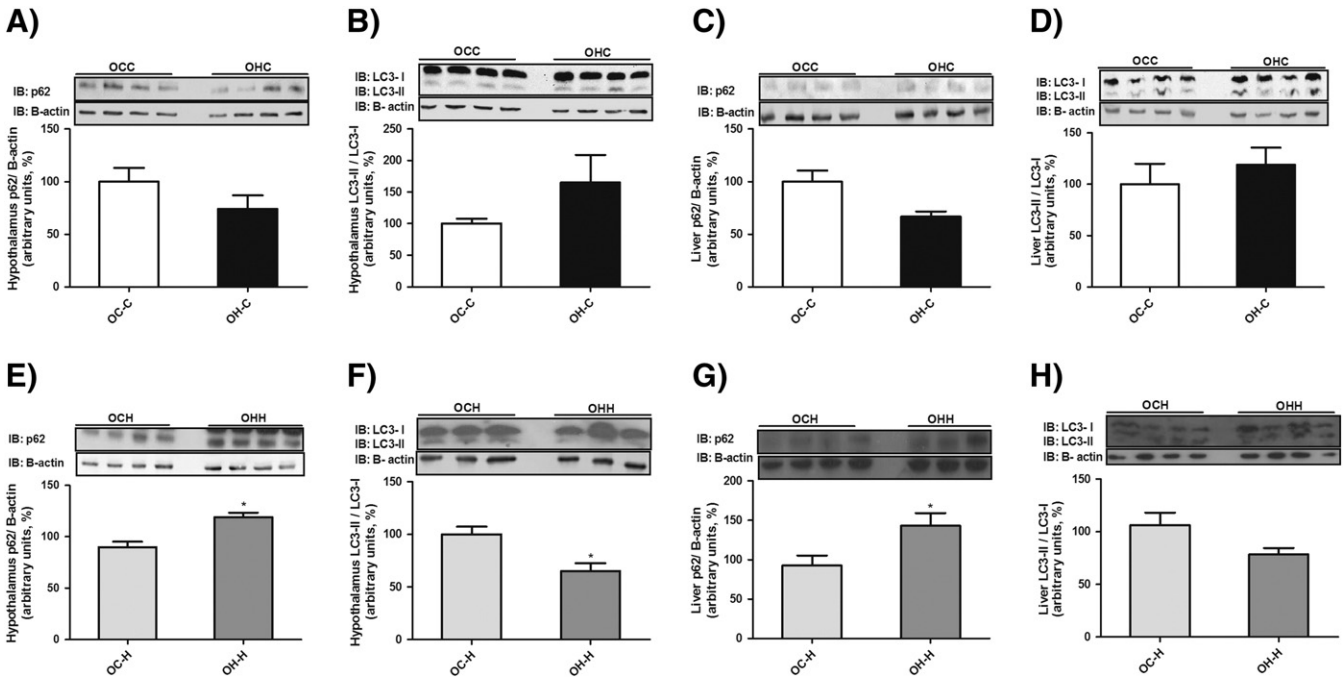

Fig. 11. Protein content of autophagy markers in hypothalamus and liver of male offspring (OC-C *vs.* OH-C) or (OCH *vs.* OHH) at adulthood (d82). Representative western blotting of hypothalamus. **(A and C)** p62/β-actin. **(B and D)** LC3-II/LC3-I. Representative western blotting of liver. **(E and G)** p62/β-actin. **(F and H)** LC3-II/LC3-I. Values are mean ± SEM, $n = 3–6$ pups per group. *$P<.05$; *OC-C *vs.* OH-C or *OC-H *vs.* OH-H.

changes in our experimental model. However, additional studies evaluating microRNAs that can modulate the autophagy pathway or acetylation and methylation of autophagy genes should be conducted to confirm this idea. We also suspect that the impairment of autophagy markers in the hypothalamus and liver during initial life and after reexposure to an HFD may contribute to metabolic disturbances and an obese phenotype in offspring, providing another avenue to be studied in future research.

In summary, the results from this study describe that offspring from obese dams show impairment of liver autophagy proteins at birth and after lactation, both in hypothalamus and liver tissues. Surprisingly, the offspring of obese dams receiving a control diet after weaning until day 82 showed no impairment of autophagy proteins in both tissues analyzed. Thus, we believe that exposure to lipids is likely an essential condition for enhancing the modulation of autophagy proteins in adulthood in our experimental model. However, we cannot exclude the possibility that the obesogenic intrauterine environment and the breast milk of dams with DIO could have a negative impact on autophagy activity in the offspring, as these animals showed down-regulation in the protein content of p62 and LC3-II after only 40 days of an HFD, while adults exhibited down-regulation of autophagy markers only after 12 or 16 weeks [13,14]. Although the molecular mechanisms have not been explored in our work, we believe that our findings could contribute to our understanding of the metabolic changes in the offspring. Finally, we highlight the importance of further studies using interventions and additional techniques for measuring autophagy activity, as well as to explore the molecular mechanisms involved in this process.

Acknowledgements

The present study was supported by São Paulo Research Foundation (FAPESP) process 2011/51.205-6. The funders had no direct influence over the present study. The authors do not have any conflict of interest to declare.

References

[1] WHO, World Health Organization. World Health Statistics 2012. Report of a WHO consultation on obesity. Geneva: WHO; 2012.
[2] Godfrey KM, Barker DJ. Fetal programming and adult health. Public Health Nutr 2001;4(2B):611–24.
[3] Hales CN, Barker DJ. The thrifty phenotype hypothesis. Br Med Bull 2001;60:5–20.
[4] Ravelli AC, van Der Meulen JH, Osmond C, Barker DJ, Bleker OP. Obesity at the age of 50 y in men and women exposed to famine prenatally. Am J Clin Nutr 1999; 70(5):811–6.
[5] Martin-Gronert MS, Ozanne SE. Early life programming of obesity. Med Wieku Rozwoj 2013;17(1):7–12.
[6] De Duve C, Wattiaux R. Functions of lysosomes. Annu Rev Physiol 1966;28: 435–92.
[7] Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature 2008;451(7182):1069–75. http://dx.doi.org/10.1038/nature06639.
[8] Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell 2011; 147(4):728–41. http://dx.doi.org/10.1016/j.cell.2011.10.026.
[9] Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol 2010;221(1):3–12.
[10] Schneider JL, Cuervo AM. Liver autophagy: much more than just taking out the trash. Nat Rev Gastroenterol Hepatol 2014;11(3):187–200. http://dx.doi.org/10.1038/nrgastro.2013.211.
[11] Behrends C, Sowa ME, Gygi SP, Harper JW. Network organization of the human autophagy system. Nature 2010;466(7302):68–76. http://dx.doi.org/10.1038/nature09204.
[12] Koga H, Kaushik S, Cuervo AM. Altered lipid content inhibits autophagic vesicular fusion. FASEB J 2010;24(8):3052–65. http://dx.doi.org/10.1096/fj.09-144519.
[13] Meng Q, Cai D. Defective hypothalamic autophagy directs the central pathogenesis of obesity *via* the IkappaB kinase beta (IKKbeta)/NF-kappaB pathway. J Biol Chem 2011;286(37):32324–32. http://dx.doi.org/10.1074/jbc.M111.254417.
[14] Portovedo M, Ignacio-Souza L, Bombassaro B, Coope A, Reginato A, Razolli DN, et al. Saturated fatty acids modulate autophagy's proteins in the hypothalamus. PLoS One 2015 Mar 18;10(3):e0119850. http://dx.doi.org/10.1371/journal.pone.0119850.
[15] Czaja MJ, Ding WX, Donohue Jr TM, Friedman SL, Kim JS, Komatsu M, et al. Functions of autophagy in normal and diseased liver. Autophagy 2013;9(8): 1131–58. http://dx.doi.org/10.4161/auto.25063.
[16] Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, et al. Autophagy regulates lipid metabolism. Nature 2009;458(7242):1131–5. http://dx.doi.org/10.1038/nature07976.
[17] Yang L, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. Cell Metab 2010;11(6):467–78. http://dx.doi.org/10.1016/j.cmet.2010.04.005.
[18] Benatti RO, Melo AM, Borges FO, Ignacio-Souza LM, Simino LA, Milanski M, et al. Maternal high-fat diet consumption modulates hepatic lipid metabolism and microRNA-122 (miR-122) and microRNA-370 (miR-370) expression in offspring. Br J Nutr 2014;111(12):2112–22. http://dx.doi.org/10.1017/S0007114514000579.
[19] Melo AM, Benatti RO, Ignacio-Souza LM, Okino C, Torsoni AS, Milanski M, et al. Hypothalamic endoplasmic reticulum stress and insulin resistance in offspring of mice dams fed high-fat diet during pregnancy and lactation. Metabolism 2014; 63(5):682–92. http://dx.doi.org/10.1016/j.metabol.2014.02.002.
[20] Lee MO. Determination of the surface area of the white rat with application to the expression of metabolic results. Am J Physiol 1929;89:24–33.
[21] Russell JA. Milk yield, suckling behaviour and milk ejection in the lactating rat nursing litters of different sizes. J Physiol 1980;303:403–15.
[22] Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008;132(1): 27–42. http://dx.doi.org/10.1016/j.cell.2007.12.018.
[23] Mizushima N, Levine B. Autophagy in mammalian development and differentiation. Nat Cell Biol 2010;12(9):823–30. http://dx.doi.org/10.1038/ncb0910-823.
[24] Tsukada M, Ohsumi Y. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. FEBS Lett 1993;333(1–2):169–74.
[25] Alvarez VE, Kosec G, Sant'Anna C, Turk V, Cazzulo JJ, Turk B. Autophagy is involved in nutritional stress response and differentiation in Trypanosoma cruzi. J Biol Chem 2008;283(6):3454–64.
[26] Juhász G, Csikós G, Sinka R, Erdélyi M, Sass M. The *Drosophila* homolog of Aut1 is essential for autophagy and development. FEBS Lett 2003;543(1–3):154–8.
[27] Reynolds CM, Vickers MH, Harrison CJ, Segovia SA, Gray C. High fat and/or high salt intake during pregnancy alters maternal meta-inflammation and offspring growth and metabolic profiles. Physiol Rep 2014;2(8):e12110. http://dx.doi.org/10.14814/phy2.12110.
[28] Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 2005;169(3):425–34.
[29] Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, et al. The role of autophagy during the early neonatal starvation period. Nature 2004; 432(7020):1032–6.
[30] Hung TH, Hsieh TT, Chen SF, Li MJ, Yeh YL. Autophagy in the human placenta throughout gestation. PLoS One 2013;8(12):e83475. http://dx.doi.org/10.1371/journal.pone.0083475.
[31] Jansson N, Rosario FJ, Gaccioli F, Lager S, Jones HN, Roos S, et al. Activation of placental mTOR signaling and amino acid transporters in obese women giving birth to large babies. J Clin Endocrinol Metab 2013;98(1):105–13. http://dx.doi.org/10.1210/jc.2012-2667.
[32] Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 2010;22(2):124–31. http://dx.doi.org/10.1016/j.ceb.2009.11.014.
[33] Frias AE, Morgan TK, Evans AE, Rasanen J, Oh KY, Thornburg KL, et al. Maternal high fat diet disturbs uteroplacental hemodynamics and increases the frequency of stillbirth in a nonhuman primate model of excess nutrition. Endocrinology 2011 Jun;152(6):2456–64. http://dx.doi.org/10.1210/en.2010-1332.
[34] Bouret SG. Early life origins of obesity: role of hypothalamic programming. J Pediatr Gastroenterol Nutr 2009(48 Suppl. 1):S31–8.
[35] Sun B, Purcell RH, Terrillion CE, Yan J, Moran TH, Tamashiro KL. Maternal high-fat diet during gestation or suckling differentially affects offspring leptin sensitivity and obesity. Diabetes 2012;61:2833–41.
[36] Vogt MC, Paeger L, Hess S, Steculorum SM, Awazawa M, Hampel B, et al. Neonatal insulin action impairs hypothalamic neurocircuit formation in response to maternal high-fat feeding. Cell 2014;156(3):495–509. http://dx.doi.org/10.1016/j.cell.2014.01.008.
[37] Coupé B, Ishii Y, Dietrich MO, Komatsu M, Horvath TL, Bouret SG. Loss of autophagy in pro-opiomelanocortin neurons perturbs axon growth and causes metabolic dysregulation. Cell Metab 2012;15(2):247–55.
[38] Priego T, Sánchez J, García AP, Palou A, Picó C. Maternal dietary fat affects milk fatty acid profile and impacts on weight gain and thermogenic capacity of suckling rats. Lipids 2013;48(5):481–95. http://dx.doi.org/10.1007/s11745-013-3764-8.
[39] Dall'Armi C, Devereaux KA, Di Paolo G. The role of lipids in the control of autophagy. Curr Biol 2013;23(1):R33–45. http://dx.doi.org/10.1016/j.cub.2012.10.041.
[40] Carlsson SR, Simonsen A. Membrane dynamics in autophagosome biogenesis. J Cell Sci 2015;128(2):193–205.
[41] Mei S, Ni HM, Manley S, Bockus A, Kassel KM, Luyendyk JP, et al. Differential roles of unsaturated and saturated fatty acids on autophagy and apoptosis in hepatocytes. J Pharmacol Exp Ther 2011;339(2):487–98. http://dx.doi.org/10.1124/jpet.111.184341.
[42] Joven J, Guirro M, Mariné-Casadó R, Rodríguez-Gallego E, Menéndez JA. Autophagy is an inflammation related defensive mechanism against disease.

Adv Exp Med Biol 2014;824:43–59. http://dx.doi.org/10.1007/978-3-319-07320-0_6.
[43] Rother E, Kuschewski R, Alcazar MA, Oberthuer A, Bae-Gartz I, Vohlen C, et al. Hypothalamic JNK1 and IKKa activation and impaired early postnatal glucose metabolism after maternal perinatal high-fat feeding. Endocrinology 2012;153:770–81.
[44] Ashino NG, Saito KN, Souza FD, Nakutz FS, Roman EA, Velloso LA, et al. Maternal high-fat feeding through pregnancy and lactation predisposes mouse offspring to molecular insulin resistance and fatty liver. J Nutr Biochem 2012;23(4):341–8. http://dx.doi.org/10.1016/j.jnutbio.2010.
[45] Burgueño AL, Cabrerizo R, Gonzales Mansilla N, Sookoian S, Pirola CJ. Maternal high-fat intake during pregnancy programs metabolic-syndrome-related phenotypes through liver mitochondrial DNA copy number and transcriptional activity of liver PPARGC1A. J Nutr Biochem 2013;24(1):6–13. http://dx.doi.org/10.1016/j.jnutbio.2011.12.008.
[46] Sullivan EL, Smith MS, Grove KL. Perinatal exposure to high-fat diet programs energy balance, metabolism and behavior in adulthood. Perinatal exposure to high fat diet programs energy balance, metabolism and behavior in adulthood. Neuroendocrinology 2011;93(1):1–8. http://dx.doi.org/10.1159/000322038.